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FEBS Letters 571 (2004) 50–54 FEBS 28606

Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-κB without affecting IκB degradation

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Received 6 May 2004; revised 16 June 2004; accepted 20 June 2004

Available online 2 July 2004

Edited by Robert Barouki

Abstract 4-Methyl-N^1-(3-phenyl-propyl)-benzene-1,2-diamine (JSH-23) is a novel chemically synthetic compound. The aromatic diamine JSH-23 compound exhibited inhibitory effect with an IC_{50} value of 7.1 μM on nuclear factor (NF)-κB transcriptional activity in lipopolysaccharide (LPS)-stimulated macrophages RAW 264.7, and interfered LPS-induced nuclear translocation of NF-κB without affecting IκB degradation. This mechanism of action is very rare for controlling NF-κB activation. Furthermore, the compound inhibited not only LPS-induced expressions of tumor necrosis factor-α, interleukin (IL)-1β, IL-6 and inducible nitric oxide synthase and cyclooxygenase-2 but also LPS-induced apoptosis of the RAW 264.7 cells.
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Keywords: Aromatic diamine compound; NF-κB; IκB; Pro-inflammatory cytokine; LPS-inducible enzyme

1. Introduction

Nuclear factor (NF)-κB is a transcription factor with heterodimer or homodimer form of rel family proteins such as RelA (p65), RelB, cRel, p50 and p52 [1]. NF-κB is sequestered in the cytoplasm, bound to inhibitory κB (IκB) proteins such as IκBα, IκBβ, IκBε, p105 and p100 [2]. Lipopolysaccharide (LPS), a major component of the outer membranes of Gram-negative bacteria, can trigger a variety of inflammatory reactions by binding to Toll-like receptor 4 [3,4]. Downstream signaling components of the receptor include MyD88, interleukin (IL)-1 receptor-associated kinase, and tumor necrosis factor (TNF) receptor-associated factor 6, which activates IκB kinase (IKK) complex [5,6]. Activation of IKK complex results in phosphorylation of IκB, which marks for ubiquitination followed by proteasome-mediated degradation [7]. IκB degradation unmasks the nuclear localization signal (NLS) of NF-κB, allowing the transcription factor to translocate to the nucleus, and then NF-κB binds to the promoter region of immune and inflammatory genes for transcriptional regulation [8,9].

In the present study, novel aromatic diamine 4-Methyl-N^1-(3-phenyl-propyl)-benzene-1,2-diamine (JSH-23) compound (Fig. 1) was discovered to inhibit NF-κB transcriptional activity in LPS-stimulated macrophages RAW 264.7. The compound inhibited nuclear translocation of NF-κB p65 without affecting IκBα degradation, which is a very rare mode of action. Furthermore, JSH-23 compound caused down-regulation of LPS-inducible cytokines and enzymes, and inhibited LPS-induced apoptosis of the RAW 264.7 cells.

2. Materials and methods

2.1. Materials

LPS (*E. coli* 055:B5) was purchased from Sigma–Aldrich and fetal bovine serum (FBS) from Invitrogen. Antibodies against NF-κB p65 or IκBα were obtained from Santa Cruz Biotech. Chemical preparation of JSH-23 compound (purity, ⩾98%) was described elsewhere [10].

2.2. Cell culture

Macrophages RAW 264.7 were cultured in DMEM (10 mg/ml Dulbecco's modified Eagle's medium, 10 mM HEPES, 24 mM $NaHCO_3$, 143 U/ml benzylpenicillin potassium, and 100 μg/ml streptomycin sulfate, pH 7.1) containing 10% FBS at 37 °C with 5% CO_2. The RAW 264.7 cells harboring pNF-κB-secretary alkaline phosphatase (SEAP)-NPT reporter construct [11] were grown in the same conditions except supplement of 0.5 mg/ml geneticin to the media.

2.3. Measurement of NF-κB transcriptional activity

Macrophages RAW 264.7 transfected stably with reporter plasmid of pNF-κB-SEAP-NPT were treated with 1 μg/ml LPS and/or sample for 16 h. As the reporter, SEAP activity in the cell-free culture media was measured as described in our previous work [11].

