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Suppressive Effects of Furonaphthoquinone NFD-37 on the Production of Lipopolysaccharide-Inducible Inflammatory Mediators in Macrophages RAW 264.7

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2-Methyl-2-(2-methylpropenyl)-2,3-dihydronaphthoquinone[2,3-*b*]furan-4,9-dione (NFD-37) is a synthetic furonaphthoquinone compound. In this study, we determined that NFD-37 could inhibit the lipopolysaccharide (LPS)-induced production of inflammatory mediators in macrophages RAW 264.7. This compound inhibited LPS-induced nitric oxide (NO) or prostaglandin (PG) E_2 production in dose-dependent manners, with IC_{50} values of 7.2 μM and 5.3 μM, respectively. As the positive controls, pyrrolidine dithiocarbamate (30 μM) exhibited a 57% inhibition of NO production, and NS-398 (1 μM) manifested a 48% inhibition of PGE_2 production. The inhibitory effects of NFD-37 on NO and PGE_2 production were determined to occur in conjunction with the suppression of inducible NO synthase or cyclooxygenase-2 expression. NFD-37 also inhibited the production of LPS-inducible tumor necrosis factor-α, interleukin (IL)-1β and IL-6, at IC_{50} values of 4.8-8.9 μM. We also determined the anti-inflammatory efficacy of NFD-37 using carrageenin-induced paw edema in experimental mice.

Key words: Furonaphthoquinone, NO, PGE_2, Cytokine, Anti-inflammation

INTRODUCTION

Inflammation occurs as a defensive mechanism of the host to foreign invasions, most notably microbial infections. In living systems, NO is generated from L-arginine *via* the catalytic activity of NO synthase (NOS). Both endothelial and neuronal NOS have been shown to generate moderate quantities of NO, which is then primarily involved in the mediation of physiological responses, including vasodilation and neurotransmission (Prast and Philippu, 2001; Ignarro, 2002). NO is also generated in the immune system, by inducible NOS (iNOS), where it facilitates the eradication of foreign invaders (MacMicking *et al.*, 1997).

Prostaglandin (PG) and thromboxane are produced by the biosynthetic pathway, originating from arachidonic acid, in which cyclooxygenase (COX) catalyzes a rate-limiting step, resulting in the formation of a ring between the C-8 and C-12 atoms of the substrate. Like NOS, COX appears in two distinct isoforms, referred to as COX-1 and COX-2. COX-1 is a constitutive enzyme, which mediates physiological responses, including cytoprotection of the stomach, and the regulation of renal blood flow (Arakawa *et al.*, 1998; Brater, 2002). By way of contrast, COX-2 is an inducible enzyme, which produced during inflammation or upon exposure to mitogenic stimuli (Crofford *et al.*, 1994; Masferrer *et al.*, 1995). Thus, the inhibition of COX-2 is thought to result in favorable anti-inflammatory, analgesic and antipyretic effects, whereas the inhibition of COX-1 has been theorized to induce undesirable gastrointestinal and renal side effects (Bovill, 2003; Becker *et al.*, 2004).

During the inflammatory process, the host generates several inflammatory cytokines, including tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6, all of which are known to play important roles in pathogenesis. The inflammatory cytokines, iNOS, and COX-2, can be induced by stimulation with the lipopolysaccharide (LPS) of Gram-negative bacteria or with cell-wall teichoic acids and muramyl peptides, although their induction appears to

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be dependent on the cell type involved (Guha and Mackman, 2001; Jones *et al.*, 2005). Biologically, IL-1 and TNF are closely related, and mimic host responses to infection, inflammation, injuries or immunological challenges, although the structures and receptors of these two cytokines are clearly distinct (Watkins *et al.*, 1999; Hopkins, 2003). IL-1 and TNF, furthermore, have been shown to function synergistically in nearly every tested *in vitro* and *in vivo* model of local or systemic inflammation (van den Berg *et al.*, 1999).

