KE-298 and Its Active Metabolite KE-758 Suppress Nitric Oxide Production by Murine Macrophage Cells and Peritoneal Cells from Rats with Adjuvant Induced Arthritis

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ABSTRACT. ***Objective***. To analyze the effects of KE-298 and KE-758 on lipopolysaccharide (LPS) induced nitric oxide (NO) production by the RAW264.7 murine macrophage cell line, and the effect of KE-758 on spontaneous NO production by peritoneal cells from rats with adjuvant induced arthritis.

Methods. The amount of NO was determined using Griess reagents. The proteins for inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) were detected by Western blot, then mRNA for interferon-ß (IFN)-ß, IFN regulatory factor-1 (IRF-1), and iNOS were detected by RT-PCR. Degradation of iNOS mRNA was analyzed using Northern blot. Nuclear factor-κB (NF-κB) in nuclear extracts was determined by EMSA. Adjuvant arthritis in rats was induced by inoculating heat killed *Mycobacterium butyricum* SC in the tail.

Results. KE-298 and KE-758 suppressed NO production by LPS activated RAW264.7 cells by inhibiting iNOS gene expression. Neither LPS induced NF-κB activation nor degradation of iNOS mRNA was affected by KE-758 treatment. LPS induced IFN-ß and IRF-1 gene expression were markedly suppressed by KE-758. In rats with adjuvant induced arthritis, enhanced NO and iNOS production by cultured peritoneal cells and the development of arthritis were suppressed by KE-758.

Conclusion. KE-758 suppressed LPS induced iNOS gene expression by murine macrophage cells by inhibiting IFN-ß/IRF-1 expression. The potential of KE-758 to inhibit iNOS production might partly explain its efficacy on adjuvant induced arthritis in rats. (J Rheumatol 2001;28:1229–37)

Key Indexing Terms:
DISEASE MODIFYING ANTIRHEUMATIC DRUGS
INDUCIBLE NITRIC OXIDE SYNTHASE
INTERFERON REGULATORY FACTOR–1
RAW264.7 CELLS
INTERFERON-ß
ADJUVANT ARTHRITIS

KE-298, which was originally developed as a new disease modifying antirheumatic drug (DMARD), showed beneficial effects in patients with rheumatoid arthritis (RA) in a clinical study[1]. KE-298 inhibited inflammatory cytokine production by human peripheral blood mononuclear cells[2], interleukin 6, and matrix metalloproteinase (MMP) production by RA synovial fibroblast-like cells[3,4], and progression of the disease in various animal models of arthritis[5-7]. Upon oral administration, KE-298 is rapidly absorbed and transformed into its active metabolite KE-758, which circulates in blood.

NO is a multifunctional molecule generated by nitric oxide synthase (NOS), and isoforms of NOS have been characterized[8]. Constitutively expressed NOS (cNOS), which is mainly found in neurons and endothelial cells, transiently produces small amounts of NO, which appears to have beneficial effects in many physiological processes[8]. In contrast, the inducible isoform of NOS (iNOS) by cytokines and/or endotoxin is found in many cells including endothelial cells, macrophages, neutrophils, chondrocytes, and synoviocytes, and generates much larger amounts of NO over longer periods of time, and contributes to the cytotoxic and cytostatic effects of cytokines and/or endotoxin.

Recently, excessive production of NO generated by iNOS has been described in RA[9,10]. NO also seems to play an important role in the pathogenesis of arthritis in animal models including adjuvant induced arthritis in rats[11,12], and treatment with an inhibitor of NOS such as N^G-monomethyl-L-arginine (L-NMMA) or N-methylester-L-arginine (L-NAME) has been shown to significantly suppress the progression of arthritis in laboratory animals[13,14].

We examined the effects of KE-298 and KE-758 on lipopolysaccharide (LPS) induced NO and iNOS production by the RAW264.7 murine macrophage cell line. We also

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investigated the effect of KE-758 on NO and iNOS production by peritoneal cells in rats with adjuvant induced arthritis *ex vivo*.

MATERIALS AND METHODS

Reagents. The chemical structures of KE-298 [2-acetylthiomethyl-4-(4-methylphenyl)-4-oxobutanoic acid] and KE-758 [2-mercaptomethyl-4-(4-methylphenyl)-4-oxobutanoic acid], the main metabolite of KE-298 in serum, are shown in Figure 1. KE-298 and KE-758 were synthesized in our laboratory[15]. For *in vitro* experiments, KE-298 was dissolved in ethanol and diluted with culture medium or distilled water (DW), and KE-758 was dissolved in culture medium or DW. For the *ex vivo* experiment, KE-758 was dissolved in saline.

RAW264.7 cell culture. RAW264.7 cells, an Abelson leukemia virus transformed murine macrophage cell line, were obtained from the American Type Culture Collection (Rockville, MD, USA).

For NO production, RAW264.7 cells [2×10^5/0.2 ml of RPMI-1640 (Gibco BRL, Rockville, MD, USA) supplemented by 10% heat inactivated fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 μg/ml)] were stimulated with 100 ng/ml of *Escherichia coli* 026:B6 lipopolysaccharide (Difco, Detroit, MI, USA) in the presence of KE-298 (0, 10, 30, 100, 200, 300 μg/ml), KE-758 (0, 10, 30, 100, 200, 300 μg/ml), or L-NAME (0, 30, 300 μg/ml) in 96 well plates (Corning Costar, Acton, MA, USA), and incubated 24 h at 37°C in an atmosphere of 5% CO_2 in air. After incubation, the supernatants were collected and assayed for nitrite (NO_2–) instead of NO as described below.

For iNOS and COX-2 production, RAW264.7 cells (2×10^6/2.0 ml RPMI-1640) were stimulated with LPS in the presence of KE-758 in a 60 mm dish (Corning Costar), and incubated 24 h at 37°C in an atmosphere of 5% CO_2 in air. After incubation, the cells were lysed in buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer) and stored at –20°C until assayed for iNOS protein.

For interferon-ß (IFN-ß), IFN regulatory factor-1 (IRF-1), and iNOS mRNA expression, RAW264.7 cells (2×10^6/2.0 ml RPMI-1640) were stimulated with LPS in the presence of KE-758 in a 60 mm dish, and incubated for 2, 4, and 16 h, respectively, at 37°C in an atmosphere of 5% CO_2 in air. After incubation, total RNA was isolated using ISOGEN reagent (Nippon Gene, Tokyo, Japan).

Cellular viability. Cellular viability was assayed by Cell Counting Kit-F (Dojindo, Kumamoto, Japan) in a 96 well plate. Briefly, cultured RAW264.7 cells were washed twice and 100 μl of PBS and 10 μl of ×50 diluted Calcein-AM solution was added to each well, followed by incubation for 30 min, and the fluorescent intensity (λex = 490 nm, λem = 515 nm) was read.

