Inhibition of Nitric Oxide and Tumor Necrosis Factor-α (TNF-α) Production by Propenone Compound through Blockade of Nuclear Factor (NF)-κB Activation in Cultured Murine Macrophages

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Lipopolysaccharide (LPS)-stimulated macrophages produce large amounts of nitric oxide (NO) by inducible nitric oxide synthase (iNOS). This is an important mechanism in macrophage-induced septic shock and inflammation. In the present study, we tested a synthetic propenone compound, 1-furan-2-yl-3-pyridin-2-yl-propenone (FPP-3) for its ability to inhibit the production of tumor necrosis factor-α (TNF-α) and an inducible enzyme, iNOS, in the LPS-stimulated murine macrophage-like cell line, RAW264.7. FPP-3 consistently inhibited nitric oxide (NO) and TNF-α production in a dose dependent manner, with IC_{50} values of 10.0 and 13.1 μM, respectively. Western blotting probed with specific anti-iNOS antibodies showed that the decrease in quantity of the NO product was accompanied by a decrease in the iNOS protein level. In cells transiently transfected with nuclear factor (NF)-κB promoter-luciferase reporter construct, this compound clearly inhibited the LPS-stimulated NF-κB activation. Moreover, this compound inhibited IκB-α degradation in a concentration and time-dependent manner. These results indicate that FPP-3 inhibits NO production *via* inhibition of degradation of IκB-α through NF-κB activation.

Key words   propenone compound; nitric oxide; inducible nitric oxide synthase; tumor necrosis factor-α (TNF-α); nuclear factor (NF)-κB

Lipopolysaccharide (LPS), which is a component of the cell walls of gram-negative bacteria, is one of the most potent macrophage activators. LPS induces the production of pro-inflammatory cytokines and small mediators, such as nitric oxide (NO) and PGE_2 in macrophages. Macrophage activation is known to play an important role in the inflammatory process.[1] Increasing evidence also suggests that NO, a bioactive free radical, is involved in various pathophysiological processes including inflammation and septic shock.[2,3] Therefore, NO production might reflect the inflammation process and provide a measure for assessing the effects of drugs on this process. NO production is regulated by NO synthases (NOSs) and three NOS isoforms have been identified and cloned. The neural (nNOS) and the endothelial (eNOS) isoforms are constitutively expressed in selected tissues, and their enzymatic activity is regulated by changes in the intracellular free Ca^{2+} concentration. A third member of the NOS family is type II NOS (inducible NOS, iNOS), which is produced in large quantities in response to inflammatory stimuli such as LPS and interferon-γ (IFN-γ).[4,5]

Proinflammmatory cytokines (tumor necrosis factor-α (TNF-α), IL-1β and IL-6) have been suggested to induce tissue damage, and are considered to be an important initiator of the inflammatory responses. The expression of these cytokines depends on the activation of the transcriptional factor, nuclear factor (NF)-κB. NF-κB is a critical intracellular mediator of the inflammatory cascade because these pro-inflammatory molecules are regulated at the transcription level.[6—10]

In a study of anti-inflammatory activities of chemically synthetic propenone compound, 1-furan-2-yl-3-pyridin-2-yl-propenone (FPP-3) (Fig. 1) was found to inhibit LPS-stimulated NO and TNF-α production in RAW264.7 macrophages

in a preliminary *in vitro* test. The aim of the present study was to evaluate the mechanism by which FPP-3 inhibits NO and TNF-α production in macrophages. The results clearly demonstrated that treatment with FPP-3 decreases LPS-stimulated iNOS protein expression and TNF-α production through the inhibition of NF-κB activation.