IL-6 is a typical pleiotropic cytokine, and appears to perform an important function in the immune system, the hematopoietic system, and in the process of inflammation, in addition to its known effects on the nervous system, endocrine system, and in bone metabolism (Kamimura *et al.*, 2003). Circulatory concentrations of IL-6 are tightly regulated, and IL-6 is normally maintained at low levels under normal conditions in young and healthy individuals. However, IL-6 production has been demonstrated to increase rapidly over the course of inflammatory process. In fact, several previous studies have documented that IL-6 is implicated in the pathogenesis of a variety of human disorders, including rheumatoid arthritis and inflammatory bowel disease (Koss *et al.*, 2000; Wong *et al.*, 2003). Therefore, LPS-inducible iNOS, COX-2, and cytokines are thought to be attractive therapeutic targets in the treatment of human disorders associated with inflammation

2-Methyl-2-(2-methylpropenyl)-2,3-dihydronaphthoquinone[2,3-*b*]furan-4,9-dione (NFD-37) is a synthetic furonaphthoquinone compound (Fig. 1). In this study, we have determined that NFD-37 exerts a suppressive effect on the generation of inflammatory mediators, including NO, PGE_2, and cytokines, in LPS-stimulated macrophages RAW 264.7. The anti-inflammatory potential of this compound was also documented in a carrageenin-challenged mouse edema model.

MATERIALS AND METHODS

Materials

The furonaphthoquinone NFD-37 (>98% purity) was prepared according to the method described in our previous study (Lee *et al.*, 2000). The fetal bovine serum (FBS) and culture media were acquired from Invitrogen (Carlsbad, U.S.A.). Antibodies against iNOS, COX-2, COX-1, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotech (Santa Cruz, U.S.A.). The other chemicals used in this study, including LPS (*Escherichia coli* 055:B5), pyrrolidine dithiocarbamate (PDTC), and NS-398 were all purchased from Sigma-Aldrich (St. Louis, U.S.A.).

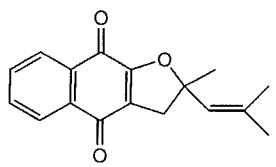

Fig. 1. Chemical structure of NFD-37

Cell culture

Macrophages RAW 264.7 were cultured in DMEM (13.4 mg/mL Dulbecco's modified Eagle's medium, 24 mM $NaHCO_3$, 10 mM HEPES, 143 U/mL benzylpenicillin potassium, and 100 μg/mL streptomycin sulfate, at pH 7.1) containing 10% FBS, and were maintained at 37°C, with an atmosphere containing 5% CO_2.

NO quantification

Macrophages RAW 264.7 were pre-treated for 2 h with NFD-37, then stimulated with LPS (1 μg/mL) for 24 h. Levels of nitrite, a stable metabolite of NO, were determined by the Griess reaction. In brief, cell-free culture media (100 μL) was allowed to react with a 1:1 mixture (100 μL) of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine in water, after which the absorbance values were measured at 540 nm. The nitrite contents were calculated from a standard curve, constructed using known sodium nitrite concentrations.

ELISA

Macrophages RAW 264.7 were pre-treated for 2 h with NFD-37, then stimulated with LPS (1 μg/mL) for 24 h. Quantities of PGE_2, TNF-α, IL-1β, and IL-6 in the cell-free culture media were determined using ELISA kits obtained from Amersham-Pharmacia (San Francisco, U.S.A.).

Western immunoblot analysis

Macrophages RAW 264.7 were pre-treated for 2 h with NFD-37, then stimulated with LPS (1 μg/mL) for 18 h. The cells were then lysed in a buffer (1% Triton X-100, 350 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM PMSF). Equal amounts of the cell lysates were resolved on SDS-acrylamide gel by electrophoresis, and then transferred to PVDF membranes (Millipore, Bedford, U.S.A.). The membranes were blocked for 2 h with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST buffer), and were subsequently incubated overnight at room temperature with anti-iNOS antibody (1:500 dilution), anti-COX-2 antibody (1:250 dilution), anti-COX-1 antibody (1:250 dilution), or anti-GAPDH antibody (1:1000 dilution). After washing with TBST buffer, the blots were incubated for 2 h with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2500 dilution) at room temperature. After extensive washing with TBST buffer, the blots were

allowed to react with ECL reagent (Amersham-Pharmacia, San Francisco, U.S.A.) and exposed to X-ray film. Signals on the films were photographed, after which their intensities were determined with a Kodak image analyzer.

