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Journal of Cellular Biochemistry 112:3594–3603 (2011)

Journal of Cellular Biochemistry

Anti-Inflammatory Effect of Pelubiprofen, 2-[4-(oxocyclohexylidenemethyl)-phenyl]propionic Acid, Mediated by Dual Suppression of COX Activity and LPS-Induced Inflammatory Gene Expression via NF-κB Inactivation

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ABSTRACT

Pelubiprofen is a non-steroidal anti-inflammatory drugs (NSAIDs) that is related both structurally and pharmacologically to ibuprofen. Anti-inflammatory properties of ibuprofen are due to its ability to both decrease prostaglandin synthesis by inhibiting the activities of cyclooxygenases (COXs) and IκB kinase-β (IKK-β). However, the exact mechanisms that accounts for the anti-inflammatory effects of pelubiprofen are not reported. In this study, we investigated the molecular mechanisms how pelubiprofen modulates the inflammatory mediators in LPS-induced macrophages and carrageenan-induced acute inflammatory rat model. Pelubiprofen potently diminished PGE_2 productions through inhibition of COX enzyme activity (IC_{50} values for COX-1 and COX-2 are 10.66 ± 0.99 and 2.88 ± 1.01 μM, respectively), but also reduced the expressions of COX-2, inducible nitric oxide (iNOS), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 at transcriptional level in LPS-induced RAW 264.7 cells. In addition, pelubiprofen attenuated the LPS-induced transcription activity and the DNA binding activity of NF-κB, which was accompanied by a parallel reduction of degradation and phosphorylation of inhibitory kappa B-α (IκB-α) and consequently by decreased nuclear translocation of NF-κB. Furthermore, pelubipofen inhibited the LPS-induced phosphorylation of IKK-β and transforming growth factor-β activated kinase-1 (TAK1). In acute inflammatory rat model, pretreatment with pelubiprofen inhibited carrageenan-induce edema, neutrophil migration, PGE_2 production, and p65, a subunit of NF-κB, nuclear translocation in inflamed paw. Taken together, our data indicated that pelubiprofen is involved in the dual inhibition of COX activity and TAK1-IKK-NF-κB pathway, revealing molecular basis for the anti-inflammatory properties of pelubiprofen. J. Cell. Biochem. 112: 3594–3603, 2011.

KEY WORDS: CYTOKINES; CYCLOOXYGENASE; NITRIC OXIDE; NUCLEAR FACTOR-κB; PAW EDEMA; PELUBIPROFEN

Non-steroidal anti-inflammatory drugs (NSAIDs) constitute an important class of drugs treating various inflammatory disease, such as rheumatoid arthritis (RA) and osteoarthritis (OA), and various pain diseases [Callejas et al., 2003]. It is well established that the effects of NSAIDs are primarily mediated by inhibition of enzymatic activity of cyclooxygenase (COX), which convert arachidonic acid to prostaglandins (PGs) [Tegeder et al., 2001b]. The two isoenzymes, COX-1 and COX-2, are encoded by different genes and have distinct physiological functions. COX-1 is expressed constitutively in most tissues [O'Neill and Ford-Hutchinson, 1993],

Grant sponsor: The Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea; Grant number: A090487.

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Received 28 June 2011; Accepted 19 July 2011 • DOI 10.1002/jcb.23290 • © 2011 Wiley Periodicals, Inc.
Published online 1 August 2011 in Wiley Online Library (wileyonlinelibrary.com).

and is believed to be involved in the release of PGs with cellular "house-keeping" functions, such as the maintenance of gastrointestinal integrity and vascular homeostasis. On the other hand, COX-2 is undetectable in most tissues under normal conditions, but is induced as an intermediate-early gene in a limited repertoire of cells, such as, monocytes, macrophages, neutrophils, and endothelial cells, after exposure to a noxious stimulus, such as, bacterial lipopolysaccharides (LPS), growth factors, cytokines, or phorbol esters [Niiro et al., 1997]. The COX-dependent PGs fulfill different physiological functions, and inhibition of COX may lead to undesired effects, such as gastointestinal and renal toxicity [Jaksch et al., 2008]. It has been postulated that troublesome problem of NSAIDs arises from inhibition of the COX-1 isoform. New generations of anti-inflammatory drugs have been developed to enhance the anti-inflammatory and analgesic activities of classic NSAIDs, and to reduce the adverse effects of these agents. Selective COX-2 inhibitors are viewed enthusiastically because they match traditional NSAIDs in terms of efficacy [Tegeder et al., 2001b], but circumvent constitutively active COX-1 and are comparatively free of stomach-associated complications. However, selective COX-2 inhibitors including rofecoxib and valdecoxib were recently withdrawn because of their adverse cardiovascular side effects [Jaksch et al., 2008]. Moreover, a mechanism unrelated to inhibition of prostaglandin synthesis has been found for NSAIDs. Acetylsalicylic acid, which is commonly considered to act through the inhibition of COX-mediated prostaglandin synthesis, also has been shown to block NF-κB activity by preventing inhibitory kappa B-α (IκB-α) phosphorylation and degradation after stimulation with TNF-α or LPS [Kopp and Ghosh, 1994; Weber et al., 1995]. Other antirheumatic drugs, such as ibuprofen [Scheuren et al., 1998; Palayoor et al., 1999], salicylic acid [Grilli et al., 1996], and sulfasalzine [Wahl et al., 1998], have been also reported to inhibit NF-κB activation.

