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Dual inhibitory effects of furonaphthoquinone compound on enzyme activity and lipopolysaccharide-induced expression of cyclooxygenase-2 in macrophages

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Abstract

2-Methyl-2-(2-methylpropenyl)-2,3-dihydronaphtho[2,3-*b*]furan-4,9-dione (NFD-37) is a synthetic furonaphthoquinone compound. In the present study, the NFD-37 compound was found to inhibit prostaglandin (PG) E_2 production in lipopolysaccharide (LPS)-stimulated macrophages RAW 264.7. NFD-37 compound exhibited a preferred inhibition on enzyme activity of cyclooxygenase (COX)-2 over COX-1. Further, NFD-37 compound attenuated LPS-induced synthesis of both mRNA and protein of COX-2, and suppressed LPS-induced COX-2 promoter activity in the macrophages, indicating that the furonaphthoquinone compound could down-regulate LPS-induced COX-2 expression at the transcription level. Even though COX-2 promoter behaves as a sophisticated biosensor for host defense, nuclear factor (NF)-κB activation has been evidenced to play a major mechanism for LPS-induced COX-2 expression in macrophages. NFD-37 compound exhibited a dose-dependent inhibitory effect on LPS-induced phosphorylation of inhibitory κBα (IκBα) protein, and subsequently inhibited IκBα degradation, DNA binding activity of NF-κB complex as well as NF-κB transcriptional activity in macrophages RAW 264.7. In another experiment, NFD-37 compound inhibited both COX-2 promoter activity and GST-IκBα phosphorylation elicited by an expression vector encoding IκB kinase β. Taken together, NFD-37 compound inhibited enzyme activity of COX-2 but also suppressed COX-2 expression depending on NF-κB activation, and thus could provide an invaluable tool to investigate pharmacological potential in the excess PG-related disorders.

Keywords: Furonaphthoquinone compound; Prostaglandin E_2; Cyclooxygenase-2; Nuclear factor-κB

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been widely used for the relief of inflammation and pain. Recently, aspirin and some of other NSAIDs have received attention to prevent cardiovascular disease and colon cancer [1–3]. It is generally accepted that anti-inflammatory and analgesic efficacy of NSAIDs is dependent on their inhibitory effects on the enzyme activity of cyclooxygenase (COX), a key enzyme in the biosynthetic pathway of prostaglandins (PGs) starting from arachidonic acid [4]. Two distinct isoforms of COX were identified in the early 1990s. COX-1 is constitutively expressed in nearly all tissues and provides PGs to maintain physiological functions such as cytoprotection of the stomach and regulation of renal blood flow [4,5]. In contrast, COX-2 is inducible in the immune cells such as macrophages and synoviocytes in response to infection, injury or other stress and produces excessive amounts of PGs that serve to sensitize nociceptors and induce inflammatory states [6,7]. PGs also contributed

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to tumor growth by inducing angiogenesis and inhibiting apoptosis [8–10]. More recently, celecoxib, a selective COX-2 inhibitor, has been approved by the FDA for the treatment of arthritis and colon cancer [11,12]. Even though COX inhibition could be important for pharmacological activity of NSAIDs, several lines of evidence suggest that some NSAIDs could modulate COX-independent cellular responses including nuclear factor (NF)-κB activation, which are also involved in anti-inflammatory and antitumor activity of the drugs [13,14].

Lipopolysaccharide (LPS) is a major constituent of the outer membrane in Gram-negative bacteria and is recognized by Toll-like receptor 4 (TLR4) on immune cells such as macrophages [15]. Specific binding of LPS to TLR4 can trigger signaling pathways to activate NF-κB [16]. Under normal conditions, NF-κB is retained in the cytoplasm as an inactive complex, bound to inhibitory κB protein (IκB) [17]. Upon stimulation with LPS, cytoplasmic IκBα can be phosphorylated on its Ser-32 and -36 residues by the IκB kinase (IKK) complex, which marks for ubiquitination followed by 26S proteasome-mediated degradation [18,19]. NF-κB such as p65 or p50 subunit, freed IκB, unmasks its nuclear localization signal motif and now moves into the nucleus [20]. NF-κB complex, homo- or hetero-dimeric forms, then binds to the promoter regions of immune and inflammatory genes including COX-2 for transcriptional activation [21].

