Arch Pharm Res Vol 27, No 10, 1053-1059, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Anti-Inflammatory Effects of N^1-Benzyl-4-Methylbenzene-1,2-Diamine (JSH-21) Analogs on Nitric Oxide Production and Nuclear Factor-kappa B Transcriptional Activity in Lipopolysaccharide-Stimulated Macrophages RAW 264.7

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(Received July 25, 2004)

N^1-Benzyl-4-methylbenzene-1,2-diamine (JSH-21) and its analogs were chemically synthesized and their anti-inflammatory potentials investigated. JSH-21 inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated macrophages RAW 264.7 in a dose-dependent manner, with an IC_{50} value of 9.2 μM, where pyrrolidine dithiocarbamate and parthenolide as positive controls exhibited IC_{50} values of 29.3 and 3.6 μM, respectively. The inhibitory effect of JSH-21 on the NO production was attributable to its down-regulatory action on LPS-inducible NO synthase (iNOS), which was documented by iNOS promoter activity. In the mechanism of the anti-inflammatory action, JSH-21 exhibited inhibitory effects on LPS-induced DNA binding activity and transcriptional activity of nuclear factor-kappa B (NF-κB). Structural analogs of JSH-21 also inhibited both the LPS-induced NO production and NF-κB transcriptional activity, where diamine substitution at positions 1 and 2 of JSH-21 seems to play an important role in the anti-inflammatory activity.

Key words: N^1-Benzyl-4-methylbenzene-1,2-diamine, Chemical preparation, Anti-inflammatory effect, Nitric oxide, Nuclear factor-kappa B, Lipopolysaccharide

INTRODUCTION

Nitric oxide (NO) is a short-lived free radical produced from L-arginine by a NO synthase (NOS) catalyzed reaction in living systems (Palmer *et al.*, 1988). NO plays an important role in the regulation of many physiological functions, such as vasodilation, neurotransmission, neurotoxicity, wound repair, and inflammation (Liaudet *et al.*, 2000; Prast and Philippu, 2001; Ignarro, 2002). Endothelial and neuronal NOS produce moderate amounts of NO, which primarily mediate physiological responses. Meanwhile, NO is also synthesized in the immune system by inducible NOS (iNOS), where it facilitates the killing of invading microorganisms (MacMicking *et al.*, 1997). However, high-output NO by iNOS can provoke septic shock, autoimmune disorders and inflammatory diseases (Bogdan, 2001; Blantz and Munger, 2002). Indeed, NO production in macrophages is related to the level of iNOS protein. The induction of iNOS by lipopolysaccharide (LPS) occurs through the Toll-like receptor (TLR) 4-dependent signaling cascade, resulting in nuclear factor-kappa B (NF-κB) activation (Jones *et al.*, 2001).

NF-κB complex exist in the cytoplasm as homodimer or heterodimer in quiescent forms bound to inhibitory IκB proteins (Beg *et al.*, 1992; Verma *et al.*, 1995; Baeuerle and Baltimore, 1996). However, LPS treatment of macrophages could turn on the TLR4 signaling pathway to activate the IκB kinase (IKK) complex, resulting in phospholation of IκB proteins. The phosphorylated IκB proteins are then subjected to ubiquitination followed by 26S proteasome-mediated degradation. The NF-κB complex, now free of IκB, are moved to the nucleus for transcriptional regulation of pro-inflammatory cytokines, iNOS, cyclooxygenase-2 (COX-2), adhesion molecules, apoptotic and anti-apoptotic proteins (Collins *et al.*, 1995; Tian and Brasier, 2003).

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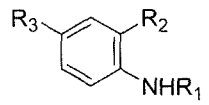
Several inhibitors of NF-κB activation have been reported to control the expression of inflammatory and immune genes, which are linked to inflammatory disorders, such as rheumatoid arthritis, asthma, septic shock, atherosclerosis and cancer (Blantz and Munger, 2002; Cavaillon *et al.*, 2003; Moreland *et al.*, 2004). Lactacystin, PS341 and MG132 are reported as inhibitors of proteasome-mediated IκB degradation (Dick *et al.*, 1997), and PS341 was recently approved, as Bortezomib (Velcade; Millenium Pharmaceuticals), by the FDA for treatment of multiple myelomas (Paramore and Frantz, 2003). As an antioxidant, *N*-acetylcysteine is reported to inhibit NF-κB activation mainly by interfering with the upstream signals that lead to IκB phosphorylation (Zafarullah *et al.*, 2003). The natural product, calagualine, inhibits the binding of NF-κB-inducing kinase (NIK) to TRAF2 sites and subsequently inhibits the NF-κB activation induced by TNF-α (Manna *et al.*, 2003). Sesquiterpene lactones, such as parthenolide and ergolide, inhibit the IKK complex that phosphorylates IκB proteins (Hehner *et al.*, 1999). Glucocorticoids, like dexamethasone, inhibit NF-κB activation by either interference of the glucocorticoid receptor-mediated DNA binding activity of NF-κB or enhanced synthesis of IκB proteins (Auphan *et al.*, 1995).