2.4. Electrophoretic mobility shift assay of NF-κB

Macrophages RAW 264.7 were treated with 1 μg/ml LPS plus sample for 1 h. The ^{32}P-labeled oligonucleotide containing κB sequence (Promega) was reacted with nuclear extracts (10 μg) of the RAW 264.7 cells and then resolved on non-denaturing 6% polyacrylamide gel by electrophoresis. The gel was dried and exposed to X-ray film.

2.5. Western immunoblot of NF-κB or IκB

Macrophages RAW 264.7 were treated with 1 μg/ml LPS and/or sample for 1 h (NF-κB) or for 10 min-8 h (IκB). Western immunoblot for NF-κB p65 was carried out with nuclear extracts and that of IκBα with cytoplasmic extracts. Western immunoblot conditions were described in our previous work [12]. The blots were finally reacted with

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doi:10.1016/j.febslet.2004.06.056

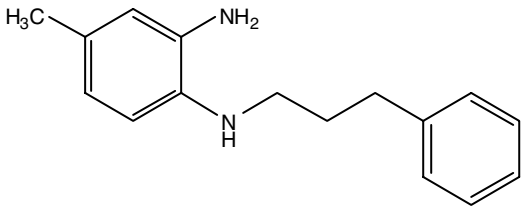

Fig. 1. Chemical structure of aromatic diamine JSH-23 compound.

ECL detection reagents (Amersham-Pharmacia) and exposed to X-ray film.

2.6. Semi-quantitative RT-PCR

Macrophages RAW 264.7 were treated with 1 μg/ml LPS and/or sample for 6 h. Total RNA was purified from the cells using a Fast-RNA Kit (Bioneer) and then subjected to semi-quantitative RT-PCR using an RNA PCR Kit (Bioneer). Primer sequences and RT-PCR conditions were described in our previous work [12]. RT-PCR products were resolved on 1.5% agarose gel by electrophoresis and then stained with ethidium bromide.

2.7. Measurement of apoptosis

Apoptosis was analyzed by 4′,6-diamidino-2-phenylindole (DAPI) staining [13]. Macrophages RAW 264.7 were treated with 1 μg/ml LPS and/or sample for 24 h. The cells were stained with 1 μg/ml DAPI for 30 min at 37 °C and then analyzed using fluorescence microscopy with excitation at 300–500 nm. Cells with nuclei containing clearly condensed chromatin or cells with fragmented nuclei were scored as an apoptosis index.

2.8. Cytotoxicity measurement

Macrophages RAW 264.7 were incubated with various concentrations of JSH-23 compound for 24 h. The cells were treated with WST-1 solution as described in our previous work [14] and absorbance was measured at 450 nm.

2.9. Statistical analysis

Resulting values were otherwise expressed as means ± S.E.M. Data were analyzed by ANOVA followed by the Student's *t* test. $P < 0.01$ was considered significant.

3. Results

3.1. Inhibitory effect on NF-κB transcriptional activity

NF-κB transcriptional activity was monitored using macrophages RAW 264.7 transfected stably with a reporter plasmid containing four copies of the κB sequence and SEAP as the reporter. Treatment of LPS to the transfected RAW 264.7 cells increased SEAP expression to about 3-fold, compared with the resting cells, indicating that cellular NF-κB is transcriptionally functional (Fig. 2). JSH-23 compound inhibited LPS-induced SEAP expression in a dose-dependent manner, corresponding to 23 ± 3% inhibition at 3 μM, 68 ± 3% at 10 μM and 103 ± 4% at 30 μM (Fig. 2). Parthenolide, an inhibitor of IKK complex on NF-κB activation [15], also exhibited an IC_{50} value of 3.5 μM on LPS-induced SEAP expression. However, JSH-23 alone did not cause the SEAP expression (Fig. 2). The inhibitory effect of JSH-23 compound on LPS-induced reporter expression was not attributable to its cytotoxicity, because the compound at <100 μM did not show significant cytotoxic effects on the RAW 264.7 cells (Fig. 3).