2-Methyl-2-(2-methylpropenyl)-2,3-dihydronaphtho[2,3-*b*]furan-4,9-dione (NFD-37) is a synthetic furonaphthoquinone compound (Fig. 1). Phytochemical or chemically synthetic furonaphthoquinone analogs have been reported to exhibit antitumor activity against lung cancer cells and antimicrobial activity against *Helicobacter pylori* [22,23]. However, pharmacological mechanism of the furonaphthoquinone analogs remains to be determined. In the present study, furonaphthoquinone NFD-37 compound was found to inhibit PGE_2 production in LPS-stimulated macrophages RAW 264.7. NFD-37 compound inhibited the enzyme activity of COX-2 over COX-1, but also suppressed LPS-induced COX-2 expression at the transcription level. Further, NFD-37 compound inhibited LPS-induced NF-κB activating pathway. These results could provide a mechanism for pharmacological activity of the furonaphthoquinone analogs, in addition to anti-inflammatory potential of NFD-37 compound.

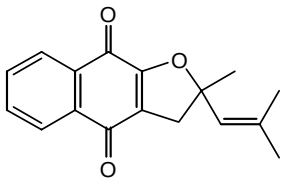

Fig. 1. Chemical structure of NFD-37 compound.

Materials and methods

Materials and cell culture. Furonaphthoquinone NFD-37 compound (purity, >98%) was prepared as described in our previous work [24]. Antibodies against COX-2, COX-1 IκBα or IKKβ were purchased from Santa Cruz Biotech (Santa Cruz, USA), and antibody against phospho-IκBα (Ser-32/36) was from Cell Signaling Tech (Berverly, USA). Fetal bovine serum (FBS) and Lipofectamine were purchased from Invitrogen (Carlsbad, USA). The other reagents including LPS (*Escherichia coli* 05:B5) and NS-398 were otherwise obtained from Sigma–Aldrich (St. Louis, USA). Macrophages RAW 264.7 were cultured in a medium (10 mg/ml DMEM, 10 mM Hepes, 24 mM $NaHCO_3$, 143 U/ml benzylpenicillin potassium, and 100 μg/ml streptomycin sulfate, pH 7.1) containing 10% FBS and maintained at 37 °C with 5% CO_2 atmosphere. Macrophages RAW 264.7 harboring pNF-κB-secretory alkaline phosphatase (SEAP)-NPT [25] were cultured in the same conditions, except supplement of geneticin (500 μg/ml) to the media.

PGE_2 ELISA. Macrophages RAW 264.7 were pre-treated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 24 h. Amounts of PGE_2 in the culture media were quantified using an ELISA kit (Amersham-Pharmacia, San Francisco, USA).

Measurement of enzyme activity of COX-1 or -2. COX activity was measured by the initial rate of oxygen consumption in the presence of arachidonic acid as a substrate. A typical reaction mixture consisted of 0.1 M Tris–HCl (pH 8.0), 100 μM arachidonic acid, 25 μg hemoglobin, and purified COX-1 or COX-2 (Cayman Chem., Ann Arbor, USA).

Western immunoblot analysis. Macrophages RAW 264.7 were pretreated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 5 min (phospho-IκBα), 20–80 min (IκBα) or 18 h (COX-2). Lysates of the cells were subjected to Western immunoblot analysis as described in our previous work [26]. The blots were finally reacted with ECL detection reagents (Amersham-Pharmacia, San Francisco, USA) and exposed to X-ray film.

Semi-quantitative RT-PCR. Macrophages RAW 264.7 were pretreated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 6 h. Total RNA of the cells was subjected to semi-quantitative RT-PCR using an RNA PCR kit (Bioneer, Taejon, Korea). Primer sequences and RT-PCR conditions were described in our previous work [27]. Briefly, total RNA was reverse-transcribed at 42 °C and then subjected to 30 cycles of PCR consisting of 30-s denaturation at 94 °C, 30-s annealing at 60 °C, and 90-s extension at 72 °C. The RT-PCR products were resolved by agarose gel electrophoresis and then stained with ethidium bromide.