Compound	R_1	R_2	R_3
JSH-21		$-NH_2$	$-CH_3$
JSH-22		$-NH_2$	$-CH_3$
JSH-23		$-NH_2$	$-CH_3$
JSH-24		$-NH_2$	-Cl
JSH-26		$-NO_2$	$-CH_3$
JSH-30		$-NO_2$	-Cl

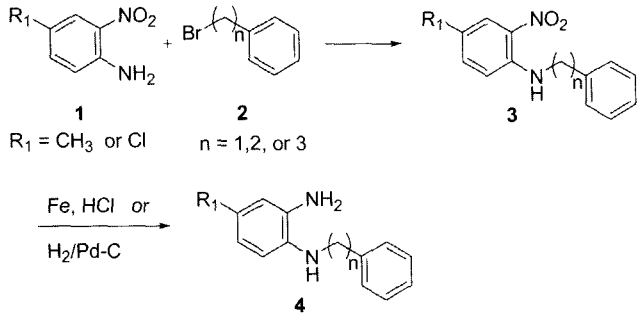
Fig. 1. The chemical structures of JSH-21 and its analogs. Compounds are *N*'-benzyl-4-methylbenzene-1,2-diamine (JSH-21), 4-methyl-*N*'-phenethylbenzene-1,2-diamine (JSH-22), 4-methyl-*N*'-(3-phenylpropyl) benzene-1,2-diamine (JSH-23), *N*'-benzyl-4-chlorobenzene-1,2-diamine (JSH-24), *N*-benzyl-4-methyl-2-nitroaniline (JSH-26), and *N*-benzyl-4-chloro-2-nitroaniline (JSH-30).

In our ongoing study to discover anti-inflammatory agents, *N*'-benzyl-4-methylbenzene-1,2-diamine (JSH-21) and its analogs (Fig. 1) were found to inhibit NO production and NF-κB transcriptional activity in LPS-stimulated macrophages RAW 264.7. Chemical preparations of JSH-21 analogs, and their anti-inflammatory potentials, have been documented in this study.

MATERIALS AND METHODS

Chemical preparation of *N*-alkylated benzene-1,2-diamine analogs

N'-Benzyl-4-methylbenzene-1,2,-diamine and its analogs have been chemically prepared, according to the reported principles (Perrin *et al.*, 1982; Yuste *et al.*, 1982; Brown and Rizzo, 1996).



N'-Benzyl-4-methylbenzene-1,2-diamine (4a, JSH-21)

Hydrochloric acid (36%, 0.5 mL) was slowly added to a mixture of *N*-benzyl-4-methyl-2-nitroaniline (**3a**, 2.0 g, 8.3 mmol) and Fe powder (4.6 g) in 20% ethanol (20 mL) at room temperature. The mixture was refluxed for 1 h and then filtered without cooling. The filtrate was evaporated under vacuum, and subjected to flash column chromatography to isolate the product (**4a**, 0.9 g), with a yield of 51.4%; Violet solid; Rf=0.3 (hexane:ethyl acetate=3:1); m.p. 111.9-112.4°C; IR (KBr) 3450, 3360, 3050, 2940, 1620 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.41-7.13 (m, 5H), 6.38 (m, 2H), 5.87 (d, *J*=7.9 Hz, 1H), 4.21 (s, 2H), 3.67 (br, NH), 2.17 (s, 3H).