There is much evidence suggesting that some of the NSAIDs effects are independent of COX inhibition because the concentration of these drugs required for other activity are higher than those necessary to inhibit PG synthesis [Tegeder et al., 2001b; Takada et al., 2004]. Similarly, extensive research has shown that aspirin and some other NSAIDs have protective effects against colon cancer, cardiovascular, and alzheimer's diseases [Takada et al., 2004] via the inhibition of mitogen-activated protein kinases (MAPKs) and IκB kinases (IKKs), which impairs the NF-κB-dependent transcription genes [Tegeder et al., 2001b; Callejas et al., 2003].

NF-κB is an inducible-transcription factor that plays a critical role in inflammatory and stress responses, proliferation, differentiation, and apoptosis [Karin and Ben-Neriah, 2000]. It is generally found that p50/p65 heterodimer in an inactive state binds with inhibitor protein, IκB-α in the cytoplasm. By following cellular stimulation, IκB-α is phosphorylated at specific amino-terminal serine residues (Ser 32 and Ser 36 for IκB-α), which is mediated by IKK complex, then undergoes poly-ubiquitination and proteasomal degradation, allowing NF-κB to translocate to the nucleus and regulate expression of target genes, many of which regulate inflammation and cell growth [Ben-Neriah, 2002; Okamoto et al., 2008].

Pelubiprofen, 2-[4-(Oxocyclohexylidene methyl)phenyl]propionic acid, is one of the 2-arylpropionic acid class of NSAIDs along with ibuprofen, flurbiprofen, and naproxen. It has wide variety of indications proposed: OA, RA, musculoskeletal pain, post-operative trauma, backache, neck-shoulder syndrome, and dental pain. Like other NSAIDs, the anti-inflammatory and antinociceptive activities of the marketed pelubiprofen racemate have been traditionally explained by its COX-mediated inhibition of prostaglandin synthesis. However, it has not been previously reported to explore the molecular mechanism of anti-inflammatory acticity of pelubiprofen. As a part of our ongoing screening program to evaluate the anti-inflammatory potentials of new compounds, we investigated the molecular mechanisms of pelubiprofen for its anti-inflammatory properties in LPS-induced macrophages and then in carrageenan-induced paw edema models.

MATERIALS AND METHODS

CHEMICALS

Pelubiprofen (Chemical structure shown in Fig. 1A) was gifted from DAEWON PHARM CO., LTD (Seoul, Korea). Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY). iNOS, COX-2, p65, antiphospho-IκB-α, antiphospho-IKK-α/β, antiphospho-transforming growth factor-β activated kinase-1(TAK1), IκB, IKK-α, IKK-β, TAK1, poly(ADP ribose)polymerase (PARP), β-actin monoclonal antibodies, and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The enzyme immunoassay (EIA) kits for PGE_2, TNF-α, interleukin-1β (IL-1β), and IL-6 were obtained from R&D Systems (Minneapolis, MN). Random oligonucleotide primers and M-MLV reverse transcriptase were purchased from Promega (Madison, WI). dNTP Mix and ex Taq were obtained from TaKaRa (Seoul, Korea). Inducible nitric oxide (iNOS), COX-1, COX-2, TNF-α, IL-1β, IL-6, and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT), sulfanilamide, aprotinin, leupeptin, phenylmethanesulfonylfluoride (PMSF), dithiothreitol (DTT), L-N^6-(1-iminoethyl)lysine (L-NIL), NS-398, LPS (*Escherichia coli*, serotype 0111:B4), Triton X-100, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

CELL CULTURE AND SAMPLE TREATMENT

The RAW 264.7 macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37°C in DMEM medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO_2. Cells were incubated with pelubiprofen at concentrations of 15, 30, or 60 μM, or with positive controls (L-NIL or NS-398), and then stimulated with LPS (1 μg/ml) for the indicated time. Various concentrations of test compounds dissolved in DMSO were added together with LPS. The final concentration of DMSO did not exceed 0.1%.