Measurement of COX-2 promoter activity. Macrophages RAW 264.7 were transiently transfected with pCOX-2-luciferase (Luc) construct [28] and pSV-β-galactosidase control vector (Promega, Madison, USA) using Lipofectamine. The transfected RAW 264.7 cells were pretreated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 16 h. Lysates of the cells were subjected to luciferase assay and β-galactosidase assay using kits from Promega (Madison, USA). In another experiment, macrophages RAW 264.7 were transiently transfected with pCOX-2-Luc construct [28] in combination with an expression vector encoding IKKβ [29] using Lipofectamine. The transfected cells were treated with NFD-37 compound for 16 h and then subjected to luciferase assay.

Measurement of NF-κB transcriptional activity. Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT construct [25] were pretreated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 16 h. Aliquots of the cell-free culture media were heated at 65 °C for 5 min and then reacted with an assay buffer (500 μM of 4-methylumbelliferyl phosphate, 2 mM diethanolamine, and 1 mM $MgCl_2$) in the dark at room temperature for 1 h. SEAP activity was measured as relative fluorescence units (RFU) with emission at 449 nm and excitation at 360 nm. In another experiment, macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT construct [25] were transiently

transfected with an expression vector encoding IKKβ [29] using Lipofectamine. The transfected cells were treated with NFD-37 compound for 16 h and then subjected to SEAP assay.

Electrophoretic mobility shift assay (EMSA). Macrophages RAW 264.7 were pre-treated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 1 h. Nuclear extracts of the cells were reacted with [^{32}P]-labeled oligonucleotide (5′-AGTTGAGGGGACTTTCC-CAGGC-3′) in a binding buffer (10 mM Tris, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 0.05 μg/μl poly(dI–dC), and 4% glycerol, pH 7.5) on ice for 10 min. The complexes between oligonucleotide and nuclear protein were resolved on non-denaturing acrylamide gel by electrophoresis. The gels were dried and then exposed to X-ray film.

In vitro kinase assay of IKKβ. Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT construct [25] were transiently transfected with an expression vector encoding IKKβ [29] using Lipofectamine. The transfected cells were treated with NFD-37 compound for 16 h. Lysates (200 μg protein) of the cells were incubated on ice for 2 h with anti-IKKβ antibody (2 μg) and protein A beads (Amersham-Pharmacia, San Francisco, USA). In vitro kinase assays were carried out with the immunoprecipitates and bacterially synthesized GST-IκBα (2 μg) in an assay buffer (20 mM Hepes, 2 mM $MgCl_2$, 2 μM ATP, 5 μCi [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 300 μM Na_3VO_4, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT, pH 7.7) at 30 °C for 1 h. The reaction mixtures were resolved on SDS–acrylamide gel by electrophoresis and then subjected to autoradiography.

Statistical analysis. Data are expressed as means ± SEM and were analyzed by ANOVA, followed by the Dunnet test. A value of $p < 0.01$ was considered significant.

Results

Furonaphthoquinone NFD-37 compound inhibited LPS-induced PGE_2 production in macrophages RAW 264.7

Macrophages RAW 264.7 in resting state released 267 ± 55 pg/ml of PGE_2 during incubation for 24 h, whereas PGE_2 production was increased to 1464 ± 51 pg/ml when the cells were stimulated with LPS alone for the same period (Fig. 2A). NFD-37 compound inhibited LPS-induced PGE_2 production in a dose-dependent manner, corresponding to 40% inhibition at 1 μM, 69% at 3 μM, and 94% at 10 μM showing an IC_{50} value of 1.7 μM (Fig. 2A). As a positive control, NS-398, a selective inhibitor of COX-2, also exhibited dose-dependent inhibitory effect with an IC_{50} value of 0.4 μM on LPS-induced PGE_2 production (Fig. 2A). Neither NFD-37 nor NS-398 at the effective concentrations showed any cytotoxic effect in macrophages (data not shown).