4-Methyl-*N*'-phenethylbenzene-1,2-diamine (4b, JSH-22)

4-Methyl-2-nitroaniline (5.0 g, 32.9 mmol) in dry toluene (100 mL) was treated with NaH (2.6 g, 65.8 mmol, 60% in oil) for 5 min at room temperature. (2-Bromoethyl) benzene (9.1 g, 49.35 mmol) was slowly added, and the resulting mixture heated at 70°C for 30 h. After cooling, the mixture

was concentrated under vacuum. The residue was dissolved in dichloromethane and washed with water three times. The organic layer was dehydrated with anhydrous sodium sulfate and evaporated under vacuum. The residue was subjected to the flash column chromatography to obtain *N*-phenethyl-4-methyl-2-nitroaniline (**3b**, 4.54 g), with a yield of 54.0%; Orange solid; Rf=0.45 (hexane: ethyl acetate=5:1); m.p. 79.4-79.8°C; IR (KBr) 2950-2830, 1635, 1570, 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.84 (br, NH), 7.31-6.92 (m, 8H), 3.91 (m, 2H), 3.05 (m, 2H), 2.44 (s, 3H). A mixture of **3b** (2.0 g, 7.8 mmol) and Pd-C (40 mg) in dichloromethane (40 mL) was shaken under 30 psi of H_2 gas for 20 h and then filtered with the aid of a celite pad. The filtrate was evaporated to give the product (**4b**, 1.6 g), with a yield of 91.0%; Orange solid; Rf=0.41 (hexane:ethyl acetate=1:1); m.p. 104.4-105.0°C; IR (KBr) 3450, 3360, 3050, 2940, 1620 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.21-7.28 (m, 6H), 6.40 (m, 2H), 3.70 (br, NH), 3.11 (m, 2H), 2.76 (t, *J*=7.2 Hz, 2H).

4-Methyl-N^1-(3-phenylpropyl)benzene-1,2-diamine (4c, JSH-23)

Employing the same method used for the preparation of **3b**, *N*-phenylpropyl-4-methyl-2-nitroaniline (**3c**) was prepared with 4-methyl-2-nitroaniline (3.0 g, 19.7 mmol) and 1-bromo-3-phenylpropane (4.7 g, 23.6 mmol), with a yield of 2.13 g, 39.8%; Orange solid; Rf=0.58 (hexane:ethyl acetate=5:1); m.p. 94.9-95.2°C; IR (KBr) 3400, 1635, 1570, 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.97 (br, 1H), 7.47-7.23 (m, 6H), 6.70 (d, *J*=8.8 Hz, 1H), 3.29 (m, 2H), 2.77 (t, *J*=7.4 Hz, 2H), 2.25 (s, 3H), 2.17-1.88 (m, 2H). Using the method for the preparation of **4b**, compound **3c** (2.0 g, 7.4 mmol) was reduced to the product (**4c**), with a yield of 1.67 g, 94.0%; Orange solid; Rf=0.41 (hexane:ethyl acetate=1:1); m.p. 104.4-105.0°C; IR (KBr) 3450, 3360, 3050, 2940, 1620 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 7.21-7.28 (m, 6H), 6.40 (m, 2H), 5.88 (d, *J*=7.9 Hz, 1H), 3.70 (br, NH), 3.11 (m, 2H), 2.76 (t, *J*=7.2 Hz, 2H), 2.00 (m, 2H).

N^1-Benzyl-4-chlorobenzene-1,2-diamine (4d, JSH-24)

Using the method for the preparation of **4a**, *N*-benzyl-4-chloro-2-nitroaniline (**3d**, 2.0 g, 7.6 mmol) was reduced to the product (**4d**), with a yield of 1,15 g, 65.1%; Dark brown solid; Rf=0.24 (hexane:ethyl acetate=5:1); m.p. 115.3-117.5°C; IR (KBr) 3350, 3070-3030, 2970, 2920, 2880, 2850, 1595, 1500 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.32 (m, 6H), 6.42 (m, 2H), 5.81 (m, 1H), 4.21 (s, 2H), 3.77 (br, NH).