Semi-quantitative RT-PCR

Macrophages RAW 264.7 were pre-treated for 2 h with NFD-37, and stimulated for 6 h with LPS (1 μg/mL). Total RNA of the cells was then subjected to semi-quantitative RT-PCR, using an RNA PCR kit (Bioneer, Taejon, Korea). The primer sequences and RT-PCR conditions used in this experiment were described in our previous study (Shin *et al.*, 2004). In brief, total RNA was reverse-transcribed at 42°C, and then subjected to 30 cycles of PCR, each consisting of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C, and 90 seconds of extension at 72°C. The RT-PCR products were then resolved on agarose gel by electrophoresis, and stained with ethidium bromide. Signals on the gels were photographed under UV irradiation, and their intensities were measured using a Kodak image analyzer.

Measurement of iNOS promoter activity

Macrophages RAW 264.7 were transiently transfected with piNOS-luciferase (Luc) construct (Lowenstein *et al.*, 1993) and pSV-β-galactosidase control vector (Promega, Madison, U.S.A.), using Lipofectamine (Invitrogen, Carlsbad, U.S.A.). The transfected cells were pre-treated for 2 h with NFD-37, and stimulated for 18 h with LPS (1 μg/mL). The cell lysates were then subjected to a luciferase assay and a β-galactosidase assay, using the appropriate kits, which were purchased from Promega (Madison, U.S.A.).

Cytotoxicity measurement

Macrophages RAW 264.7 were incubated for 24 h with various concentrations of NFD-37. The cells were then treated with WST-1 solution (Dojindo Lab, Kumamoto, Japan), after which their absorbance values were determined at 450 nm.

Paw edema measurement

Male ICR mice, weighing 25 ± 3 g each, were allowed to acclimate to an animal room, and were given *ad libitum* access to a standard diet and water. The mice were divided into groups of 7 mice each, and NFD-37 was orally administered to the mice. After 2 h, 10 μL of either 2% carrageenin or saline was injected into a subplantar site in the right or left hind paw, respectively. The paw volumes were measured with a plethysmometer at zero time and at 6 h after the carrageenin challenge, and edema (%) was calculated according to the following formula: 100 x [(Rt-Ro)-(Lt-Lo)]/Ro, in which Ro and Rt represent the volumes of the right paw at either zero time or at 6 h after the carrageenin injection, and Lo and Lt are the volumes of the left paw at zero time or at 6 h after saline injection.

Statistical analysis

All data were expressed as means ± S.E.M., and analyzed by Student's *t*-tests. A P value of < 0.01 was considered to be significant.

RESULTS AND DISCUSSION

In the present study, we determined the inhibitory effect of NFD-37 on NO production by measuring the nitrite levels in the culture media of LPS-stimulated macrophages RAW 264.7. The macrophages, in resting state, released 7.7 ± 2.5 μM of nitrite during 24 h of incubation, whereas, upon exposure to LPS alone, the macrophages increased nitrite production, of up to 48.8 ± 4.1 μM (Fig. 2A). NFD-37 inhibited LPS-induced nitrite production in a dose-dependent manner, corresponding to a 30% inhibition at 3 μM, 63% at 10 μM, and 96% at 30 μM, with an IC_{50} value of 7.2 μM (Fig. 2A). As a sup-pressor of