NFD-37 compound showed a preferred inhibitory effect on the enzyme activity of COX-2 over COX-1

We next investigated whether NFD-37 compound could inhibit the enzyme activity of cell-free COX isozymes. NFD-37 compound inhibited the COX-2 activity in a dose-dependent manner, corresponding to 34% inhibition at 3 μM, 55% at 10 μM, and 84% at 30 μM showing

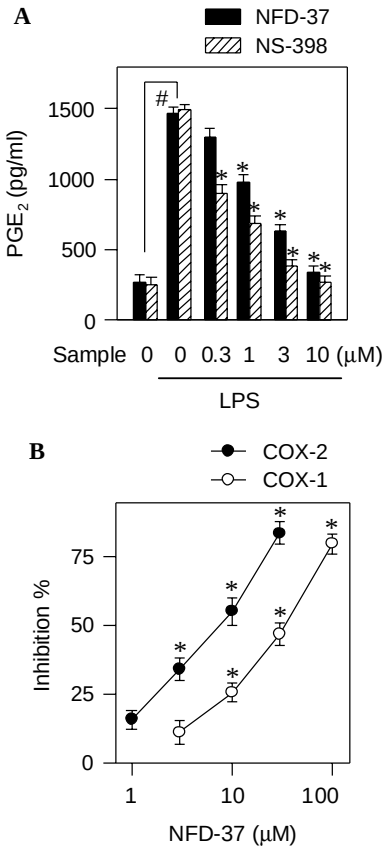

Fig. 2. Effects of NFD-37 compound on LPS-induced PGE_2 production and enzyme activity of COX isozymes. Macrophages RAW 264.7 were pre-treated with NFD-37 for 2 h and stimulated with LPS (1 μg/ml) for 24 h. Amounts of PGE_2 in the culture media were measured by ELISA (A). Enzyme activity of purified COX-1 or COX-2 was analyzed by the initial rate of oxygen consumption and the effect of NFD-37 compound is represented as inhibition % (B). Values are means ± SEM of three independent experiments. $^{\#}p < 0.01$ vs. media alone-treated group (A). $^{*}p < 0.01$ vs. LPS alone-treated group (A) or enzyme alone-treated group (B).

an IC_{50} value of 4.8 μM (Fig. 2B). NFD-37 compound also exhibited dose-dependent inhibitory effect with an IC_{50} value of 36.8 μM on COX-1 activity (Fig. 2B).

NFD-37 compound down-regulated LPS-induced COX-2 expression at the transcription level

To investigate whether NFD-37 compound could affect LPS-induced COX-2 expression, Western immunoblot analysis was carried out. COX-2 protein was hardly detectable in resting macrophages RAW 264.7, but pronounced amounts of COX-2 protein were induced upon exposure to LPS alone (Fig. 3A). However, synthesis of COX-1 protein was not changed by treatment of LPS and NFD-37 compound (Fig. 3A). NFD-37 compound inhibited LPS-induced synthesis of COX-2 protein in a dose-dependent manner, corresponding to 22% inhibition at 3 μM, 61% at 10 μM, and 91% at 30 μM showing