N-Benzyl-4-methyl-2-nitroaniline (3a, JSH-26)

A mixture of 4-methyl-2-nitroaniline (3.0 g, 19.7 mmol) and benzyl bromide (5.1 g, 29.6 mmol) in dichloromethane (150 mL) was refluxed in the presence of pyridine (4.7 g, 59.1 mmol) for 16 h. After cooling, the mixture was washed with water three times. The organic layer was dehydrated with anhydrous sodium sulfate and evaporated under vacuum. The residue was subjected to the flash column chromatography to obtain the product (**3a**, 4.31 g), with a yield of 90.3%; Orange solid; Rf=0.53 (hexane:ethyl acetate = 5:1); m.p. 87.4-88.5°C; IR (KBr) 3400, 1635, cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.33 (br, NH), 7.99 (s, 1H), 7.34-7.17 (m, 6H), 6.72 (d, *J*=8.8 Hz, 1H), 4.54 (d, *J*=5.6 Hz, 2H), 2.25 (s, 3H).

N-Benzyl-4-chloro-2-nitroaniline (3d, JSH-30)

Using the method for the preparation of **3a**, the product (**3d**) was obtained from 4-chloro-2-nitroaniline (3.0 g, 17.4 mmol) and benzyl bromide (3.6 g, 20.9 mmol), with a yield of 3.1 g, 67.1%; Orange solid; Rf=0.46 (hexane:ethyl acetate = 5:1); m.p. 78.0-78.2°C; IR (KBr) 3400, 1622, 1563, 1510 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.42 (br, NH), 8.20 (m, 1H), 7.47-7.26 (m, 6H), 6.77 (m, 1H), 4.55 (d, *J*=5.6 Hz, 2H).

NO quantification

Murine macrophages RAW 264.7 were cultured in DMEM (10 mg/mL Dulbecco's modified Eagle's medium, 24 mM $NaHCO_3$, 10 mM HEPES, 143 units/mL benzylpenicillin potassium, 100 μg/mL streptomycin sulfate, pH 7.1) containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. The cells were treated with LPS (*E. coli* 055:B5, 1 μg/mL) for 24 h. Nitrite, as the stable metabolite of NO, was measured using Griess reagent (Archer, 1993). Briefly, the cell-free culture supernatant (100 mL) was reacted with a 1:1 mixture (100 mL) of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine in water, and the absorbance at wavelength 540 nm then measured. The nitrite content was calculated from a standard curve constructed with known concentrations of sodium nitrite.

Measurement of iNOS promoter activity

Macrophages RAW 264.7 were cultured in 100 mm culture dishes with DMEM containing 10% FBS. When the cells were confluent, the media were replaced with serum-free Opti-MEM (Invitrogen). Then the RAW 264.7 cells were transfected with the iNOS-luciferase reporter plasmid (Lowenstein *et al.*, 1993) and pSV-β-galactosidase control vector (Promega) using LipofectAMINE™ (Invitrogen). After incubation at 37°C with 5% CO_2 for 5 h, the transfected RAW 264.7 cells were supplied with DMEM containing 10% FBS, and further incubated for 5 h. The transfected RAW 264.7 cells were detached and equal numbers dispensed into 6-well culture plates and then incubated for 24 h. The transfected RAW 264.7 cells were

then treated with LPS (1 μg/mL) for 16 h, and harvested in a passive lysis buffer (Promega). Equal amounts (40 μg) of protein were subjected to luciferase and β-galactosidase assays, using the Luciferase Reporter Assay System (Promega) and the β-Galactosidase Enzyme Assay System (Promega), respectively.

Measurement of NF-κB transcriptional activity

NF-κB transcriptional activity was monitored using macrophages RAW 264.7 stably transfected with a reporter plasmid, pNF-κB-secretory alkaline phosphatase (SEAP)-NPT, which encodes four copies of the NF-κB binding sites (Moon *et al.*, 2001). The cells were grown in DMEM supplemented with 10% FBS and geneticin (500 μg/mL) at 37°C with 5% CO_2 for 24 h, and then treated with LPS (1 μg/mL) for 16 h. Cell-free supernatants were taken, and heated at 65°C for 5 min. After mixing with 1×SEAP assay buffer (2 M diethanolamine, 1 mM $MgCl_2$) containing 4-methylumbelliferyl phosphate (0.5 mM), the fluorescence of the supernatants was measured as relative fluorescence units with emission and excitation at 449 and 360 nm, respectively.