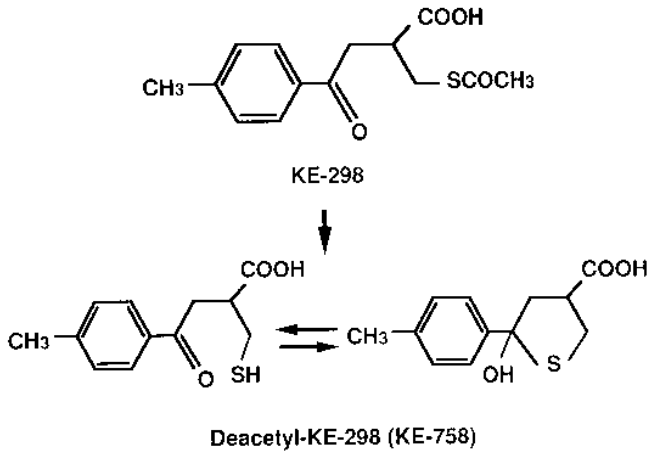

Figure 1. Chemical structures of KE-298 and its metabolite KE-758. The molecular weights of KE-298 and KE-758 are 280.34 kDa and 255.34 kDa, respectively. Upon oral administration, KE-298 is rapidly absorbed, and immediately transformed into KE-758 by deacetylation, which circulates in blood.

Animal experiments. Eight-week-old female Sprague-Dawley (SD) rats (Charles River, Shizuoka, Japan) were used for the experiments. The rats were allocated to 4 groups as follows: Group 1, the rats were given no treatment during the experiments (normal group, n = 12); Group 2, these rats served as control of adjuvant induced arthritis and were daily administered saline intravenously (iv, control group, n = 12); Groups 3 and 4, the rats were daily given KE-758 iv, 10 mg/kg and 30 mg/kg, respectively (each test group: n = 12).

The rats were inoculated subcutaneously in the tail with 0.6 mg of heat killed *Mycobacterium butyricum* (Difco) suspended in 0.1 ml of liquid paraffin. Edema in the hind paw was measured using a plethysmograph, TK-112 (Unicom, Yachiyo, Japan). The edema volumes of both hind paws were obtained from differences in the volume of both footpads on Day 0 and on Day 18.

Further, peritoneal cells were collected on Day 21 by washing the peritoneal cavity of rats with 10 ml of saline containing 0.1% heparin (Sigma, St. Louis, MO, USA) and 10% heat inactivated FBS. The cells were washed 3 times with RPMI-1640 and suspended in culture medium (RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin). Peritoneal cells (2×10^6/1.0 ml/well RPMI-1640) were seeded in 24 well plates (Corning Costar), and incubated 24 h at 37°C in an atmosphere of 5% CO_2 in air. After incubation, the supernatants obtained by centrifugation at 1500 rpm for 5 min were assayed for nitrite, and the cells were lysed in lysis buffer and stored at –20°C until assayed for iNOS protein as described below.

Nitrite determination. The amount of nitrite in cell-free culture supernatants was measured using Griess reagent[16]. Briefly, 0.1 ml of supernatant was mixed with 0.1 ml of Griess reagent [0.1 % N-1-naphtylethylenediamine 2-sulfate (Wako Pure Chemical Industries, Osaka, Japan) and 1% sulfanylamide (Wako) in 0.1% sulfuric acid] and after 30 min incubation, the absorbance at 550 nm was read. Then nitrite concentration was calculated using a $NaNO_2$ standard curve.

Assays for NOS enzyme activity. Enzyme activity of NOS was determined using an assay kit for NOS activity (Cerep, Celle L'Evescault, France). Briefly, the lysate from RAW264.7 cells (a protein concentration of 37.5 μg/200 μl) was incubated for 3 h at 37°C with 100 mM of L-arginine in the presence of KE-298, KE-758, or L-NAME, and the conversion of L-arginine to nitrite was monitored. The nitrite generated in the reaction mixture was assayed using Griess reagent. Although this assay detects total NOS, the lysate from RAW264.7 cells mainly contains iNOS and this assay is regarded to detect iNOS.

Western blot analysis. RAW264.7 cells or peritoneal cells were lysed in lysis buffer. Then 10 μg of total protein was separated on 7.5% SDS-PAGE gel using Laemmli's buffer system and transferred to a nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK). Nonspecific binding was blocked with Block Ace (Yukijirushi Industries, Sapporo, Japan) and incubated sequentially with a specific anti-iNOS (1:250) or anti-cyclooxygenase-2 (COX-2) (1:250) murine monoclonal antibody (Mab; Transduction Laboratories, Lexington, KY, USA) and then with horseradish peroxidase conjugated goat anti-mouse Ig antibody (1:1000) (PharMingen, San Diego, CA, USA). Protein bands were visualized with the ECL system (Amersham). The amounts of iNOS and COX-2 were evaluated by densitometer (Quantity One, version 3.0) (Toyobo, Osaka, Japan).

Reverse transcription-coupled PCR (RT-PCR) analysis. The primer sequences for iNOS and ß-actin were purchased from Toyobo, and those for IFN-ß and IRF-1 were synthesized by Sawady Technology (Tokyo, Japan). The sense and antisense oligonucleotides for iNOS, IFN-ß, IRF-1, and ß-actin are listed in Table 1. RT-PCR was performed as described[17,18]. The cycle conditions for amplification of cDNA were 1 min at 94°C, 1 min at 65°C (iNOS) or 55°C (IFN-ß, IRF-1, ß-actin), and 1 min at 72°C for 30

Table 1. Primer sequences.

Factor	Primer	Sequence
iNOS (5′→3′)	Sense primer	CCCTTCCGAAGTTTCTGGCAGCAGC
	Antisense primer	GGCTGTCAGAGCCTCGTG GCTTTGG
IFN-ß (5′→3′)	Sense primer	CTCCAGCTCCAAGAAAGGACG
	Antisense primer	GAAGTTTCTGGTAAGTCTTCG
IRF-1 (5′→3′)	Sense primer	CAGAGGAAAGAGAGAAAGTCC
	Antisense primer	CACACGGTGACAGTGCTGG
ß-actin (5′→3′)	Sense primer	ATCTGGCACCACACCTTCTQACAATGAGCTGCG
	Antisense primer	CGTCATACTCC TGCTTGCTGATCCACATCTGC

cycles (iNOS, IRF-1, ß-actin) or 35 cycles (IFN-ß). The amounts of iNOS, IFN-ß, and IRF-1 mRNA were evaluated by Quantity One, version 3.0, and the results were normalized by the amount of ß-actin mRNA.