MATERIALS AND METHODS

Materials   2-Acetylfuran and 2-pyridinecarboxaldehyde were purchased from Aldrich Chemical Co. (St. Louis, MO, U.S.A.) and were reagent grade. Thin-layer chromatography (TLC) and column chromatography were performed with Kieselgel 60 F_{254} (Merck) and silica gel Kieselgel 60 (230—240 mesh, Merck), respectively. Compounds were visualized on TLC plates with UV light and ρ-anisaldehyde solution. Nuclear magnetic resonance (NMR) spectra were taken on a Bruker AMX 250 MHz, and tetramethylsilane was used as an internal standard. Chemical shifts (δ) were recorded in ppm, and coupling constants (*J*) in Hz. RPMI 1640 and phosphate-buffer saline were obtained from GIBCO-BRL (Grand Island, NY, U.S.A.). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT, U.S.A.). Rabbit polyclonal iNOS antibody, and anti-rabbit IgG peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). LPS (from *Escherichia coli* 0111:B4, γ-irradiated), and other chemicals
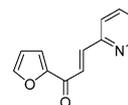

Fig. 1.   Chemical Structure of FPP-3

from Sigma Chemical Co. (St. Louis, MO, U.S.A.). FPP-3 was dissolved in dimethyl sulfoxide (DMSO) before addition to cell cultures: final concentration of DMSO was 0.05%. Control with DMSO alone was run in all cases.

Synthesis of 1-Furan-2-yl-3-pyridin-2-yl-propenone (FPP-3) To a solution of 2-acetylfuran (5.0 ml, 49.9 mmol) and 2-pyridinecarboxaldehyde (5.2 ml, 54.9 mmol) in absolute ethanol (30 ml) under nitrogen at 25 °C was added a catalytic amount of solid sodium hydroxide (34 mg), and the mixture was vigorously stirred at 25 °C for 3 h. Water (80 ml) was added to the solution to quench the reaction. The mixture was extracted with CH_2Cl_2 (120 ml) and the organic layer was washed with water (80 ml, 2 times) and saturated aqueous NaCl solution (80 ml), and dried with Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography with an elution of ethyl acetate and hexane (EtOAc : n-hexane=1 : 2, v/v) to give FPP-3 (3.84 g) as a yellow crystal in 38.7% yield. TLC (EtOAc : n-hexane=1 : 2, v/v), Rf=0.17. ^{1}H-NMR (250 MHz, CDCl$_3$): δ 8.70 (ddd, J=4.8, 1.7, 0.9 Hz, 1H, pyridine H-6), 7.97 (d, J=15.4 Hz, 1H, –CH=CH–CO), 7.84 (d, J=15.4 Hz, 1H, –CH=CH–CO–), 7.75 (dt, J=7.7, 1.8 Hz, 1H, pyridine H-4), 7.68 (dd, J=1.7, 0.7 Hz, 1H, furan H-5), 7.48 (dt, J=7.8, 1.2 Hz, 1H, pyridine H-3), 7.42 (dd, J=3.6, 0.7 Hz, 1H, furan H-3), 7.31 (ddd, J=7.6, 4.8, 1.1 Hz, 1H, pyridine H-5), 6.61 (dd, J=3.6, 1.7 Hz, 1H, furan H-4). The purity of this compound is above 99% analyzed by HPLC and ^{1}H-NMR.

Cell Culture RAW264.7 cells were cultured in RPMI supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 10% fetal calf serum. Cells were grown at 37 °C, 5% CO_2 in fully humidified air and subcultured 2 times a week. They were then seeded in 96-well plates at 1×10^6 cells/ml or 6-well plates at 1×10^6 cells/ml. The cells were stimulated for intervals ranging from 1 to 24 h in the presence of LPS with or without FPP-3. LPS was diluted with culture medium to a final concentration of 200 ng/ml.

Assay of NO Synthesis Synthesis of NO was determined by assaying culture supernatants for nitrite, the stable reaction product of NO with molecular oxygen. Briefly, 100 μl of culture supernatant was allowed to react with 100 μl of Griess reagent (1% sulfanilamide, 0.1% naphthyethylenediamine dihydrochloride, and 2.5% phosphoric acid) at room temperature for 10 min. The optical density of the assay sample was measured spectrophotometrically at 570 nm. Fresh culture medium served as the blank in all experiments. Nitrite concentration was calculated from a standard curve derived from the reaction of $NaNO_2$ under the assay condition.

TNF-α Determination RAW264.7 cells were preincubated with FPP-3 for 30 min before the addition of LPS (200 ng/ml) unless otherwise stated. TNF-α level in the cell culture supernatants was measured by a mouse cytokine ELISA kit according to the manufacturer's instructions (Genzyme).