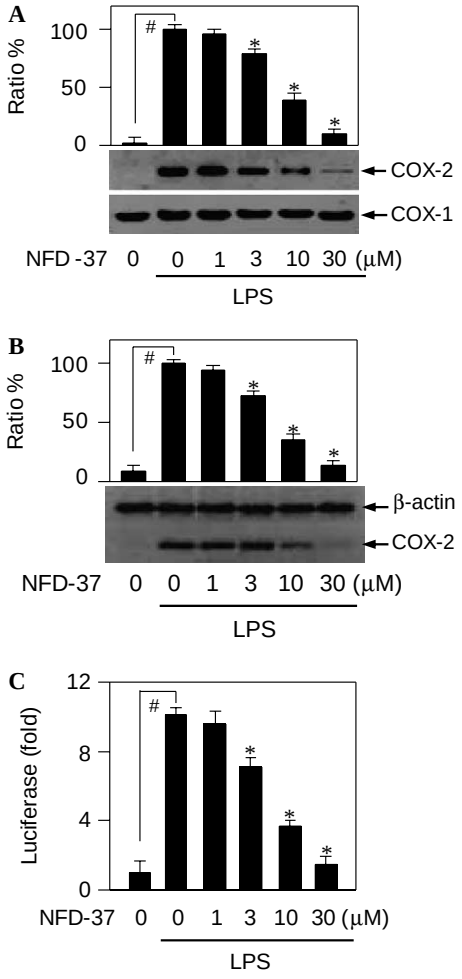

Fig. 3. Effect of NFD-37 compound on LPS-induced COX-2 expression. Macrophages RAW 264.7 were pre-treated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 18 h (A) or 6 h (B). Cell lysates were subjected to Western immunoblot analysis. One of similar results is represented and relative ratio % is also shown, where COX-2 signal was normalized to COX-1 signal (A). Total RNA of the cells was subjected to semi-quantitative RT-PCR. One of similar results is represented and relative ratio % is also shown, where COX-2 signal was normalized to β-actin signal (B). Macrophages RAW 264.7 were transiently transfected with pCOX-2-Luc construct and pSV-β-galactosidase control vector. The transfected cells were pre-treated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 16 h. Cell lysates were subjected to luciferase assay and β-galactosidase assay. Luciferase expression as a reporter of COX-2 promoter activity is represented as relative fold, where luciferase activity was normalized to β-galactosidase activity (C). Values are means ± SEM of three independent experiments. $^{\#}p < 0.01$ vs. media alone-treated group. $^{*}p < 0.01$ vs. LPS alone-treated group.

an IC_{50} value of 7.6 μM (Fig. 3A). To further understand whether inhibitory effect of NFD-37 compound on COX-2 expression had taken place at the transcription level, semi-quantitative RT-PCR was carried out. COX-2 transcript at steady state was hardly detectable in resting macrophages RAW 264.7, but markedly increased when the cells were stimulated with LPS alone (Fig. 3B). However, amounts of housekeeping β-actin transcript were not affected by treatment of LPS and NFD-37 compound (Fig. 3B). NFD-37 compound inhibited LPS-induced synthesis of COX-2 transcript in a dose-dependent manner, corresponding to 28% inhibition at 3 μM, 65% at 10 μM, and 87% at 30 μM (Fig. 3B). Transcriptional regulation of COX-2 expression by NFD-37 compound was further documented by COX-2 promoter activity. The promoter activity was determined using macrophages RAW 264.7 transfected transiently with pCOX-2-Luc construct encoding COX-2 promoter (−963/+1) fused to luciferase gene as a reporter [28]. Upon exposure to LPS alone, the transfected cells increased luciferase expression up to 10-fold over the basal level (Fig. 3C). NFD-37 compound inhibited LPS-induced luciferase expression in a dose-dependent manner, corresponding to 33% inhibition at 3 μM, 70% at 10 μM, and 95% at 30 μM (Fig. 3C).

NFD-37 compound inhibited LPS-induced NF-κB transcriptional activity in macrophages

NF-κB is a transcription factor that plays a major mechanism in the LPS-induced expression of pro-inflammatory proteins including COX-2 in macrophages [21,30]. NF-κB transcriptional activity was monitored using macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT construct encoding four copies of the κB sequence fused to the SEAP gene as a reporter [25]. Upon exposure to LPS alone, the transfected cells increased the SEAP expression up to 4-fold over the basal level (Fig. 4A), indicating that cellular NF-κB is transcriptionally functional. NFD-37 compound inhibited LPS-induced SEAP expression in a dose-dependent manner, corresponding to 35% inhibition at 3 μM, 74% at 10 μM, and 87% at 30 μM showing an IC_{50} value of 5.7 μM (Fig. 4A).