Electrophoretic mobility shift assay (EMSA)

Macrophages RAW 264.7 were treated with LPS (1 μg/mL) for 1 h. Equal amounts (10 μg) of nuclear protein were reacted with ^{32}P-labeled oligonucleotide (Promega) in a binding buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 μg/mL poly(dI-dC), 4% glycerol, pH 7.5) for 10 min on ice. The nucleotide sequences of the oligonucleotides were as follows: NF-κB, AGTTGAGGGGACTTTCCCAGGC; OCT-1, TGTCGAATGCAAATCACTAGAA; AP-1 (*c*-Jun), CGCTTGATGAGTCAGCCGGAA; and CREB, AGAGATTGCCTGACGTCAGAGAGCTAG. The complex between oligonucleotide and nuclear protein was resolved on non-denaturing 6% acrylamide gel by electrophoresis. The gel was dried and exposed to X-ray film to detect the mobility shift of oligonucleotide due to its binding with the transcription factor.

Statistical analysis

Results are expressed as mean ± S.E.M. Data were analyzed by ANOVA followed by the Students *t* test. $P < 0.01$ was considered significant.

RESULTS AND DISCUSSION

Inhibitory effect of JSH-21 on NO production was determined in LPS-stimulated macrophages RAW 264.7 by measuring the nitrite accumulation in the culture media. Resting RAW 264.7 cells released 9.5±3.1 μM of nitrite during incubation for 24 h, whereas the cells treated with LPS alone released a pronounced amount of nitrite, 61.2±3.2 μM (Fig. 2A). No significant difference in the nitrite accumulation was found between resting RAW 264.7 cells and the cells treated with JSH-21 (30 μM) alone (Fig. 2A). JSH-21 inhibited the LPS-induced nitrite accumulation in a dose-dependent manner, corresponding to 24.5±3.7% inhibition at 3 μM, 53.5±4.9% at 10 μM and 74.5±3.5% at 30 μM, with an IC_{50} value of 9.2 μM (Fig. 2B). As positive controls, pyrrolidine dithiocarbamate (PDTC, Sherman *et al.*, 1993) and parthenolide (Hehner *et al.*, 1999) also inhibited the LPS-induced nitrite accumulation in dose-dependent manners, with IC_{50} values of 29.3 and 3.6 μM, respectively (Fig. 2B).

As shown in Table I, structural analogs of JSH-21 also showed dose-dependent inhibitory effects on the LPS-induced nitrite accumulation. 4-Methyl-*N*^{1}-phenethylbenzene-1,2-diamine (JSH-22) and 4-methyl-*N*^{1}-(3-phenylpropyl)benzene-1,2-diamine (JSH-23) exhibited IC_{50} values of 16.5 and 14.4 μM, respectively. Another analog, *N*^{1}-benzyl-4-chlorobenzene-1,2-diamine (JSH-24), exhibited an IC_{50} value of 38.2 μM. On the other hand, nitroaniline derivatives, such as *N*-benzyl-4-methyl-2-nitroaniline (JSH-26) and *N*-benzyl-4-chloro-2-nitroaniline (JSH-30), showed very weak or no inhibitory effects on the LPS-induced nitrite accumulation. JSH-21 and its analogs did not exhibit significant cytotoxic effects to macrophages RAW 264.7, even at 100 μM (data not shown).

To examine whether inhibitory effect of JSH-21 on the LPS-induced nitrite accumulation was attributed to its influence on iNOS expression, iNOS promoter activity was analyzed using macrophages RAW 264.7 transfected transiently with an iNOS-luciferase plasmid containing 1,592 bp of the murine iNOS promoter fused to luciferase

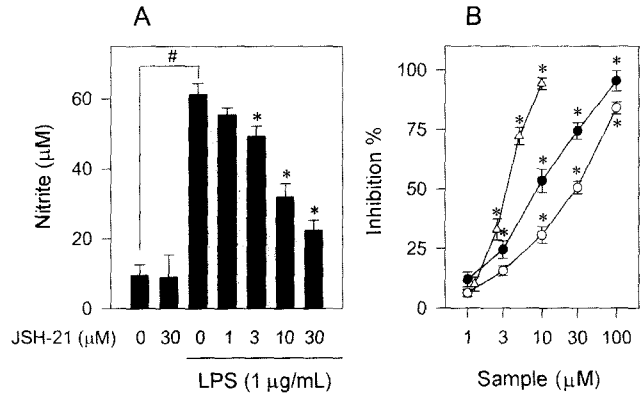


Fig. 2. Inhibition of LPS-induced NO production by JSH-21. Macrophages RAW 264.7 were treated with LPS plus JSH-21 for 24 h, and nitrite accumulation in the cell-free culture media measured (A). Effects of JSH-21 (●), PDTC (○) and parthenolide (△) on LPS-induced nitrite accumulation are represented as a percentage of inhibition (B). Values are mean±S.E.M. (n=3). $^{\#}P<0.01$ vs. media alone-treated group. $^{*}P<0.01$ vs. LPS alone-treated group.