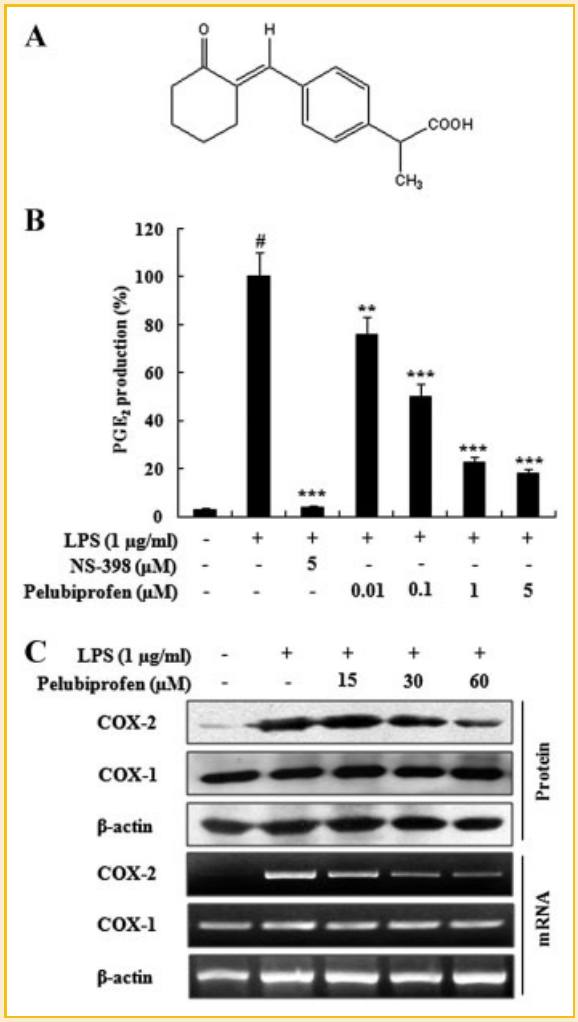

Fig. 1. The effects of pelubiprofen on LPS-induced PGE_2 production and COX-2 expression in RAW 264.7 cells. Chemical structure of pelubiprofen (A). Cells were treated with LPS 1 μg/ml alone or in the presence of various concentrations (0.01–5 μM) of pelubiprofen for 24 h. Levels of PGE_2 in culture media were quantified using EIA kits. NS-398 (5 μM) was used as a positive control. Values shown are means ± S.D. of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-induced group; significant differences between groups were determined using ANOVA and Dunnett's post-hoc test (B). Lysates or total RNA were prepared from control or LPS (1 μg/ml)-induced cells alone or LPS plus with pelubiprofen (15, 30, or 60 μM) for 24 or 4 h. Total cellular proteins were resolved by SDS–PAGE, transferred to PVDF membranes, and detected with specific antibodies. COX-2-specific sequences (721 bp) and COX-1-specific sequences (140 bp) were detected by agarose gel electrophoresis, as described in Materials and Methods section. β-actin was performed to verify that the protein or initial cDNA contents of samples were similar (C). The experiment was repeated three times and similar results were obtained.

MTT ASSAY FOR CELL VIABILITY

RAW 264.7 cells were plated at a density of 10^5 cells/well in 96-well plates. To determine the appropriate concentration of pelubiprofen, which has no effect on cell viability, cytotoxicity studies were performed at 24 h following treatment of cells with various concentrations of pelubiprofen. Viabilities were determined using colorimetric MTT assays, as described previously [Kim et al., 2008].

PGE_2, TNF-α, IL-1β, AND IL-6 ASSAY

RAW 264.7 cells were pretreated with pelubiprofen for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. Levels of PGE_2, TNF-α, IL-1β, and IL-6 in the culture media were quantified using enzyme immunoassay (EIA) kits (R&D Systems).

COX ENZYME ACTIVITY ASSAY

Human recombinant COX-2 proteins were incubated with various concentrations of pelubiprofen for 10 min at 37°C before initiating the reaction by the addition of arachidonic acid. The reaction was terminated with a mixture of 0.1 N HCl and stannous chloride to convert all PGE_2 to $PGF_{2\alpha}$. The prostanoid products were quantified via EIA using a broadly specific antibody that binds to all the major PG compounds using COX Inhibitor Screening Assay (Cayman, MI).