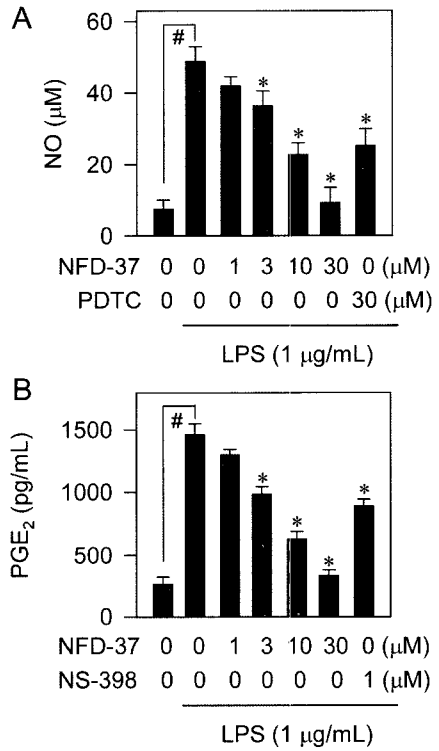

Fig. 2. Inhibitory effect of NFD-37 on LPS-induced NO or PGE_2 production. Macrophages RAW 264.7 were pre-treated for 2 h with NFD-37, and stimulated with LPS for 24 h. The amounts of NO in the culture media were measured by Griess reaction (A) and PGE_2 levels were determined by ELISA (B). Values are expressed as means ± S.E.M. from five independent experiments (A) or from three independent experiments (B). $^{\#}P<0.01$ vs. media alone-treated group. $^{*}P<0.01$ vs. LPS alone-treated group.

iNOS expression, PDTC (30 μM) also manifested a 57% inhibition on LPS-induced nitrite production (Fig. 2A).

Macrophages RAW 264.7, in resting state, released 267 ± 60 pg/mL of PGE_2 during 24 h of incubation. However, upon exposure to LPS alone, the macrophages increased PGE_2 production, of up to 1464 ± 47 pg/mL (Fig. 2B). NFD-37 inhibited the LPS-induced production of PGE_2 in a dose-dependent manner, corresponding to a 40% inhibition at 3 μM, 71% at 10 μM, and 94% at 30 μM, with an IC_{50} value of 5.3 μM (Fig. 2B). NS-398 (1 mM), a specific inhibitor of COX-2 activity, also manifested a 48% inhibition of LPS-induced PGE_2 production (Fig. 2B).

In order to ascertain whether or not the inhibitory effects of NFD-37 on NO or PGE_2 production were attributable to its influence on LPS-inducible iNOS or COX-2 expression, we conducted Western immunoblot analysis. Both iNOS and COX-2 proteins were present at minimal levels in the macrophages RAW 264.7 in their resting state, but theses levels were remarkably increased upon exposure to LPS alone (Fig. 3A and 3B). NFD-37 was shown to attenuate LPS-induced iNOS protein synthesis in a dose-dependent manner, corresponding to a 32% inhibition at 3 μM, 67% at 10 μM, and 85% at 30 μM (Fig. 3A). Moreover, the compound inhibited the LPS-induced synthesis of COX-2 protein, also in a dose-dependent manner, corresponding to a 22% inhibition at 3 μM, 71% at 10 μM, and 92% at 30 μM (Fig. 3B). However, both GAPDH and COX-1 protein syntheses in the RAW 264.7 cells were unaffected by both LPS and NFD-37 treatments (Fig. 3A and 3B).