Table I. Inhibitory effects of JSH-21 analogs on LPS-induced NO production

Compound	Inhibition %				IC_{50} (μM)
	100 μM	30 μM	10 μM	3 μM	
JSH-21	95.4±4.3*	74.5±3.5*	53.5±4.9*	24.5±3.7*	9.2
JSH-22	94.7±4.9*	90.4±2.1*	30.6±6.2*	8.1±2.1	16.5
JSH-23	86.2±4.5*	79.8±4.4*	41.5±5.1*	18.7±7.6	14.4
JSH-24	94.3±2.7*	44.1±3.9*	16.5±3.6	n.d.	38.2
JSH-26	9.6±3.3	13.9±4.2	n.d.	n.d	>100
JSH-30	34.1±3.8*	19.2±4.0	n.d.	n.d.	>100
PDTC					29.3
Parthenolide					3.6

Values are mean±S.E.M. (n=3). *P < 0.01 vs. LPS alone-treated group. n.d.; not determined.

as the reporter (Lowenstein *et al.*, 1993). Treatment of LPS to the transfected RAW 264.7 cells increased luciferase expression, 35-fold over the basal level (Fig. 3A). No significant difference in the luciferase expression was found between resting RAW 264.7 cells and the cells treated with JSH-21 (30 μM) alone (Fig. 3A). JSH-21 inhibited the LPS-induced luciferase expression in a dose-dependent manner, corresponding to 33.1±2.7% inhibition at 3 μM, 68.2±3.1% at 10 μM, and 90.4±3.4% at 30 μM, with an IC_{50} value of 6.4 μM (Fig. 3B). PDTC, as a suppressor of iNOS transcription (Sherman *et al.*, 1993), also inhibited LPS-induced luciferase expression in a dose-dependent manner, with an IC_{50} value of 19.7 μM (Fig. 3B).

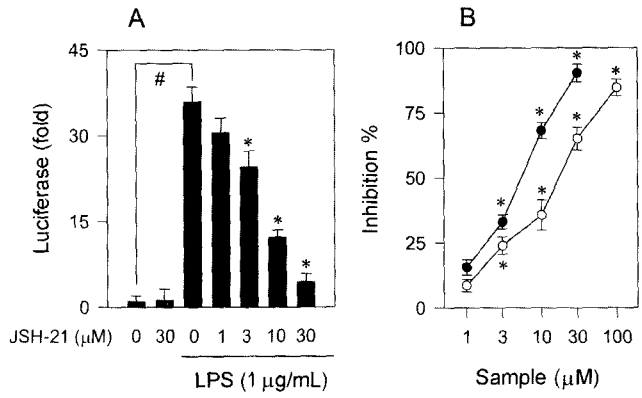


Fig. 3. Inhibition of LPS-induced iNOS promoter activity by JSH-21. Macrophages RAW 264.7, transfected transiently with iNOS-luciferase reporter plasmid and pSV-β-galactosidase control vector, were treated with LPS plus JSH-21 for 16 h. The luciferase and β-galactosidase activities were measured with the lysates. Luciferase expression as the iNOS promoter activity is represented as relative fold, where the luciferase activity was normalized to that of β-galactosidase (A). Effects of JSH-21 (●) or PDTC (○) on LPS-induced luciferase expression are also represented as a percentange of inhibition (B). Values are mean±S.E.M. (n=3). #P<0.01 vs. media alone-treated group. *P<0.01 vs. LPS alone-treated group.