3.2. Inhibitory effect on DNA binding activity of NF-κB

To elucidate the mechanism, we next determined whether JSH-23 compound could inhibit DNA binding activity of NF-κB in LPS-stimulated macrophages RAW 264.7. Treatment of

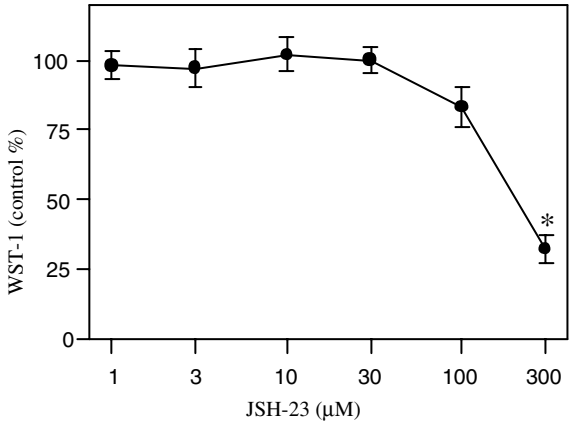

Fig. 3. Cytotoxic effect of JSH-23 compound. Macrophages RAW 264.7 were treated with various concentrations of JSH-23 compound for 24 h. Proliferation of the cells was analyzed with WST-1 method, and is represented as control %, compared with that of media alone-treated group. Data are means ± S.E.M. ($n = 5$). $^{*}P < 0.01$ vs. media alone-treated group.

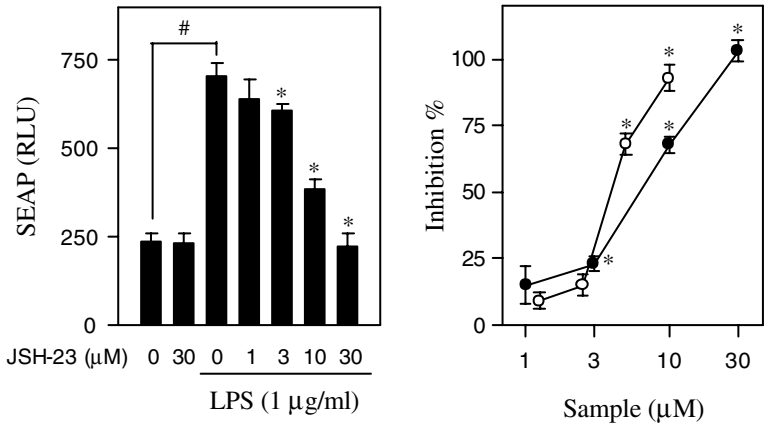

Fig. 2. NF-κB mediated reporter gene expression. Macrophages RAW 264.7 harboring reporter construct pNF-κB-SEAP-NPT were treated with LPS and/or JSH-23 compound for 16 h. As the reporter, SEAP expression is shown as relative fluorescence units for NF-κB transcriptional activity (left panel). Effects of JSH-23 compound (solid circle) or parthenolide (open circle) on LPS-induced NF-κB transcriptional activity are shown as inhibition % (right panel). Data are means ± S.E.M. ($n = 3$). $^{\#}P < 0.01$ vs. media alone-treated group. $^{*}P < 0.01$ vs. LPS alone-treated group.

LPS to the cells induced a marked increase in DNA binding activity of NF-κB complex within 1 h (Fig. 4). Under the same conditions, JSH-23 compound decreased LPS-induced DNA binding activity of NF-κB in a dose-dependent manner (Fig. 4).

3.3. Inhibitory effect on nuclear translocation of NF-κB

To further investigate whether JSH-23 compound could affect nuclear translocation of NF-κB, Western immunoblot for NF-κB p65 was carried out with nuclear extracts of LPS-stimulated RAW264.7 cells. Nuclear amount of NF-κB p65 was markedly increased upon exposure to LPS for 1 h (Fig. 5). Treatment of JSH-23 compound to LPS-stimulated RAW 264.7 cells decreased nuclear content of NF-κB p65 in a dose-dependent manner, corresponding to $49 \pm 4\%$ inhibition at 3 μM, $75 \pm 7\%$ at 10 μM and $95 \pm 8\%$ at 30 μM (Fig. 5).