We also conducted semi-quantitative RT-PCR, in an attempt to determine whether NFD-37 could affect LPS-induced synthesis of iNOS or COX-2 transcript in macrophages RAW 264.7. The quantities of iNOS and COX-2

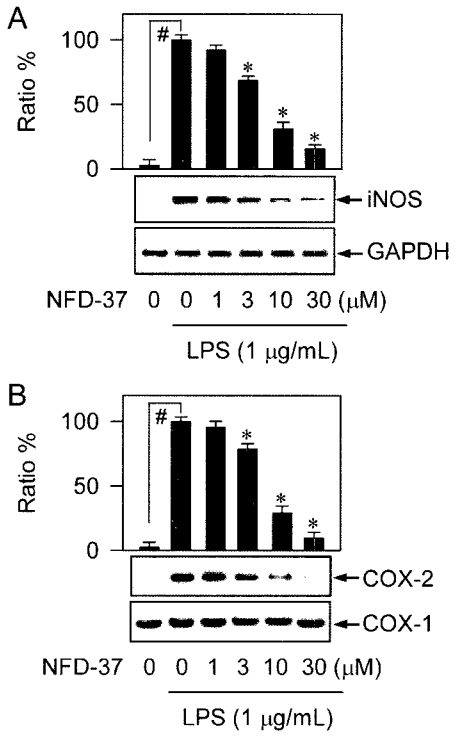

Fig. 3. Inhibitory effect of NFD-37 on LPS-induced synthesis of iNOS or COX-2 protein. Macrophages RAW 264.7 were pre-treated for 2 h with NFD-37, and stimulated with LPS for 18 h. The cell lysates were subjected to Western immunoblot analysis with either anti-iNOS antibody (A) or anti-COX-2 antibody (B). One of the similar results is represented, and the relative ratio % is also shown, where the iNOS signal was normalized to a GAPDH signal (A) or the COX-2 signal was normalized to a COX-1 signal (B). The values are expressed as means ± S.E.M. from three independent experiments. $^{\#}P<0.01$ vs. media alone-treated group. $^{*}P<0.01$ vs. LPS alone-treated group.

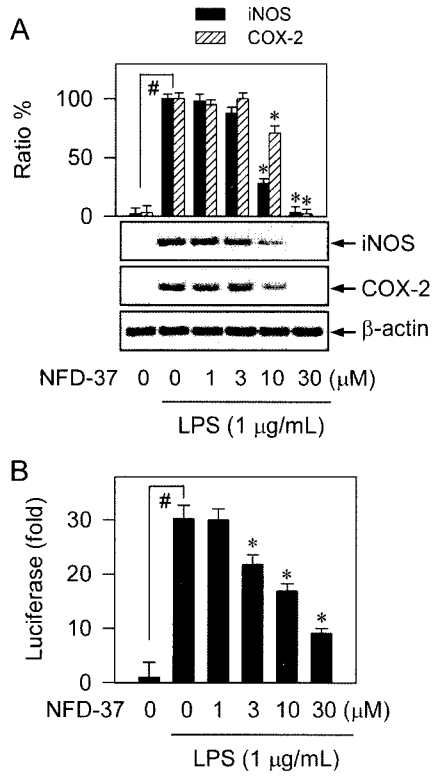

Fig. 4. Inhibitory effect of NFD-37 on LPS-induced synthesis of iNOS or COX-2 transcript, and iNOS promoter activity. Macrophages RAW 264.7 were pre-treated for 2 h with NFD-37, and stimulated with LPS for 6 h. Total RNA of the cells was then subjected to semi-quantitative RT-PCR. One of the similar results is provided, and the relative ratio % is also shown, where either the iNOS signal or the COX-2 signal was normalized to a β-actin signal (A). Macrophages RAW 264.7 transiently transfected with piNOS-Luc reporter construct and pSV-β-galactosidase control vector were pre-treated for 2 h with NFD-37, and stimulated for 18 h with LPS. Luciferase and β-galactosidase activities were measured with the cell lysates. Luciferase expression, as a reporter for iNOS promoter activity, is represented as relative fold values, in which luciferase activity was normalized to β-galactosidase activity (B). The values are expressed as means ± S.E.M. from three independent experiments (A) or from five independent experiments (B). $^{\#}P<0.01$ vs. media alone-treated group. $^{*}P<0.01$ vs. LPS alone-treated group.