NFD-37 compound inhibited LPS-induced DNA binding activity of the NF-κB complex but also LPS-induced degradation of IκBα

To elucidate the inhibitory mechanism on NF-κB activation, we performed an EMSA with a [^{32}P]-labeled oligonucleotide containing the κB sequence. Upon exposure to LPS alone, the RAW 264.7 cells markedly increased the DNA binding activity of the NF-κB complex, p65/p50 and p50/p50 (Fig. 4B). NFD-37 compound inhibited LPS-increased DNA binding activities of the NF-κB complex in a dose-dependent manner (Fig. 4B). To further understand whether NFD-37 compound could affect IκB degradation, Western immunoblot analysis with anti-IκBα antibody was carried out with cytoplasmic extracts of LPS-stimulated macrophages RAW 264.7. Upon exposure to LPS alone, IκBα phosphorylation was dramatically caused within 20 min and the amounts of cytoplasmic IκBα recovered to the

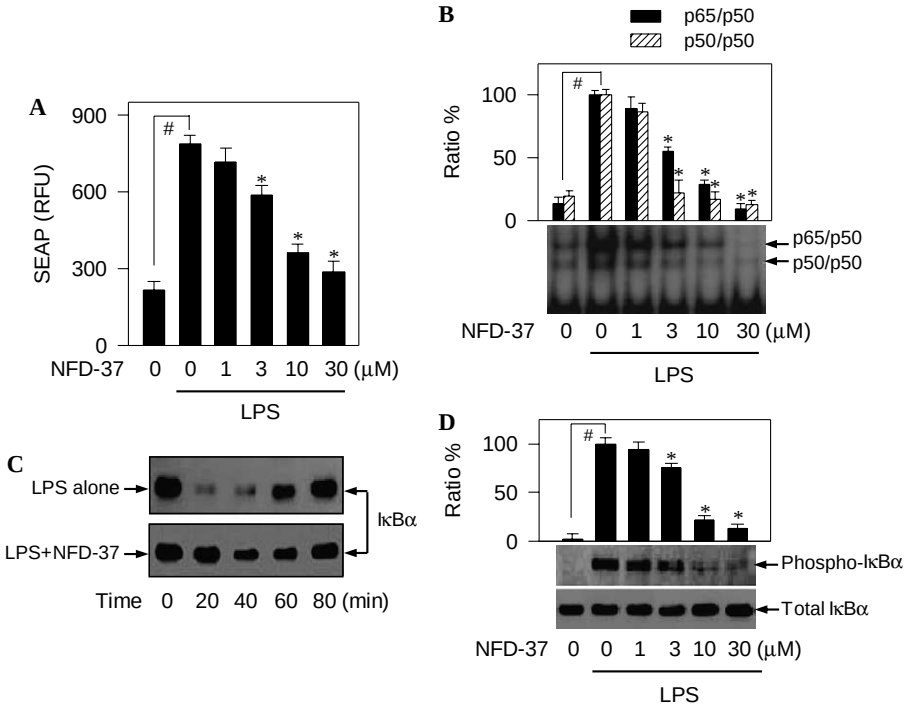

Fig. 4. Effect of NFD-37 compound on LPS-induced NF-κB signaling pathway. Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT construct were pre-treated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 16 h. SEAP expression as a reporter of NF-κB transcriptional activity was measured with the cell-free culture media and is represented as relative fluorescence units (RFU) (A). Macrophages RAW 264.7 were pre-treated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 1 h. Nuclear extracts of the cells were subjected to EMSA with [^{32}P]-labeled oligonucleotide corresponding to κB sequence. One of similar results is represented and relative ratio % is also shown (B). Macrophages RAW 264.7 were pre-treated with NFD-37 compound (10 μM) for 2 h and stimulated with LPS (1 μg/ml) for the indicated times. Cytoplasmic extracts of the cells were subjected to Western immunoblot analysis with anti-IκBα antibody (C). Macrophages RAW 264.7 were pre-treated with NFD-37 compound for 2 h and stimulated with LPS (1 μg/ml) for 5 min. Cytoplasmic extracts of the cells were subjected to Western immunoblot analysis with anti-phospho-IκBα antibody. One of similar results is represented and relative ratio % is also shown, where phospho-IκBα signal was normalized to total IκBα signal (D). Values are means ± SEM of three independent experiments. $^{\#}p < 0.01$ vs. media alone-treated group. $^{*}p < 0.01$ vs. LPS alone-treated group.