3.4. Effect on IκB degradation

Another immunoblot was carried out with cytoplasmic extracts of LPS-stimulated macrophages RAW 264.7 to know whether IκB degradation was influenced by JSH-23 compound. IκBα degradation was dramatically happened within 10 min upon exposure to LPS and JSH-23 compound (30 μM) did not inhibit LPS-induced IκBα degradation (Fig. 6). Cytoplasmic content of IκBα was recovered to basal level at 40–50 min after LPS-induced IκBα degradation, which was not inhibited or delayed until 8 h by treatment of JSH-23 compound (Fig. 6).

3.5. Inhibitory effect on expressions of pro-inflammatory cytokines and enzymes

JSH-23 compound inhibited LPS-induced NF-κB activation, and thus effect of the compound on expressions of LPS-inducible cytokines and enzymes was analyzed by semi-quantitative RT-PCR. As shown in Fig. 7, expressions of IL-1β, IL-6, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 transcripts, except that of TNF-α transcript, were hardly detectable in the resting RAW 264.7 cells. However, the cells expressed pronounced amounts of the pro-inflammatory transcripts when stimulated with LPS for 6 h. JSH-23 compound showed differential inhibitory effects on LPS-induced expressions of the pro-inflammatory transcripts; IL-6 and iNOS at $\geqslant 3$ μM, IL-1β and COX-2 at $\geqslant 10$ μM and TNF-α at $\geqslant 30$ μM (Fig. 7).

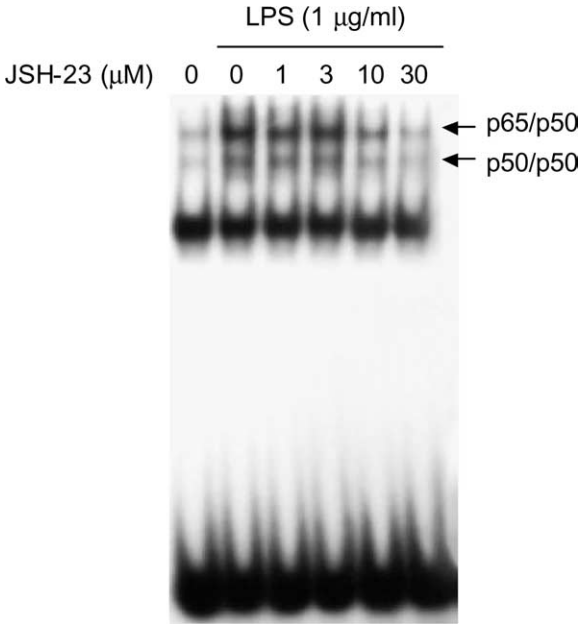

Fig. 4. EMSA of NF-κB complex. Macrophages RAW 264.7 were treated with LPS and/or JSH-23 compound for 1 h, and nuclear extracts were subjected to DNA binding activity with ^{32}P-labeled oligonucleotide specific to NF-κB. Specific DNA binding of NF-κB complex is indicated by an arrow.

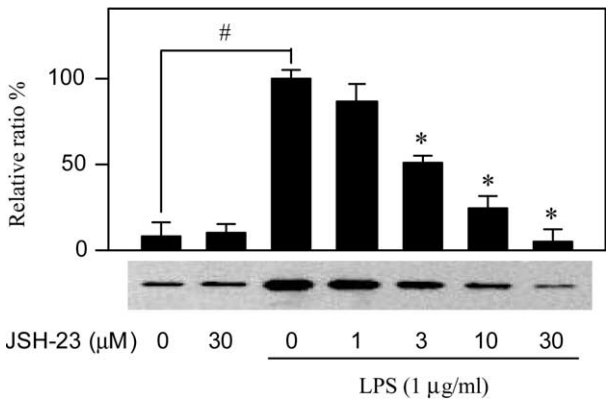

Fig. 5. Western immunoblot of NF-κB p65. Macrophages RAW 264.7 were treated with LPS and/or JSH-23 compound for 1 h, and nuclear extracts were subjected to Western immunoblot with anti-NF-κB p65 antibody. One of the similar immunoblot results is represented and relative ratio % is also shown, compared with LPS alone-treated group ($100 \pm 5\%$). Data are means ± S.E.M. ($n = 3$). $^{\#}P < 0.01$ vs. media alone-treated group. $^{*}P < 0.01$ vs. LPS alone-treated group.