Northern analysis. Total RNA was isolated using Isogen (Wako). Northern blot analysis was as described[19]. Briefly, 20 μg of RNA was subjected to electrophoresis in 1% agarose/formaldehyde gel. The gel was then transferred via capillary action onto a nylon membrane (Hybond N$^+$; Amersham). The membrane was hybridized with [α-^{32}P]-dCTP labeled mouse iNOS cDNA probe (Cayman Chemical, Ann Arbor, MI, USA). After hybridization the blot was washed thoroughly and exposed to molecular image screen (Bio-Rad, Hercules, CA, USA) for 5.0 h and RNA bands were visualized with the molecular imager GS-525 (Bio-Rad). The amount of iNOS mRNA was quantitated by multi-analyst software (Bio-Rad), and the results were normalized by reprobing the same blots with a cDNA coding for mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Electrophoretic mobility shift analysis (EMSA). Confluent RAW264.7 cells were preincubated with or without KE-758 for 30 min, and stimulated with 10 ng/ml of LPS for 30 min. After incubation, cells were collected, lysed and nuclear proteins were isolated[20]. Aliquots of nuclear protein were incubated with the nuclear factor-κB (NF-κB) specific double stranded oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3'; Promega, Madison, WI, USA), which had been labeled with ^{32}P by random priming. Binding experiments were performed with 3–12 μl (10 μg) nuclear extract, 5 ml of 5× binding buffer [50 mM HEPES, pH 7.5, 500 mM NaCl, 25% glycerol (w/v), 5 mM EDTA], and the oligonucleotides (50,000 cpm) in a total volume of 25 ml for 30 min at room temperature. Nondenaturing polyacrylamide gel (4 %) electrophoresis was performed with 1× TBE buffer, pH 8.0 (Nippon Gene), followed by autoradiography. The amounts of NF-κB were evaluated by Quantity One.

Assay for degradation of iNOS mRNA. RAW264.7 cells (2×10^6 cells/2 ml/10 cm^2 dish) were stimulated with LPS (100 ng/ml) for 16 h, and then medium was washed out and actinomycin D (Sigma) (5 μg/ml) was added with or without KE-758 (200 μg/ml). Cells were harvested at the indicated times, total RNA was extracted, and Northern analysis of iNOS and GAPDH mRNA expression was done as described above.

Statistics. The statistical significance of the differences between normal and control and the control and treated groups was determined by Student's t test and Dunnett's test, respectively. A p value < 0.05 was considered statistically significant.

RESULTS

Effect of KE-298, KE-758, and L-NAME on NO and iNOS production by LPS stimulated RAW264.7 cells. LPS stimulated the production of NO by RAW264.7 cells in a dose dependent manner; this reached a maximum in the presence of 100 ng/ml of LPS (data not shown). KE-298 and KE-758 (10–300 μg/ml) as well as L-NAME (30–300 μg/ml) suppressed the production of NO by RAW264.7 cells in the presence of 100 ng/ml of LPS, in a dose dependent manner (Figure 2). The IC_{50} of KE-298, KE-758, and L-NAME were 117.5, 131.8, and 151.4 μg/ml, respectively. Nitrite instead of NO was measured because NO synthesized by RAW264.7 cells was rapidly oxidized in the medium to nitrite. KE-298, KE-758, and L-NAME did not affect cellular viability at these doses (data not shown).

As shown in Figure 3, KE-298 and KE-758, unlike L-NAME, had no direct effect on NOS activity in cell-free extracts of RAW264.7 cells. The IC_{50} of L-NAME was 72.4 μg/ml.

Further, apparent expressions of iNOS and COX-2 protein were observed when RAW264.7 cells were stimulated with 100 ng/ml of LPS for 24 h. And a dose dependent inhibition of LPS induced iNOS expression by KE-758 (10–200 μg/ml) was shown (Figure 4a), whereas COX-2 expression was not altered by KE-758 in the same sample

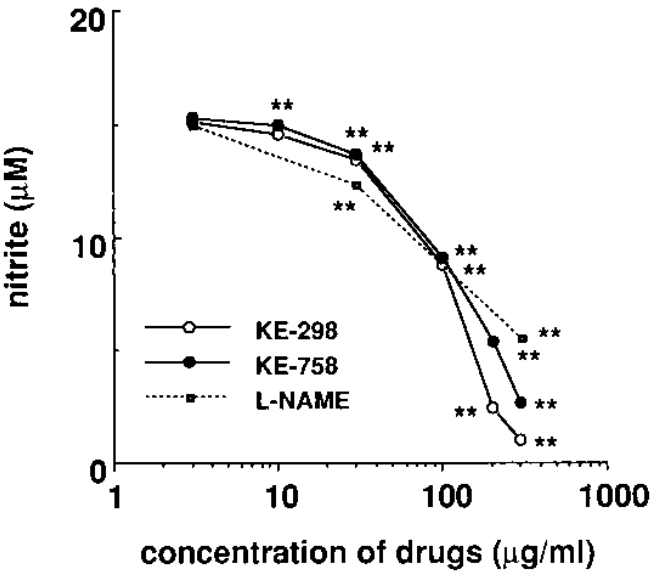

Figure 2. Effects of KE-298, KE-758, and L-NAME on LPS induced NO production by RAW264.7 cells. RAW264.7 cells (2×10^5/0.2 ml RPMI-1640) were stimulated with 100 ng/ml of LPS for 24 h in the presence or absence of drugs, as described in Materials and Methods. Nitrite in the culture supernatants was measured using Griess reagent. Each point represents the mean (n = 6). **p < 0.01 versus control (Dunnett's test).

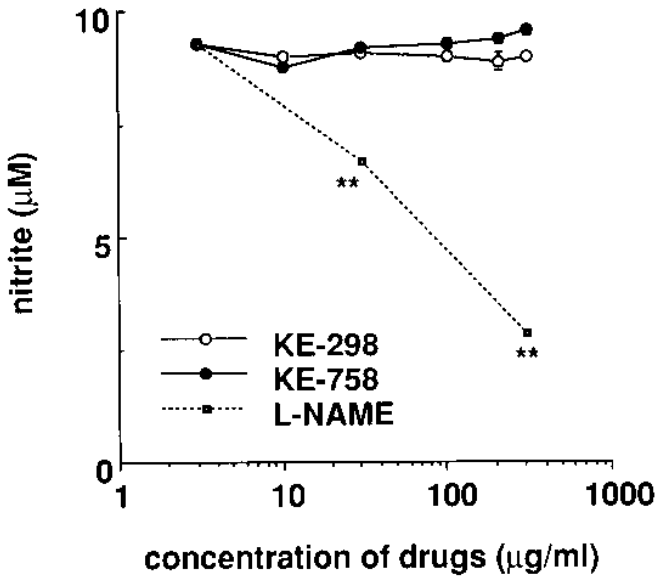

Figure 3. Direct effects of KE-298, KE-758, and L-NAME on NOS enzyme activity (by monitoring the conversion of L-arginine to L-citrulline + nitrite) in a cell-free assay system. Cell-free extracts including NOS derived from RAW264.7 cells (37.5 mg/0.2 ml) were incubated 3 h at 37°C with L-arginine (100 mM) in the presence or absence of drugs, and the generated nitrite was assayed using Griess reagent. Each point represents the mean (n = 6). **p < 0.01 versus control (Dunnett's test).