NF-κB activation has been evidenced to play a role in the mechanism of LPS-induced iNOS expression (Lowenstein *et al.*, 1993; Xie *et al.*, 1993; Jones *et al.*, 2001). To investigate whether JSH-21 could inhibit NF-κB trans-activation, LPS-induced NF-κB transcriptional activity was carried out using macrophages RAW 264.7 transfected stably with pNF-κB-SEAP-NPT plasmid, containing four copies of the κB sequence fused to SEAP as the reporter (Moon *et al.*, 2001). Treatment of LPS to the transfected RAW 264.7 cells increased the SEAP expression, 3-4 fold, compared with the resting cells harboring the reporter construct, indicating that cellular NF-κB is transcriptionally functional (Fig. 4A). No significant difference in SEAP expression was found between resting macrophages RAW 264.7 and the cells treated with JSH-21 (30 μM) alone (Fig. 4A). JSH-21 inhibited the LPS-induced SEAP expression in a dose-dependent manner, corresponding to 28.5±2.5% inhibition at 3 μM, 53.3±5.2% at 10 μM and 92.3±6.6% at 30 μM, with an IC_{50} value of 9.1 mM (Fig. 4B). Parthenolide, as an inhibitor of the IKK complex on NF-κB activation signaling (Hehner *et al.*, 1999), also inhibited the reporter gene expression in a dose-dependent manner, with an IC_{50} value of 3.5 μM (Fig. 4B).

As shown in Table II, JSH-21 analogs also showed inhibitory effects on the LPS-induced NF-κB transcriptional activity in dose-dependent manners. 4-Methyl-*N*'-phenylethylbenzene-1,2-diamine (JSH-22) and 4-methyl-*N*'-(3-phenylpropyl)benzene-1,2-diamine (JSH-23) exhibited IC_{50} values of 20.7 and 22.0 μM, respectively. Another analog, *N*'-benzyl-4-chlorobenzene-1,2-diamine (JSH-

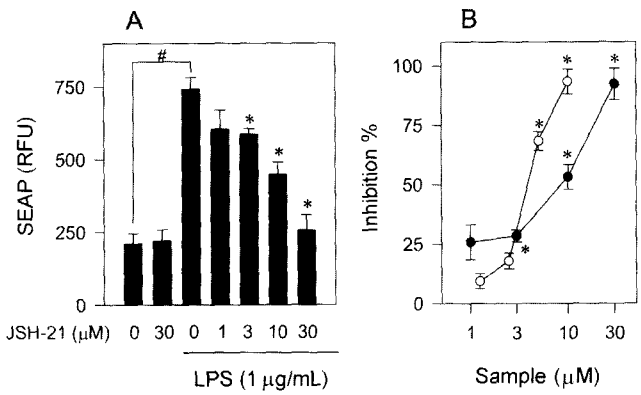


Fig. 4. Inhibition of LPS-induced NF-κB transcriptional activity by JSH-21. Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT reporter construct were treated with LPS plus JSH-21 for 16 h, and SEAP expression as the NF-κB transcriptional activity measured (A). Effects of JSH-21 (●) or parthenolide (○) on LPS-induced SEAP expression are represented as a percentage of inhibition (B). Values are mean±S.E.M. (n=3). #P<0.01 vs. media alone-treated group. *P<0.01 vs. LPS alone-treated group.

Table II. Inhibitory effects of JSH-21 analogs on LPS-induced NF-κB transcriptional activity

Compound	Inhibition %				IC_{50} (μM)
	100 μM	30 μM	10 μM	3 μM	
JSH-21	n.d.	92.3±6.6*	53.3±5.2*	28.5±2.5*	9.1
JSH-22	73.2±4.5*	58.2±4.2*	40.5±7.1*	20.0±5.4	20.7
JSH-23	92.9±5.0*	67.9±2.1*	23.2±1.6*	14.6±6.7	22.0
JSH-24	96.4±2.7*	37.0±2.2*	8.4±2.8	n.d.	45.3
JSH-26	48.1±6.3*	5.3±3.5	n.d.	n.d	>100
JSH-30	51.9±4.8*	7.8±3.1	n.d.	n.d.	97.0
Parthenolide					3.5

Values are mean±S.E.M. (n=3). *P<0.01 vs. LPS alone-treated group. n.d.; not determined.

24), exhibited an IC_{50} value of 45.3 μM.

To investigate whether inhibitory effect of JSH-21 on the LPS-induced NF-κB transcriptional activity was attributable to its influence on DNA binding activity of the transcription factor, EMSA was carried out. Treatment of LPS to macrophages RAW 264.7 induced a marked increase in the DNA binding activity of NF-κB complex within 1 h (Fig. 5). Under the same conditions, JSH-21 decreased the LPS-induced DNA binding activity of NF-κB complex, p65/p50 and p50/p50, in a dose-dependent manner (Fig. 5). The signals corresponding to NF-κB p65/p50 and p50/p50 were identified by supershift experiments (data not shown). Furthermore, LPS-induced DNA binding activity of OCT-1, AP-1 (c-Jun) or CREB were also inhibited by treatment of JSH-21 to the RAW 264.7 cells (Fig. 5).