SDS-PAGE/Immunoblot Analysis RAW264.7 cells were plated in 6-well plates (1×10^6 cells/well) and treated with LPS for 24 h. The cells were washed and scraped into cold phosphate-buffered saline (PBS) and centrifuged at $500\times g$ at 4 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM ethylenediaminetetraacetic acid [EDTA], 150 mM NaCl, 0.5% Nonidet-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) and centrifuged to yield whole-cell lysates. The proteins (20 μg) were separated by 8% reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred in 20% methanol, 25 mM Tris, and 192 mM glycine to a nitrocellulose membrane (Schleicher and Schull, Dassel, Germany). The nitrocellulose membrane was then blocked by incubation in TTBS (25 mM Tris–HCl, 150 mM NaCl, and 0.2% Tween 20) containing 5% nonfat milk. Subsequently, the membrane was incubated with anti-iNOS antibody for 4 h, washed, and finally incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase. The protein bands were visualized using an enhanced chemiluminesence (ECL) system (Amersham Corp., Newark, NJ, U.S.A.).

Transient Transfection and Reporter Activity Assay An NF-κB reporter construct, consisting of the firefly luciferase gene under the control of four tandem copies of the consensus NF-κB site p NF-κB–Luc, was used to quantify NF-κB transcriptional activity. RAW264.7 cells were transfected by Lipofectamine reagent according to the instructions of the manufacturer. Twenty-four hours later, the culture medium was replaced, and cells were pretreated with DMSO or the indicated concentrations of FPP-3 for 30 min following by stimulation with LPS (200 ng/ml). After 24 h the cells were lysed, and the luciferase activity was measured according to the manufacturer's instruction using a luminometer.

RESULTS

Effects of FPP-3 on NO Production and iNOS Protein Expression in RAW264.7 Cells To assess the effect of FPP-3 on LPS-stimulated NO production in RAW264.7 cells, cell culture medium was collected, and the concentration of produced nitrite was determined by the Griess method. FPP-3 was dissolved in DMSO before addition to cell cultures, DMSO showed neither cytotoxicity nor any significant effect on NO production at a concentration up to 0.1% (data not shown). As a result, FPP-3 inhibited in a concentration-dependent manner with an IC$_{50}$ value of 10.0 μM. The inhibitory effect of the FPP-3 on inducible NO production was examined to determine if it is a direct effect of the iNOS protein or if this inhibition is mediated by some other mechanism. LPS induced the Mr 130000 iNOS protein in the RAW264.7 cells in a dose-dependent manner over the concentration range, 10—10000 ng/ml (data not shown). As shown in Fig. 2, the iNOS protein was not detected in unstimulated RAW264.7 cells, whereas LPS (200 ng/ml) strongly induced the formation of detectable iNOS. The LPS induced iNOS protein expression was inhibited by FPP-3. Inhibition of LPS-stimulated NO production in RAW264.7 cells was not attributed to cytotoxicity as assessed by trypan blue exclusion and MTT assay (data not shown).

Effects of FPP-3 on NF-κB Transcriptional Activity in RAW264.7 To understand the effect of FPP-3 on NO production and iNOS protein expression, RAW264.7 cells were transiently transfected with a plasmid containing four tandem copies of the NF-κB consensus sequence linked to the luciferase gene. NF-κB transcriptional activity was measured
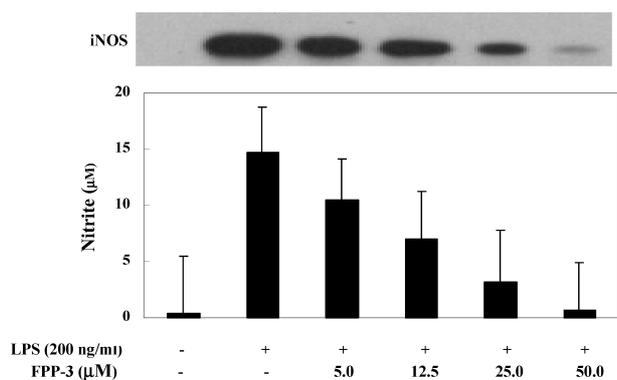
Fig. 2.   Suppression of LPS-Induced NO Production and Inhibition of Expression of iNOS Protein by FPP-3