(Figure 4b). In contrast, KE-758 itself did not affect iNOS and COX-2 protein expression by nonstimulated RAW264.7 cells (data not shown).

Effect of KE-758 on iNOS mRNA expression, iNOS mRNA degradation, and NF-κB activation in LPS stimulated RAW264.7 cells. NOS mRNA was induced when RAW264.7 cells were stimulated by 100 ng/ml of LPS for 16 h, and a dose dependent inhibition of LPS induced iNOS mRNA expression by KE-758 (10–200 μg/ml) was shown by RT-PCR (Figure 5a) and Northern blot analysis (Figure 5b), whereas control ß-actin (RT-PCR) and GAPDH (Northern blot) mRNA expression were not affected.

Figure 6 shows the effect of KE-758 on LPS induced NF-κB activation of RAW264.7 cells. The expression of NF-κB was markedly increased in nuclear extracts from cells stimulated with 100 ng/ml of LPS for 30 min. But the intensity of NF-κB complex was hardly affected when RAW264.7 cells were treated with KE-758 (10, 30, 100, 200 μg/ml).

Figure 7 shows the effect of KE-758 on iNOS mRNA stability in RAW264.7 cells. LPS (100 ng/ml) markedly activated iNOS mRNA expression after 16 h incubation, and the time dependent degradation of iNOS mRNA was observed when RAW264.7 cells were washed once and treated with actinomycin D (AcD) for a subsequent 16 h. However, the degradation of iNOS mRNA was hardly affected by KE-758 (200 μg/ml) at any time points.

Effect of KE-758 on IFN-ß and IRF-1 mRNA expression, and subsequent IRF-1 activation in LPS stimulated RAW264.7 cells. IFN-ß and IRF-1 mRNA were markedly induced when RAW264.7 cells were stimulated by 100 ng/ml of LPS for 2 h (IFN-ß) and 4 h (IRF-1), respectively, as reported[18,21], and a dose dependent inhibition of IFN-ß mRNA (Figure 8a) and IRF-1 mRNA (Figure 8b) expression by KE-758 (10-200 μg/ml) was shown by RT-PCR.

Effect of iv administration of KE-758 on the edema of foot-

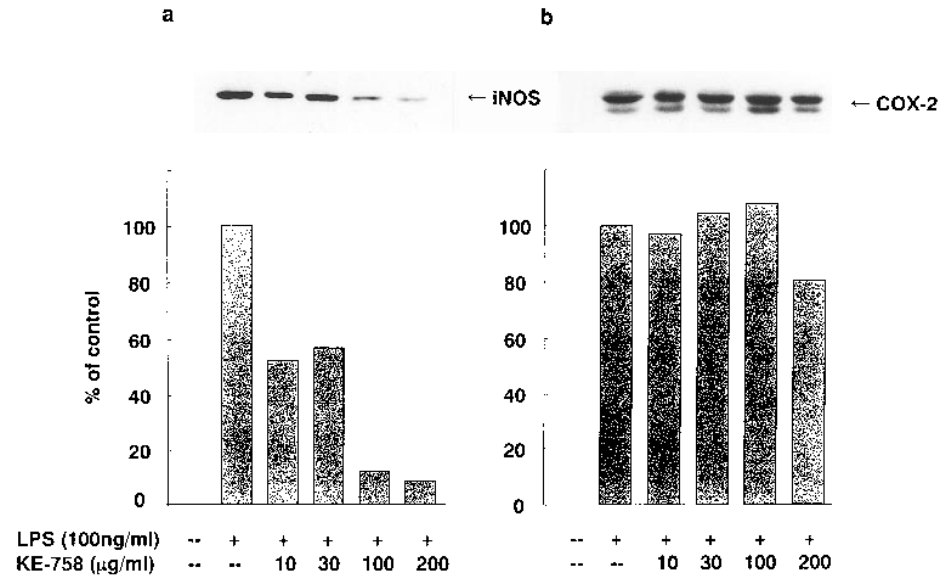

Figure 4. Effect of KE-758 on LPS induced iNOS and COX-2 protein expression in RAW264.7 cells. RAW264.7 cells (2×10^6/2.0 ml RPMI-1640) were stimulated with 100 ng/ml of LPS for 24 h in the presence or absence of drugs. The adherent cells were collected, and iNOS (a) and COX-2 (b) protein expression were examined by Western blot analysis as described in Materials and Methods.

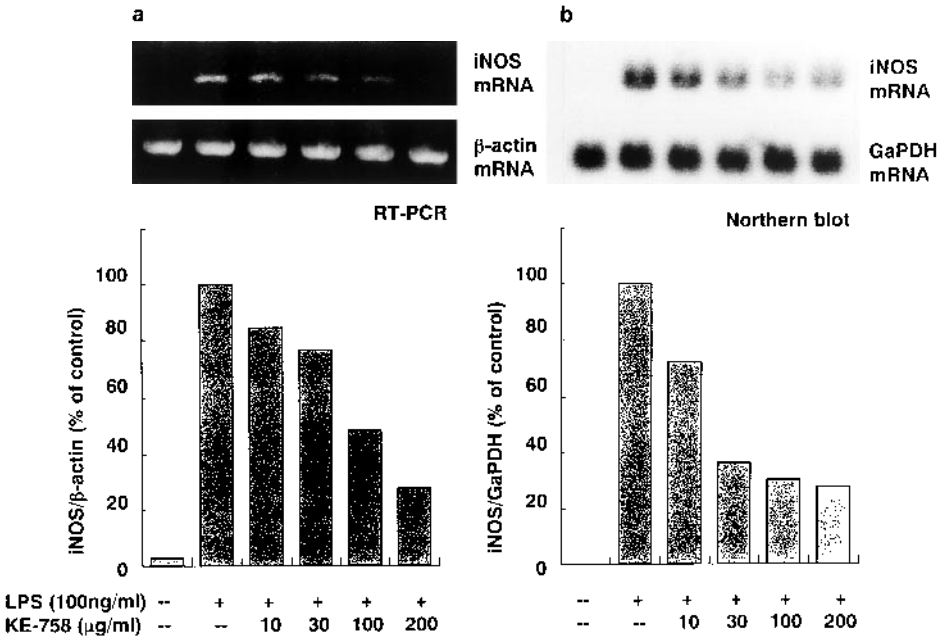

Figure 5. Effect of KE-758 on LPS induced iNOS mRNA expression in RAW264.7 cells. RAW264.7 cells (2×10^6/2.0 ml RPMI-1640) were stimulated with 100 ng/ml of LPS for 16 h in the presence or absence of KE-758, and iNOS mRNA expression was analyzed by RT-PCR (a) and Northern blot analysis (b) as described in Materials and Methods.