In the present study, chemically synthetic JSH-21 was discovered to have an inhibitory effect on NO production

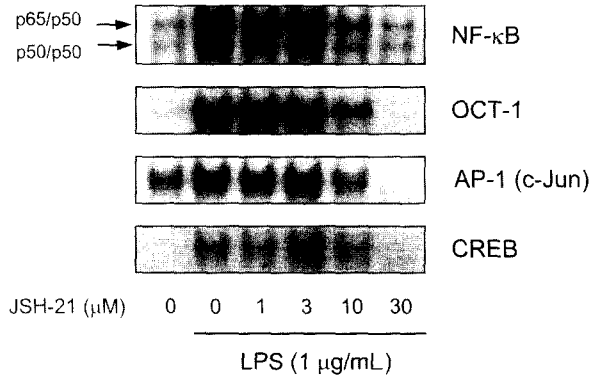


Fig. 5. EMSA for NF-κB or other transcription factors. Macrophages RAW 264.7 were pre-treated with JSH-21 for 2 h, and then stimulated with LPS for 1 h. Nuclear extracts of the cells were reacted with ^{32}P-labeled oligonucleotides specific to each transcription factor of NF-κB, OCT-1, AP-1 (c-Jun) or CREB, and then resolved on non-denaturing 6% polyacrylamide gel by electrophoresis.

in LPS-stimulated macrophages RAW 264.7 (Fig. 2). The inhibitory effect of JSH-21 was attributable to its down-regulatory action on the LPS-induced iNOS promoter activity (Fig. 3). The iNOS promoter in macrophages RAW 264.7 contains κB sites for the binding of the NF-κB transcription factor, specifically located at 55 bp and 971 bp upstream of the TATA box (Lowenstein *et al.*, 1993; Xie *et al.*, 1993). JSH-21 inhibited both LPS-induced transcriptional activity and DNA binding activity of NF-κB, in parallel, (Fig. 4 and 5). Therefore, JSH-21 exhibited an anti-inflammatory effect on the LPS-induced nitrite accumulation via iNOS up-regulation by its inhibitory effect on NF-κB activation.

JSH-21 inhibited both LPS-induced nitrite accumulation and NF-κB transcriptional activity, with similar inhibitory potency (Fig. 2 and 4). JSH-21 analogs also showed inhibitory potencies on the nitrite accumulation, which correlated to that on the NF-κB transcriptional activity in LPS-stimulated macrophages RAW 264.7 (Table I and II). The inhibitory potentials on LPS-induced nitrite accumulation and NF-κB transcriptional activity were maintained by substitution with phenethyl or phenylpropyl group, instead of benzyl group at position 1 amine of JSH-21, but was decreased 5-fold by substitution with chlorine group instead of methyl group at position 4 (Table I and II). However, the inhibitory effects were almost abolished by replacement of amino group at position 2 of JSH-21 with nitro group. Therefore, diamine substitution at positions 1 and 2 of JSH-21 would at least be required for its inhibitory potentials on both nitrite accumulation and NF-κB transcriptional activity in LPS-stimulated macrophages RAW 264.7.

Finally, NF-κB is known to play an important role in the expression of pro-inflammatory proteins, such as cytokines, adhesion molecule and COX-2 in addition to iNOS (Collins *et al.*, 1995; Tian and Brasier, 2003). The physiological functions of pro-inflammatory proteins, including iNOS, could provide benefit to living organisms. However, the aberrant or excessive production of pro-inflammatory proteins has been implicated in inflammation-related disorders, such as arthritis, cancer and sepsis (Blantz and Munger, 2002; Cavaillon *et al.*, 2003; Moreland *et al.*, 2004). As an anti-inflammatory action of JSH-21, inhibitory effects of the compound on LPS-induced nitrite accumulation and NF-κB activation have been documented in this study.

ACKNOWLEGEMENTS

This work was financially supported by Chungbuk National University Grant in 2004. pNF-κB-SEAP-NPT and iNOS-luciferase reporter constructs were supplied from Dr. Y. S. Kim and Dr. C. J. Lowenstein, respectively.

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