RAW264.7 cells were incubated for 24 h with LPS (200 ng/ml) and the indicated concentration of FPP-3. NO in the supernatant was determined by Griess reaction. Error bars represent the mean±S.D. of 3 individual experiments. RAW264.7 cells were preincubated with FPP-3 for 30 min before the addition of LPS (200 ng/ml), then incubated 24 h with vehicle or LPS (200 ng/ml) plus the indicated concentration of FPP-3. Samples were processed by SDS-PAGE and transferred to a nitrocellulose filter. The immunoblot was then probed with anti-iNOS at a dilution 1 : 1000. Similar results were obtained from 3 independent experiments.
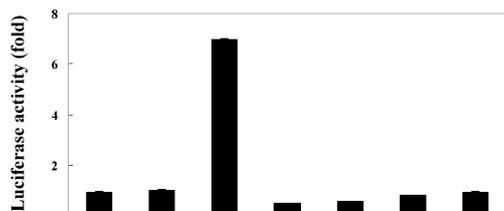
Fig. 3.   Effect of FPP-3 on NF-κB Transcriptional Activity in RAW264.7 Cells Stimulated with LPS

Cells were transiently co-transfected with NF-κB firefly luciferase reporter gene and *Renilla* luciferase reporter gene. 24 h later, cells were pretreated with DMSO or the indicated concentrations of FPP-3 for 30 min before stimulation with LPS (200 ng/ml) for 24 h or without stimulation. Cells were then lysed, and the luciferase activities were measured sequentially by a luminometer using a Dual-luciferase reporter assay system. Values (means±S.D.) are expressed as activity of the control from four independent experiments.

in terms of the activity of expressed luciferase by a luminometer. As shown in Fig. 3, LPS-stimulation resulted in a significant increase in luciferase activity. NF-κB pathway inhibitor, pyrrolidine dithiocarbamate (PDTC) as well as FPP-3 for 30 min followed by stimulation with LPS strongly inhibited this activity.

Effects of FPP-3 on IκB-α Degradation LPS-Stimulated RAW264.7 Cells   Activation of NF-κB required the phosphorylation and proteolytic degradation of IκB-α. To determine whether the inhibitory action of FPP-3 was due to its effects on IκB-α degradation, the IκB-α protein expression level was examined at the indicated dose for various time intervals after LPS-stimulation in RAW264.7 cells. As shown Fig. 4, partial degradation of IκB-α was noted within 20 min and complete degradation had occurred by 30 min after LPS-stimulation. The full reappearance of IκB-α was observed by 120 min 25 μM of FPP-3 completely inhibited a
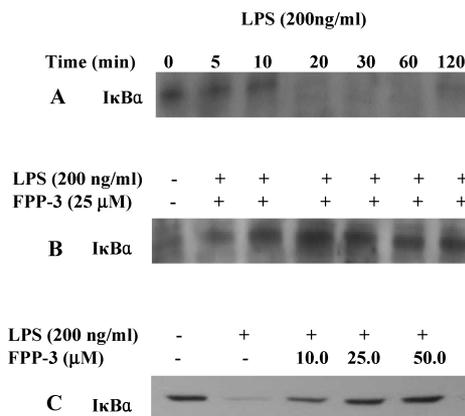

Fig. 4.   Time Course and Concentration Effect of FPP-3 on LPS-Stimulated Expression of IκB-α Protein

The RAW264.7 cells (1×10^6 cells/ml) were incubated for the indicated time with LPS (200 ng/ml) without (A) or with FPP-3 (B) and an increasing concentration of FPP-3 (C). IκB-α protein expression was measured by Western blotting as described in the experimental procedures.

Table 1.   Inhibition of TNF-α Production by FPP-3 in LPS-Stimulated RAW264.7 Cells

FPP-3 (μM)	TNF-α production (pg/ml)
Without FPP-3	1328.1±76.2
2.5	1593.1±98.8
5.0	1251.4±32.1
10.0	1071.1±30.1
25.0	705.9±25.7
30.0	560.2±22.5

RAW264.7 cells (1×10^6 cells/ml) were preincubated with FPP-3 at the indicated concentration for 30 min prior to the addition of LPS. The cells were incubated for an additional 1 h with 200 ng/ml LPS. Cell culture supernatants were measured using a commercially available mouse cytokine ELISA kit. The error bars represent the mean±S.D. of 3 individual experiments.

blockade of IκB-α degradation. These results suggest that FPP-3 inhibited IκB-α degradation through the blockade of NF-κB activation.