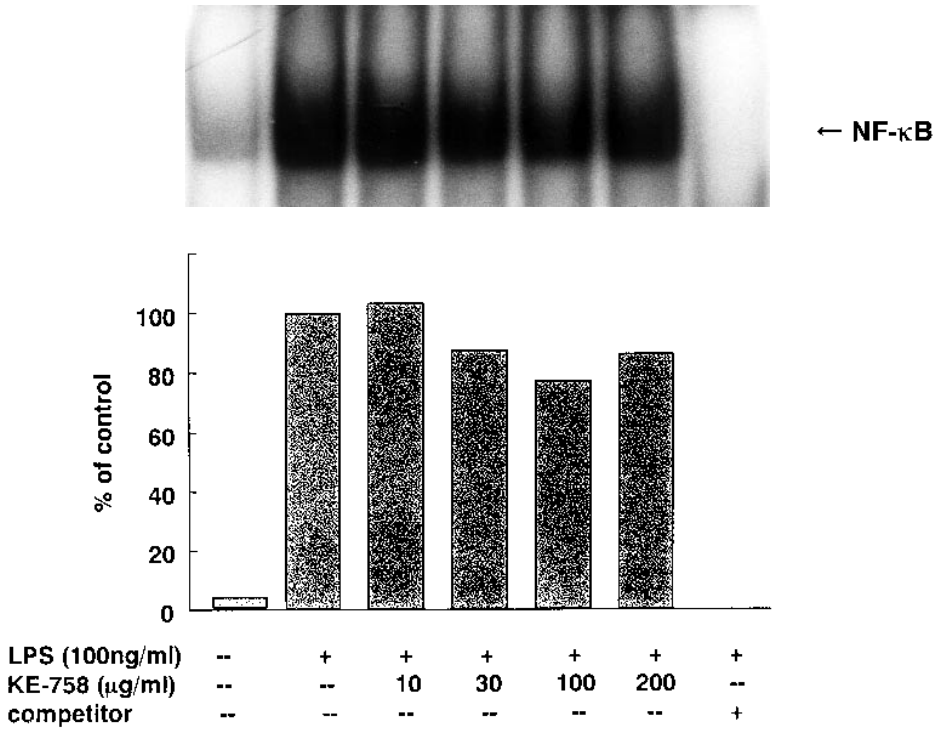

Figure 6. Effect of KE-758 on LPS induced NF-κB activation in RAW264.7 cells. Confluent RAW264.7 cells were preincubated with or without KE-758 for 30 min, and stimulated with 100 ng/ml LPS for 30 min. After incubation, cells were collected, lysed, and nuclear proteins were isolated. Activated NF-κB was detected by EMSA as described in Materials and Methods.

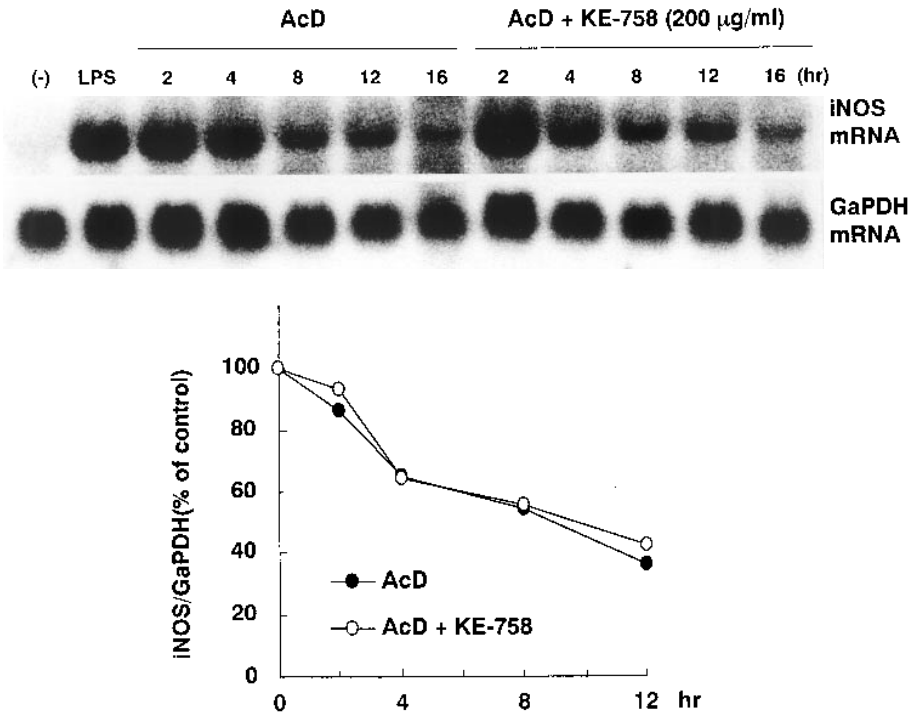

Figure 7. Effect of KE-758 on the stability of iNOS mRNA in RAW264.7 cells. RAW264.7 cells (2×10^6/2.0 ml/10 cm^2 dish) were stimulated with 100 ng/ml of LPS for 16 h, followed by actinomycin D (AcD) with or without 200 μg/ml of KE-758. Cells were harvested at the indicated times and Northern blot analysis of iNOS mRNA expression was done as described in Materials and Methods.

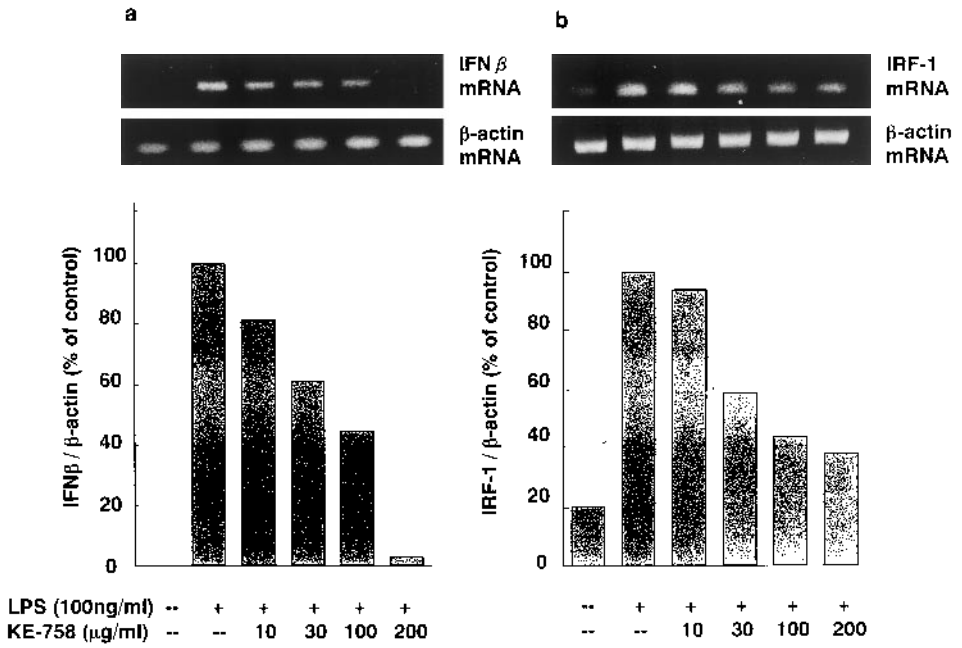

Figure 8. Effect of KE-758 on LPS induced IFN-ß and IRF-1 mRNA expression in RAW264.7 cells. RAW264.7 cells (2×10^6/2.0 ml RPMI-1640) were stimulated with 100 ng/ml of LPS for 2 h (IFN-ß) and 4 h (IRF-1) in the presence or absence of KE-758, and IFN-ß (a) and IRF-1 (b) mRNA expressions were analyzed by RT-PCR as described in Materials and Methods.