normal level at 80 min after LPS stimulation (Fig. 4C). NFD-37 compound (10 μM) showed significant inhibitory effect on LPS-induced IκBα degradation in the time-course study (Fig. 4C). IκBα should be phosphorylated on its Ser-32 and -36 residues by the IKK complex, in order to mark for ubiquitination followed by proteasome-mediated degradation [18,19]. Upon exposure to LPS alone for 5 min, IκBα phosphorylation had markedly taken place but IκBα degradation had not started yet (Fig. 4D). At the 5-min time point, NFD-37 compound inhibited LPS-induced IκBα phosphorylation in a dose-dependent manner, corresponding to 25% inhibition at 3 μM, 78% at 10 μM, and 87% at 30 μM (Fig. 4D).

NFD-37 compound inhibited COX-2 promoter activity and NF-κB activation triggered by the expression of IKKβ

To further document whether NFD-37 compound could inhibit NF-κB-dependent COX-2 promoter activity, macrophages RAW 264.7 were transiently transfected with the pCOX-2-Luc construct [28] in combination with an expression vector encoding IKKβ [29]. Luciferase expression as a reporter of COX-2 promoter activity was efficiently increased by the expression of IKKβ (Fig. 5A). NFD-37 inhibited IKKβ-elicited COX-2 promoter activity in a dose-dependent manner with an IC_{50} value of 7.1 μM (Fig. 5A). Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT construct [25] were also transiently transfected with an expression vector encoding IKKβ [29]. The transfected cells were treated with NFD-37 compound. SEAP expression as a reporter of NF-κB transcriptional activity was measured with the cell-free culture media, and GST-IκBα phosphorylation as an in vitro kinase activity was analyzed with IKKβ immunoprecipitates obtained from lysates of the cells. Both SEAP expression and GST-IκBα phosphorylation were significantly increased by the expression of IKKβ (Figs. 5B and C). NFD-37 compound exhibited dose-dependent inhibitory effects not only on IKKβ-elicited SEAP expression (Fig. 5B) but also GST-IκBα phosphorylation (Fig. 5C).

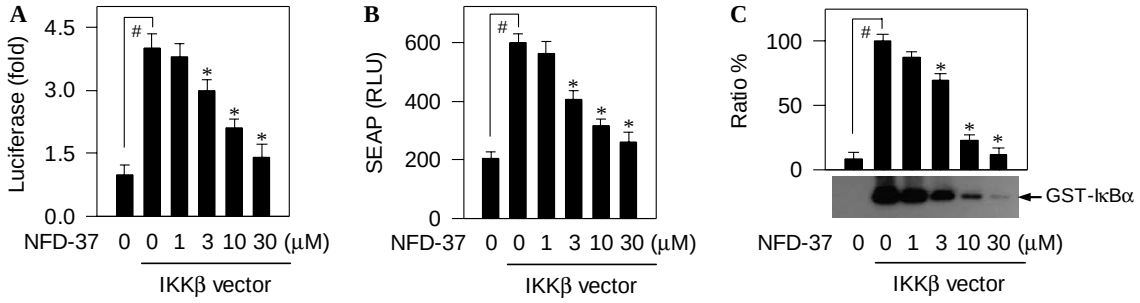

Fig. 5. Effect of NFD-37 compound on COX-2 promoter activity and NF-κB activation triggered by expression of IKKβ. Macrophages RAW 264.7 were transiently transfected with pCOX-2-Luc reporter construct in combination with an expression vector encoding IKKβ. The transfected cells were treated with NFD-37 compound for 16 h. Luciferase expression as a reporter of COX-2 promoter activity was measured with lysates of the cells and is represented as relative fold (A). Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT reporter construct were transiently transfected with an expression vector encoding IKKβ. The transfected cells were treated with NFD-37 compound for 16 h. SEAP expression as a reporter of NF-κB transcriptional activity was measured with the cell-free culture media and is represented as relative fluorescence units (RFU) (B). In vitro kinase assays with GST-IκBα as a substrate were carried out with IKKβ immunoprecipitates obtained from lysates of the cells. One of similar results is represented and relative ratio % is also shown (C). Values are means ± SEM of three independent experiments. $^{\#}p < 0.01$ vs. each reporter construct alone-transfected group. $^{*}p < 0.01$ vs. each reporter construct plus IKKβ-expression vector alone-transfected group.