transcripts were markedly increased upon exposure to LPS alone (Fig. 4A). NFD-37 reduced the LPS-induced synthesis of iNOS transcript, corresponding to a 72% inhibition at 10 μM and 97% at 30 μM. NFD-37 also attenuated the LPS-induced synthesis of COX-2 transcript, corresponding to a 29% inhibition at 20 μM and 98% at 30 μM (Fig. 4A). However, synthesis of the housekeeping b-action transcript was unaffected by both LPS and NFD-37 treatments (Fig. 4A). The transcriptional regulation of iNOS expression by NFD-37 was documented further using macrophages RAW 264.7 transfected transiently with piNOS-Luc construct encoding iNOS promoter (−1592/+183) fused to luciferase gene as a reporter (Lowenstein *et al.*, 1993). Upon exposure to LPS alone, the transfected cells increased the luciferase expression, by up to 30 times over the basal level (Fig. 4B). NFD-37 inhibited the LPS-induced luciferase expression in a dose-dependent manner, corresponding to a 29% inhibition at 3 μM, 46% at 10 μM, and 73% at 30 μM (Fig. 4B).

We next determined whether NFD-37 could affect LPS-induced productions of TNF-α, IL-1β, and IL-6. Macrophages RAW 264.7, in resting state, released 0.9 ± 0.6 ng/mL of TNF-α during 24 h of incubation. However, upon exposure to LPS alone, the macrophages increased TNF-α production, of up to 14.0 ± 0.5 ng/mL (Fig. 5A). NFD-37 inhibited LPS-induced TNF-α production in a dose-dependent manner, showing an IC_{50} value of 6.5 μM (Fig. 5A). The resting RAW 264.7 cells released 142 ± 42 pg/mL of IL-1β during 24 h of incubation, but also increased IL-1β production, of up to 2004 ± 79 pg/mL, upon exposure to LPS alone (Fig. 5B). NFD-37 showed an IC_{50} value of 8.9 μM with regard to the LPS-induced production of IL-1β (Fig. 5B). Macrophages RAW 264.7, in resting state, released 0.9 ± 4.1 ng/mL of IL-6 during 24 h of incubation, but also increased IL-6 production, of up to 49.3 ± 5.2 ng/mL, upon exposure to LPS alone (Fig. 5C). NFD-37 exhibited an IC_{50} value of 4.8 μM with regard to LPS-induced IL-6 production (Fig. 5C). The inhibitory effects exerted by NFD-37 on LPS-induced production of inflammatory mediators were not attributable to its non-specific toxicity, as this compound, at concentrations of less than 100 μM, did not exhibit significant cytotoxic effects against macrophages RAW 264.7 (Fig. 6).

The stimulation of macrophages by LPS results in the induction of many genes, and also generated a variety of inflammatory mediators. LPS responsive *cis*-acting DNA elements have been determined to exist in the 5' flanking regions of genes encoding iNOS, COX-2, and cytokines (Lowenstein *et al.*, 1993; Sanceau *et al.*, 1995; Inoue *et al.*, 1997). The transcription factors that bind to LPS responsive elements include nuclear factor (NF)-κB/Rel proteins, activator protein-1, and NF-IL-6 (Lowenstein *et al.*, 1993; Sanceau *et al.*, 1995; Inoue *et al.*, 1997). These