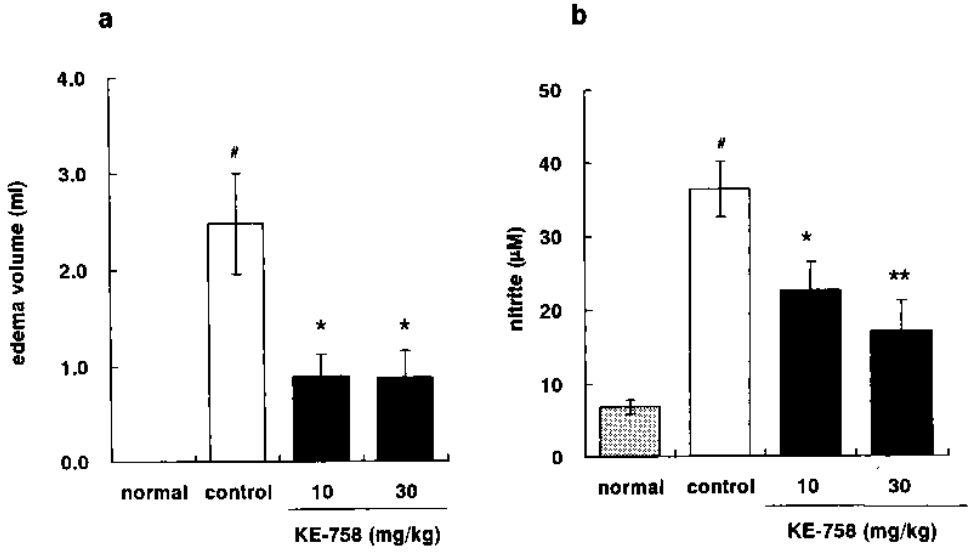

Figure 9. Effect of KE-758 on edema volume of footpads and NO production by peritoneal cells in rats with adjuvant induced arthritis. a. Arthritis was induced as described in Materials and Methods, and the edema of both hind paws was calculated from differences in the average volume of both footpads on Day 0 and on Day 18. b. Peritoneal cells (2×10^6/2.0 ml RPMI-1640) from each group of rats were cultured for 24 h as described in Materials and Methods. Spontaneous nitrite production by peritoneal cells was determined using Griess reagent. Each column and bar represents the mean ± SE (n = 12). *p < 0.05; **p < 0.01 versus control, respectively (Dunnett's test). # p < 0.01 vs normal (Student t test).

pads of rats with adjuvant induced arthritis. In control rats given heat killed *M. butyricum* to induce arthritis, the volume of the footpads gradually increased, reaching a peak on Day 18 after injection. As shown in Figure 9a, edema was suppressed in groups treated with 10 and 30 mg/kg KE-758 to 0.92 ± 0.22 ml ($p < 0.05$) and 1.14 ± 0.34 ml ($p < 0.05$), respectively, compared to that in the control group (2.48 ± 0.51 ml). Moreover, as the body weight of KE-758 treated animals was significantly restored, KE-758 was assumed to be nontoxic (data not shown).

Effect of KE-758 on spontaneous NO production and iNOS protein expression by peritoneal cells from rats with adjuvant induced arthritis. Spontaneous NO production by cultured peritoneal cells was shown in the control group (36.4 ± 3.8 μM); however, in the groups treated with 10 and 30 mg/kg of KE-758 the production was significantly decreased to 22.6 ± 3.9 μM ($p < 0.05$) and 17.0 ± 4.2 μM ($p < 0.01$), respectively (Figure 9b).

Further, spontaneous iNOS expression in cultured peritoneal cells from rats of the control group was detected, whereas in the KE-758 treated group (30 mg/kg), iNOS expression was markedly suppressed (Figure 10).

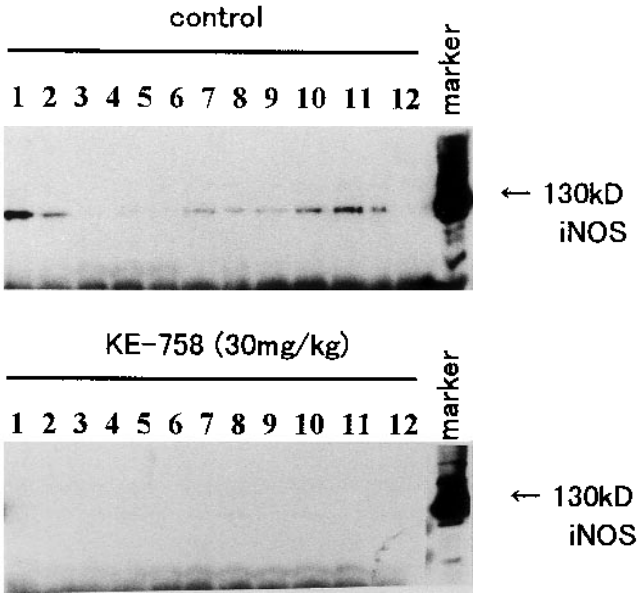

Figure 10. Effect of KE-758 on iNOS expression in peritoneal cells from rats with adjuvant induced arthritis. Peritoneal cells (2×10^6/2.0 ml RPMI-1640) from rats in each group were cultured for 24 h as described in Materials and Methods. Adherent cells were collected, and iNOS expression was examined by Western blotting.

DISCUSSION

We observed that KE-298, our newly developed antirheumatic drug, and KE-758, its active metabolite, suppressed NO production by LPS stimulated RAW264.7 cells by inhibiting iNOS mRNA expression *in vitro*. Even though optimal generation of iNOS was usually achieved using a combination of LPS + interferon-γ (IFN-γ)[22,23], only LPS induced an increase of NO, as described[24]. To simplify the experiments, we employed LPS alone.

Neither KE-298 nor KE-758 had a direct inhibitory effect

on NOS enzyme activity, while acetylating agents such as aspirin and N-acetylimidasole, as well as competitive inhibitors of L-arginine, have been reported to suppress NO production through the inhibition of iNOS catalytic activity *in vitro*[25].