Discussion

In the present study, furonaphthoquinone NFD-37 compound (Fig. 1) was discovered to show a dose-dependent inhibitory effect on PGE_2 production in LPS-stimulated macrophages RAW 264.7 (Fig. 2A). NFD-37 compound exhibited a preferred inhibition on enzyme activity of COX-2 over COX-1 (Fig. 2B). Furthermore, NFD-37 compound attenuated LPS-induced mRNA and protein syntheses of COX-2 (Figs. 3A and B) and inhibited LPS-induced COX-2 promoter activity in the macrophages (Fig. 3C). However, COX-1 synthesis was not affected by LPS and NFD-37 (Fig. 3A). Therefore, anti-inflammatory action of NFD-37 compound on PGE_2 production in LPS-stimulated macrophages RAW 264.7 turns out to be a dual mechanism to inhibit enzyme activity of COX-2 but also to suppress LPS-induced COX-2 expression at the transcription level.

A promoter region of COX-2 gene contains a canonical TATA box and various transcriptional regulatory elements, such as NF-κB, NF-interleukin 6, and cAMP responsive element [30,31]. NF-κB activation has been evidenced to play a major mechanism on LPS-induced COX-2 expression in macrophages [21,30]. NFD-37 compound inhibited LPS-induced NF-κB transcriptional activity (Fig. 4A). Further, NFD-37 compound inhibited LPS-induced DNA binding activity of the NF-κB complex (Fig. 4B), as well as LPS-induced IκBα degradation and phosphorylation (Figs. 4C and D). These results suggest that NFD-37 compound seems to inhibit NF-κB-dependent COX-2 expression and its control target would be LPS signaling molecule upstream IκBα phosphorylation on the NF-κB activating pathway. The possibility was tested by transiently transfecting macrophages RAW 264.7 with each reporter construct for COX-2 promoter activity or NF-κB transcriptional activity, in combination with an expression vector encoding IKKβ, a Ser/Thr kinase that can phosphorylate IκBα as a substrate. NFD-37 compound inhibited IKKβ-elicited COX-2 promoter activity (Fig. 5A). Further, NFD-37 compound inhibited IKKβ-elicited NF-κB transcriptional activity (Fig. 5B), as well as the expression vector-derived kinase activity to phosphorylate GST-IκBα (Fig. 5C).

NSAIDs are among the most widely used therapeutic agents for inflammation and pain, despite their potential gastrointestinal and renal side effects by inhibition of enzyme activity of housekeeping COX-1. Some of the NSAIDs have been evidenced to modulate the NF-κB activating pathway, which is not derived from their inhibitory effects on PG production. Aspirin, ibuprofen, and sulindac were reported to target IKK activity for their NF-κB inhibitory mechanisms, and fluriprofen to interfere with nuclear translocation of NF-κB complex without affecting IκB phosphorylation and degradation [32–35]. Even though cell type differences exist, indomethacin and ketoprofen were, however, reported to be inactive on the NF-κB activating pathway [14]. Celecoxib, a specific inhibitor of COX-2 activity, was also documented to down-regulate NF-κB-dependent COX-2 expression [36].

Taken together, NFD-37 compound, like celecoxib, inhibited enzyme activity of COX-2 but also suppressed COX-2 synthesis at the transcription level. The down-regulatory action of NFD-37 compound on LPS-induced COX-2 expression in macrophages was attributable to its inhibitory mechanism on IKKβ activity, a key molecule on the NF-κB activating pathway.

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