3.6. Inhibitory effect on apoptosis

Apoptosis was evaluated by DAPI staining. Apoptotic nuclei with condensed chromatin were detectable in macrophages RAW 264.7 by stimulation of LPS for 24 h but not by treatment of JSH-23 alone (Fig. 8). JSH-23 compound inhibited LPS-induced chromatin condensation in a dose-dependent

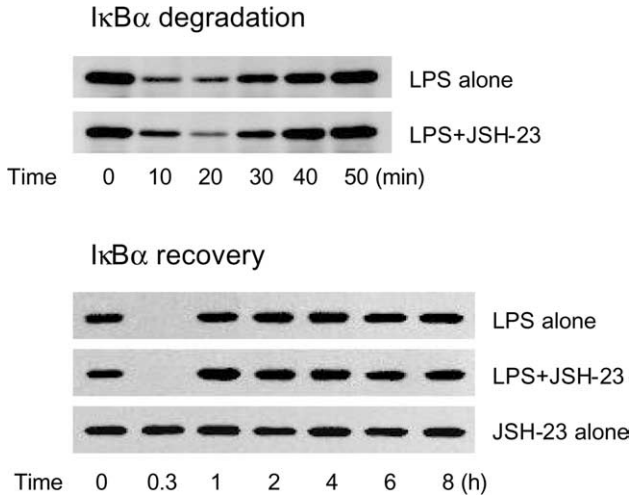

Fig. 6. Western immunoblot of IκBα. Macrophages RAW 264.7 were treated with LPS and/or JSH-23 compound (30 μM) for the indicated times, and cytoplasmic extracts were subjected to Western immunoblot with anti-IκBα antibody.

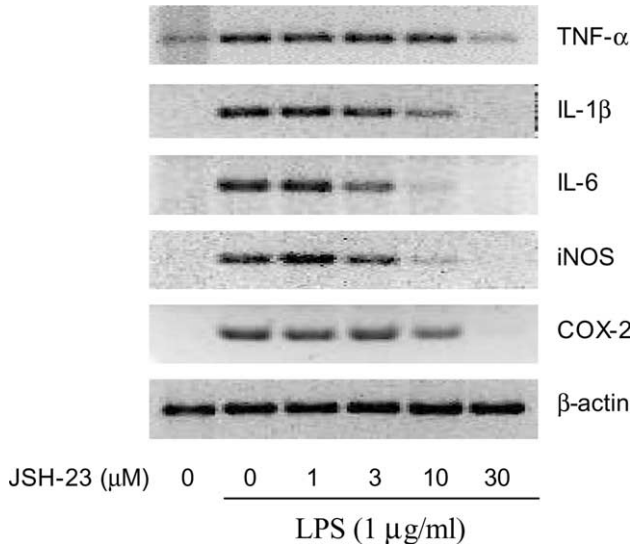

Fig. 7. RT-PCR of pro-inflammatoty cytokines (TNF-α, IL-1β and IL-6) and enzymes (iNOS and COX-2). Macrophages RAW 264.7 were treated with LPS and/or JSH-23 compound for 6 h, and total RNA was subjected to semi-quantitative RT-PCR using β-actin as the internal control.

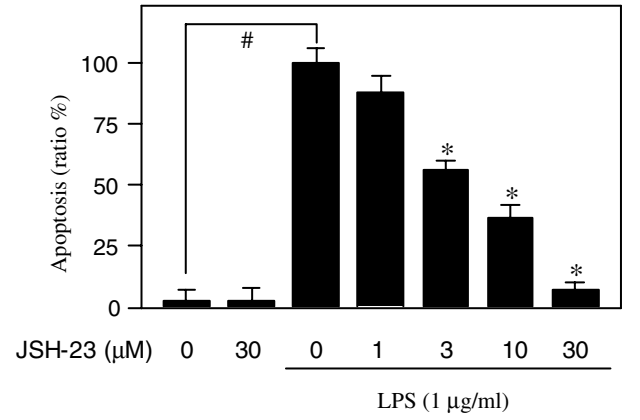

Fig. 8. Effect on LPS-induced apoptosis. Macrophages RAW 264.7 were treated with LPS and/or JSH-23 compound for 24 h, and stained with DAPI. Apoptotic score is represented as relative ratio %, compared with LPS alone-treated group. Data are means ± S.E.M. ($n = 4$). $^{\#}P < 0.01$ vs. media alone-treated group. $^{*}P < 0.01$ vs. LPS alone-treated group.