The results of Western blot, RT-PCR, and Northern blot analysis suggested that this inhibitory effect of KE-298 and KE-758 might be due to the inhibition of iNOS protein synthesis and mRNA expression. Meanwhile, auranofin[24,26], oroxylin A[27], and cyclolinteinone[28] have been reported to suppress both iNOS and COX-2 expression through the inhibition of NF-κB activation. But KE-298 and KE-758 did not affect COX-2 expression nor the NF-κB activation, which indicates the inhibition of iNOS gene expression by KE-298/758 might be through a NF-κB-independent mechanism. NF-κB has been considered as one of the key regulators of LPS induced iNOS transcription in murine macrophages[29], and inhibitors of NF-κB, such as pyrrolidine, dithiocarbamate, hydrocortisone[30], and NAC[31], have been shown to decrease LPS induced iNOS mRNA expression. It has recently been reported that orally administered NAC suppresses arthritis activity in animal models through inhibition of NF-κB activation, and it has been suggested to be useful for the treatment of chronic inflammatory diseases such as RA[31,32]. Recently, several DMARD, such as bucillamine[31], D-penicillamine[33], and gold compounds[34], have been shown to suppress NF-κB activation. However, LPS induced activation of NF-κB in RAW264.7 cells was hardly suppressed by pharmacological dose of KE-758.

As the total tyrosine phosphorylation of cytoplasmic protein of LPS stimulated RAW264.7 cells was also unaffected by KE-758 treatment in a preliminary experiment (data not shown), we focused next on the mRNA stability of iNOS. It has been shown that members of the tetracycline family of antibiotics, such as doxycycline and minocycline, suppress NO production not via direct inhibition at the enzyme level but through an augmentation of iNOS mRNA degradation, which leads to a decrease in the protein expression[17,35]. And transforming growth factor-ß has also been shown to act post-transcriptionally by destabilizing iNOS mRNA and decreasing translation[36]. Moreover, glucocorticoids such as dexamethasone have been reported to act at multiple levels including attenuation of iNOS gene transcription, prolongation of the half-life of iNOS mRNA, and reduction of the translation of iNOS mRNA, and by increasing the degradation of iNOS to regulate iNOS expression[37]. As the degradation of LPS induced iNOS mRNA expression in RAW264.7 cells, after terminating the mRNA translation by actinomycin D, was hardly affected by KE-758 in Northern blot analysis, we concluded that the suppressive effect of KE-758 on the expression of iNOS is independent of the stability of iNOS mRNA.

Recently, several reports have indicated that endogenously induced IFN-ß is an essential cofactor for LPS induced iNOS gene expression in murine macrophage cells and subsequent activation of signal transducer and activator of transcription-1α and IRF-1 in concert with NF-κB activation is critical for the murine iNOS induction[38]. Furthermore, Saito, *et al* have reported that membrane associated CD14 (mCD14) expression but not the soluble form of CD14 (sCD14) is necessary for LPS induced IFN-ß production and subsequent iNOS induction[21]. In the present study, the results of RT-PCR for LPS induced IFN-ß and IRF-1 mRNA expression in RAW264.7 cells have clearly indicated that the suppressive target of KE-758 might be located on the pathway of mCD14 dependent IFN-ß induction. To elucidate the mechanism in detail, further experiments are required.

Although several transcription factors including activator protein 1 (AP-1), nuclear factor interleukin 6, and Oct[39] have been identified in the consensus sequences of the iNOS gene promoter in murine macrophages, KE-758 did not suppress LPS induced AP-1 activation in RAW264.7 cells in our preliminary experiments (data not shown). It has been reported that increased production of NO by peritoneal cells was observed in rats with adjuvant induced arthritis, and that their disease course was modulated by the endogenously produced NO[11]. In our present *ex vivo* study, we confirmed that in peritoneal cells from rats with adjuvant induced arthritis iNOS protein expression was increased and that they spontaneously produced high levels of NO on Day 21 as reported[11]. Moreover, we observed that this enhanced NO and iNOS production by cultured peritoneal cells and progression of arthritis were suppressed by KE-758. It has also been reported that the administration of N^G-monomethyl-L-arginine, an inhibitor of NOS, is considered to suppress joint inflammation during zymosan induced gonarthritis via release of endogenous glucocorticoids[40]. Although they suggested the role of NO in T cell dependent models such as antigen induced arthritis, further studies are necessary to determine whether KE-758 might induce glucocorticoids to suppress adjuvant induced arthritis in rats.

In conclusion, KE-298/758 suppressed LPS induced NO and iNOS production in RAW264.7 cells by inhibiting the IFN-ß dependent pathway, and the potential of KE-298 to inhibit NO production might partly explain its efficacy on adjuvant induced arthritis in rats.

REFERENCES

1. Abe T, Mizushima Y, Kashiwazaki S, et al. A 16-week dose finding double blind study of KE-298, a newly developed novel DMARD, in patients with rheumatoid arthritis [abstract]. Proceedings XIXth ILAR Congress Rheum 1997;19:475-6.
2. Yasuda Y, Inoue T, Takeshita K. Pharmacological profile of KE-758, the main metabolite of the antirheumatic agent KE-298. Drugs Exp Clin Res 1996;22:1-8.
3. Takahashi S, Inoue T, Higaki M, Mizushima Y. Suppressive effect of a new anti-rheumatic drug KE-298 on TNF-α-induced matrix metalloproteinase production in RA synoviocytes. Drugs Exp Clin