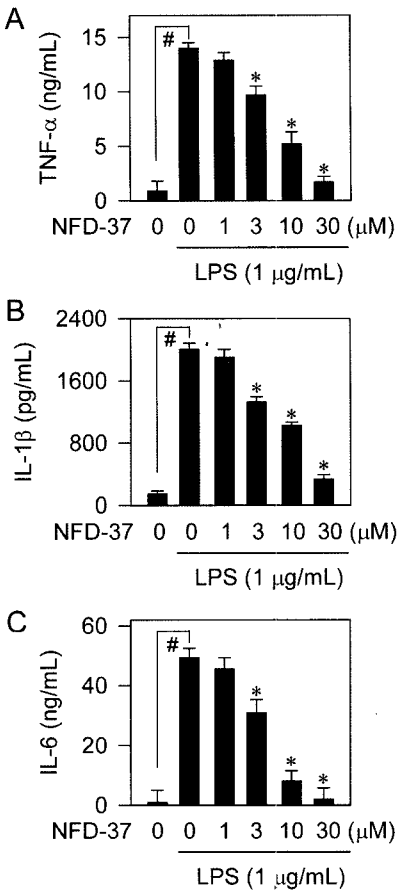

Fig. 5. Inhibitory effect of NFD-37 on LPS-induced inflammatory cytokine production. Macrophages RAW 264.7 were pre-treated for 2 h with NFD-37, and stimulated for 24 h with LPS. Amounts of TNF-α (A), IL-1β (B), or IL-6 (C) in the culture media were determined by ELISA. The values are expressed as means ± S.E.M. from three independent experiments. #P<0.01 vs. media alone-treated group. *P<0.01 vs. LPS alone-treated group.

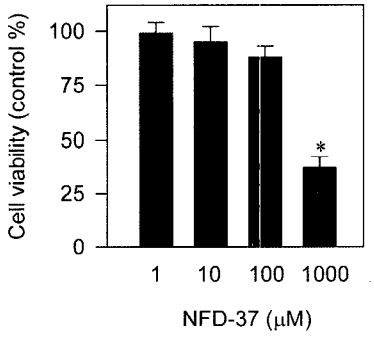

Fig. 6. Cytotoxic effect of NFD-37. Macrophages RAW 264.7 were incubated for 24 h with NFD-37. Cell viability is expressed as a control %, compared with the group treated with media alone. The values are expressed as means ± S.E.M. from five independent experiments. *P<0.01 vs. media alone-treated group.

transcription factors are activated *via* signaling pathways, and are then assembled onto adjacent sites in the

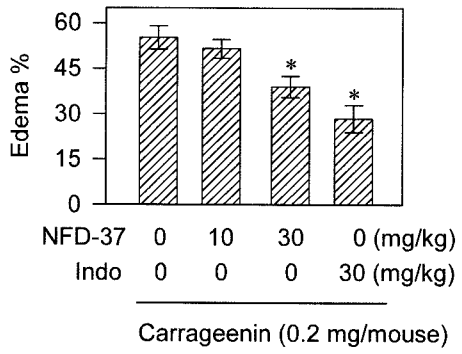

Fig. 7. Anti-inflammatory effect of NFD-37 on carrageenin-induced paw edema. Mice were pre-treated orally with NFD-37 or indomethacin (Indo) for 2 h, and then challenged with carrageenin for 6 h. Paw edema was measured and edema % values are expressed as means ± S.E.M. from two independent experiments. *P<0.01 vs. carrageenin alone-challenged group.

promoter regions of LPS-inducible genes. The NF-κB/Rel proteins appear to be a prerequisite for the expression of all LPS-inducible genes in macrophages (Guha and Mackman, 2001; Tian and Brasier, 2003).

In order to determine whether NFD-37 could exhibit anti-inflammatory effects *in vivo*, we used an acute inflammation model, involving the induction of paw edema in mice by carrageenin. In this experiment, the group challenged with carrageenin alone exhibited a 55% paw edema (Fig. 7). At the dose of 30 mg/kg (p.o.), NFD-37 exhibited anti-inflammatory effects on the carrageenin-challenged animal model, showing a 38% paw edema, and indomethacin, as a positive control, exhibited a 29% paw edema (Fig. 7). However, NFD-37, at a low dose of 10 mg/kg (p.o.), exerted insignificant anti-inflammatory effects (Fig. 7).

In conclusion, we have clearly demonstrated that NFD-37 suppresses the production of LPS-inducible inflammatory mediators, such as NO, PGE_2, TNF-α, IL-1β, and IL-6, in macrophages RAW 264.7. The compound was also exhibited anti-inflammatory efficacy on carrageenin-induced paw edema in an experimental mouse model.

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