Inhibition of TNF-α Production by FPP-3   In order to determine whether or not FPP-3 can modulate LPS-stimulated proinflammatory cytokine such as TNF-α production, the cells were pretreated with various FPP-3 concentrations for 30 min prior to LPS stimulation. The production of TNF-α by the unstimulated RAW264.7 cells was <15 pg/ml ($n=3$). Incubation of these cells with LPS (200 ng/ml) for 1 h caused a substantial increase in TNF-α production (1328 pg±76/ml; $n=3$). When RAW264.7 cells were stimulated with LPS in the presence of FPP-3 (0—30 μM), a concentration-dependent inhibition of TNF-α production with a respective IC$_{50}$ value of 13.1 μM was observed (Table 1). Inhibition of LPS-stimulated TNF-α production in RAW264.7 cells was not attributed to cytotoxicity as assessed by trypan blue exclusion and MTT assay (data not shown).

DISCUSSION

In this study a synthetic propenone compound, FPP-3, was demonstrated to inhibit the production of NO *via* a decrease in iNOS protein expression through the inhibition of NF-κB activation. Our continuing efforts to develop anti-inflammatory agents from synthetic compounds led us to synthesize

propenone compound, which was demonstrated to inhibit the production of TNF-α and NO in the LPS-stimulated RAW264.7 macrophages.

To explore the mechanism of inhibition of NO production by FPP-3, the expression of iNOS protein and NF-κB activation was examined using a Western blot and luciferase activity assay, respectively. FPP-3 inhibited the LPS-induced expression of iNOS protein expression (Fig. 2) and NF-κB promoter activation (Fig. 3) in a parallel concentration-dependent manner with IC$_{50}$ values similar to those required for inhibiting NO production. Under the same conditions, an NF-κB pathway inhibitor, PDTC, strongly inhibited NF-κB promoter activation.

The transcription factor NF-κB is a candidate for regulating the pro-inflammatory cytokines such as TNF-α and IL-6 gene because the consensus binding sites for the transcription factor are present in them.[9,11] NF-κB also regulates a large number of genes involved in inflammation, including cytokines (*e.g.*, IL-1, IL-8, G-CSF and GM-CSF), cell adhesion molecules, and enzymes (*e.g.*, NOS, COX-2).[12]

NF-κB in unstimulated cells is present as an inactive heterodimer of the p50/p56 subunits bound to the NF-κB inhibitory protein, IκB-α. The next stage of this study was to determine whether or not LPS-induced iNOS protein expression is regulated by the IκB-α degradation. As shown in Fig. 4, the complete LPS-stimulated IκB-α degradation was observed after 20 min and reappearance of IκB-α was observed by 120 min. Together with NF-κB promoter luciferase assay, FPP-3 inhibited NF-κB activation degradation through a blockade of IκB-α.

Evidence showing that proinflamatory cytokines such as TNF-α, inerleukin-1β (IL-1β) and inerleukin-6 (IL-6) mediate the development of various inflammatory diseases has been provided.[13—16] In order to determine if FPP-3 can modulate TNF-α production, the cells were pretreated with FPP-3 at various concentrations for 30 min prior to LPS stimulation. When the RAW264.7 cells were stimulated with LPS (200 ng/ml) in the presence of FPP-3, TNF-α production was inhibited with an IC$_{50}$ value of 13.1 μM (Table 1).

Although the physiological or normal production of NO played a important role in the host defense against various pathogens, the overproduction of NO can be harmful and result in septic shock, rheumatoid arthritis and autoimmune diseases.[17—19] Therefore, therapeutic agents that inhibit the biosynthesis of NO as well as TNF-α production may be useful for the relieving these inflammatory conditions.

In conclusion, the anti-inflammatory activity of FPP-3 *in vitro* could be was attributed at least in part to inhibition of the TNF-α production and iNOS protein expression. These results could also serve as an additional rationale for the use of propenone derivatives in inflammatory disorders. The precise mechanism by which FPP-3 regulates iNOS and TNF-α production in RAW264.7 cells is the subject of an ongoing investigation.

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