manner, corresponding to 44 ± 4% inhibition at 3 μM, 63 ± 5% at 10 μM and 93 ± 3% at 30 μM (Fig. 8).

4. Discussion

In the present study, novel aromatic diamine JSH-23 compound was discovered to have inhibitory effect on NF-κB transcriptional activity in LPS-stimulated macrophages RAW 264.7 (Fig. 2). To clarify the mechanism by which JSH-23 compound inhibited NF-κB activation, electrophoretic mobility shift assay (EMSA) and Western immunoblot were carried out. JSH-23 compound showed inhibitory effects, in parallel, on LPS-induced DNA binding activity and nuclear translocation of NF-κB p65 (Figs. 4 and 5). However, JSH-23 compound did not influence LPS-induced IκBα degradation and did not inhibit or delay cytoplasmic IκBα recovery until 8 h after LPS-induced IκBα degradation (Fig. 6). These results indicate that JSH-23 compound could inhibit LPS-induced nuclear translocation of NF-κB p65 without affecting IκBα degradation. In most mammalian cells, IκBα is rapidly degraded (<10 min) following phosphorylation but is quickly resynthesized (50–60 min) in an NF-κB-dependent manner [16]. Newly synthesized IκBα binds with nuclear NF-κB for an autoregulatory pathway to ensure the proper balance of NF-κB and IκBα in the cell [2]. Three NF-κB binding sites are reported in the IκBα promoter, where one site binds only NF-κB p50 complexed with itself, p65 or cRel and the other sites bind NF-κB complexes predominantly comprised of p65 and cRel homodimers or heterodimers [17]. We demonstrated that LPS-induced nuclear translocation of NF-κB p65 was dose-dependently inhibited by JSH-23 compound (Fig. 5). However, NF-κB complexes comprised p50, cRel or others which can induce IκBα transcription by themselves. Thus, JSH-23 compound presumably has differential effects on nuclear translocation of other members of NF-κB family.

Synthetic peptide SN50, containing a hydrophobic membrane-translocating region and the NLS of NF-κB p50, was reported to inhibit nuclear translocation of NF-κB in response to LPS and TNF-α [18]. In LPS-stimulated cells, various enzymes are activated, leading to the phosphorylation and subsequent degradation of IκB, freeing NF-κB to be recognized by karyopherin α [19]. Upon binding of karyopherin α to NF-κB, they interact with karyopherin β and small GTP binding protein, Ran [20]. These complexes bind to the nuclear pore and then NF-κB is transported to the nucleus. Target of synthetic peptide SN50 is the NLS on NF-κB p50 that is recognized by karyopherin α [18]. Even though molecular target of JSH-23 compound would be elucidated, this study is the first observation to show that a non-peptide compound specifically interfered LPS-induced nuclear translocation of NF-κB without affecting IκB degradation as shown in synthetic peptide SN50.

Importantly, JSH-23 compound suppressed LPS-induced expressions of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and enzymes (iNOS and COX-2) (Fig. 7). Physiological actions of the cytokines, iNOS and COX-2 may provide a benefit to the organism. However, aberrant or excessive expressions of the proteins have been implicated in inflammation-related disorders including arthritis, cancer, as well as septic shock [21]. Furthermore, JSH-23 compound inhibited LPS-induced apoptotic chromatin condensation (Fig. 8). However, JSH-23 alone did not induce apoptotic death of macrophages RAW 264.7.

Acknowledgements: This work was financially supported by grant (02-PJ2-PG3-21605-0004) from Korea Ministry of Health and Welfare and grant RRC (RCBH) from KOSEF.

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