Res 1998;24:67-71.
4. Sakane T, Suzuki N, Hirose Y, et al. Mechanisms of KE-298, 2-acetylthiomethyl-4-(4-methylphenyl)-4-oxobutanoic acid, to suppress abnormal synovial cell functions in patients with rheumatoid arthritis. J Rheumatol 1997;24:2213-20.
5. Nagai H, Takaoka Y, Mori H, Kawada K. Effect of KE-298 on experimental arthritis in mice. Pharmacology 1996;53:190-6.
6. Kameo K, Takeshita K, Yasuda Y, Matsumoto K, Tomisawa K. Preparation and antirheumatic activity of optically active 2-acetylthiomethyl-4-(4-methylphenyl)-4-oxobutanoic acid (KE-298). Chem Pharm Bull 1996;44:602-4.
7. Hashimoto M, Kobayashi K, Yamagata N, et al. Suppression of pulmonary granulomatous inflammation by immunomodulating agents. Agents Actions 1992;37:99-106.
8. Forstermann U, Gath I, Schwarz P, Closs EI, Kleinert H. Isoforms of nitric oxide synthase. Properties, cellular distribution and expressional control. Biochem Pharmacol 1995;50:1321-32.
9. Borderie D, Hilliquin P, Hernvann A, Kahan A, Menkes CJ, Ekindjian OG. Nitric oxide synthase is expressed in the lymphomononuclear cells of synovial fluid in patients with rheumatoid arthritis. J Rheumatol 1999;26:2083-8.
10. Clancy RM, Amin AR, Abramson SB. The role of nitric oxide in inflammation and immunity. Arthritis Rheum 1998;41:1141-51.
11. Ialenti A, Moncada S, Rosa MD. Modulation of adjuvant arthritis by endogenous nitric oxide. Br J Pharmacol 1993;110:701-6.
12. Cannon GW, Openshaw SJ, Hibbs JB, Hoidal JR, Huecksteadt TP, Griffiths MM. Nitric oxide production during adjuvant-induced and collagen-induced arthritis. Arthritis Rheum 1996;39:1677-84.
13. Stefanovic-Racic M, Meyers K, Meschter C, Coffey JW, Hoffman RA, Evans CH. N-monomethyl arginine, an inhibitor of nitric oxide synthase, suppresses the development of adjuvant arthritis in rats. Arthritis Rheum 1994;37:1062-9.
14. Connor JR, Manning PT, Settle SL, et al. Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. Eur J Pharmacol 1995;273:15-24.
15. Kameo K, Ogawa K, Takeshita K, Nakaike S, Tomisawa K, Sota K. Studies on anti-rheumatic agents: 3-Benzoylpropionic acid derivatives. Chem Pharm Bull 1988;36:2050-60.
16. Rockett KA, Awburn MM, Cowden WB, Clark IA. Killing of Plasmodium falciparum in vitro by nitric oxide derivatives. Infect Immun 1991;59:3280-3.
17. Amin AR, Attur MG, Thakker GD, et al. A novel mechanism of action of tetracyclines: Effects on nitric oxide synthases. Proc Natl Acad Sci USA 1996;93:14014-9.
18. Barber SA, Fultz MJ, Salkowski CA, Vogel SN. Differential expression of interferon regulatory factor 1 (IRF-1), IRF-2, and interferon consensus sequence binding protein gene in lipopolysaccharide (LPS)-responsive and LPS-hyporesponsive macrophages. Infect Immun 1995;63:601-8.
19. Schreiber E, Matthias P, Muller EE, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 1989;17:6419.
20. Church GM, Gilbert W. Genomic sequencing. Proc Natl Acad Sci USA 1984;81:1991-5.
21. Saito S, Matsuura M, Tominaga K, Kirikae T, Nakano M. Important role of membrane-associated CD14 in the induction of IFN-ß and subsequent nitric oxide production by murine macrophages in response to bacterial lipopolysaccharide. Eur J Biochem 2000;267:37-45.
22. Weisz A, Cicatiello L, Esumi H. Regulation of the mouse inducible-type nitric oxide synthase gene promoter by interferon-γ, bacterial lipopolysaccharide and MG-monomethyl- L-arginine. Biochem J 1996;316:209-15.
23. Delgado M, Munoz-Elias EJ, Gomariz RP, Ganea D. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide prevent inducible nitric oxide synthase transcription in macrophage by inhibiting NF-kB and IFN regulatory factor 1 activation. J Immunol 1999;162:4685-96.
24. Yamashita M, Niki H, Yamada M, Mue S, Ohuchi K. Induction of nitric oxide synthase by lipopolysaccharide and its inhibition by auranofin in RAW264.7 cells. Eur J Pharmacol 1997;338:151-8.
25. Amin AR, Vyas P, Attur MG, et al. The mode of action of aspirin-like drugs: Effect on inducible nitric oxide synthase. Proc Natl Acad Sci USA 1995;92:7926-30.
26. Jeon KI, Jeong JY, Jue DM. Thiol-reactive metal compounds inhibit NF-kB activation by blocking IkB kinase. J Immunol 2000;164:5981-9.
27. Chen YC, Yang LL, Lee TFJ. Oroxylin A inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor-kB activation. Biochem Pharmacol 2000;59:1445-57.
28. D'acquisto F, Lanzotti V, Carnuccio R. Cyclolinteinone, a sesterterpene from sponge Cacospongia linteiformis, prevents inducible nitric oxide synthase and inducible cyclo-oxygenase protein expression by blocking nuclear factor-kB activation in J774 macrophages. Biochem J 2000;346:793-8.
29. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kB/Rel in induction of nitric oxide synthase. J Biol Chem 1994;269:4705-8.
30. Nathan C, Xie QW. Nitric oxide synthases: Roles, tolls and controls. Cell 1994;78:915-8.
31. Tsuji F, Miyake Y, Aono H, Kawashima Y, Mita S. Effects of bucillamine and N-acetyl-L-cysteine on cytokine production and collagen-induced arthritis. Clin Exp Immunol 1999;115:26-31.
32. Fujisawa K, Aono H, Hasunuma T, Yamamoto K, Mita S, Nishioka K. Activation of transcription factor NF-kB in human synovial cells in response to tumor necrosis factor α. Arthritis Rheum 1996; 39:197-203.
33. Chandra A, Demirhan I, Arya SK, Chandra P. D-penicillamine inhibits transactivation of human immunodeficiency virus type-1 LTR by transactivator protein. FEBS Lett 1988;236:282-6.
34. Yang JP, Merin JP, Nakano T, Kato T, Kitade Y, Okamoto T. Inhibition of the DNA-binding activity of NF-kB by gold compounds in vitro. FEBS Lett 1995;361:89-96.
35. Amin AR, Patel RN, Thakker GD, Lowenstein CJ, Attur MG, Abramson SB. Post-transcriptional regulation of inducible nitric oxide synthase mRNA in murine macrophages by doxycycline and chemically modified tetracyclines. FEBS Lett 1997;410:259-64.
36. Vodovots Y, Bogdan C, Paik J, Xie QW, Nathan CF. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor ß. J Exp Med 1993;178:605-13.
37. Kunz D, Walker G, Eberhardt W, Pfeilschifter J. Molecular mechanisms of dexamethasone inhibition of nitric oxide synthase expression in interleukin 1ß-stimulated mesangial cells: Evidence for the involvement of transcriptional and posttranscriptional regulation. Proc Natl Acad Sci USA 1996;93:255-9.
38. Gao JJ, Filla MB, Fultz MJ, Vogel SN, Russell SW, Murphy WJ. Autocrine/paracrine IFN-αß mediates the lipopolysaccharide-induced activation of transcription factor stat1a in mouse macrophage: Pivotal role of stat1a in induction of the inducible nitric oxide synthase gene. J Immunol 1998;161:4803-10.
39. Goldring CEP, Reveneau S, Aigarte M, Jeannin JF. In vivo foot-printing of the mouse inducible nitric oxide synthase gene: inducible protein occupation of numerous sites including Oct and NF-IL6. Nucleic Acids Res 1996;24:1682-7.
40. van de Loo FA, Arntz OJ, van Enckevort FH, van Lent PL, van den Berg WB. Reduced cartilage proteoglycan loss during zymosan-induced gonarthritis in NOS2-deficient mice and in anti-interleukin-1-treated wild-type mice with unabated joint inflammation. Arthritis Rheum 1998;41:634-46.