NITRITE DETERMINATION

RAW 264.7 cells were plated at 5×10^5 cells/well in 24 well-plates and then incubated with or without LPS (1 μg/ml) in the absence or presence of various concentrations of pelubiprofen for 24 h. Nitrite levels in culture media were determined using the Griess reaction assay and presumed to reflect NO levels [Kim et al., 2008]. The amount of nitrite in the samples was measured with the serial dilution standard curve of sodium nitrite.

WESTERN BLOT ANALYSIS

RAW 264.7 cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in PRO-PREP™ protein extraction solution (Intron Biotechnology, Seoul, Korea) and incubated with 20 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacture's instruction. Cellular protein from treated and untreated cell extracts was electroblotted onto a PVDF membrane following separation on a 10–12% SDS–PAGE. The immunoblot was incubated with blocking solution (5% skim milk) for 1 h at RT, followed by incubation for overnight with a primary antibody at 4°C. Blots were washed four times with Tween 20/Tris-buffered saline and incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Blots were again washed three times with Tween 20/Tris-buffered saline, and then developed by enhanced chemiluminescence (Amersham Life Science).

RNA PREPARATION AND REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology). From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM deoxyribonucleotide triphosphate (dNTP), and oligo (dT_{12-18}) 0.5 μg/μl. PCR analyses were performed on aliquots of the cDNA preparations to detect iNOS, COX-2, COX-1, TNF-α, IL-1β, IL-6, and β-actin (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA). Reactions were carried out in a volume of 25 μl containing; 1 unit of Taq DNA polymerase, 0.2 mM dNTP, ×10 reaction buffer, and 100 pmol of 5′ and 3′ primers. After an

initial denaturation for 2 min at 95°C, 26 or 30 amplification cycles were performed for iNOS (1 min of 95°C denaturation, 1 min of 60°C annealing, and 1.5 min 72°C extension), COX-2 (1 min of 94°C, 1 min of 60°C, and 1 min 72°C), COX-1 (1 min of 94°C, 1 min of 55°C, and 1 min 72°C), TNF-α (1 min of 95°C, 1 min of 55°C, and 1 min 72°C), IL-1β (1 min of 94°C, 1 min of 60°C, and 1 min 72°C and IL-6 (1 min of 94°C, 1 min of 56°C, and 1 min 72°C). The PCR primers used in this study are listed below and were purchased from Bioneer: Sense strand iNOS, 5′-AATGGCAACATCAGGTCGGCCATCACT-3′, anti-sense strand iNOS, 5′-GCTGTGTGTCACAGAAGTCTCGAACTC-3′; sense strand COX-2, 5′-GGAGAGAC TATCAAGATAGT-3′, anti-sense strand COX-2, 5′-ATGGTCAGTAGACTTTTACA-3′; sense strand COX-1, 5′-TCATGTTCTGCATGTGGCTG-3′, anti-sense strand COX-1, 5′-GCAGCCATCTCCTTCTCTCC-3′; sense strand TNF-α, 5′-ATGAGCACAGAA AG CATGATC-3′, anti-sense strand TNF-α, 5′-TACAGGCTTGTCACTCGA ATT-3′; sense strand IL-1β, 5′-TGCAGAGTTCCCCAACTGGTACATC-3′; anti-sense strand IL-1β, 5′-GTGCTGCCTAATGTCCCCTTGAATC-3′; sense strand IL-6, 5′-GAGGATACCAC TCCCAACAGACC-3′, anti-sense strand IL-6, 5′-AAGTGCATCATCGTTGTTCATAC A-3′; sense strand β-actin, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′, anti-sense strand β-actin, 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′. After amplification, the PCR reactions were electrophoresized on 1–2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

NUCLEAR EXTRACTION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

RAW 264.7 cells were plated in 100-mm dishes (1×10^6 cells/ml), and treated with pelubiprofen (15, 30, or 60 μM), stimulated with LPS for 1 h, washed once with PBS, scraped into 1 ml of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as described previously [Kim et al., 2008]. Cell pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μg/ml aprotinin) and incubated on ice for 15 min. Cells were then lysed by adding 0.1% Nonidet P-40 and vortexed vigorously for 10 s. Nuclei were pelleted by centrifugation at 12,000*g* for 1 min at 4°C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate). Nuclear extracts (10 μg) were mixed with double-stranded NF-κB oligonucleotide. 5′-AGTTGAGGGGACT-TTCC-CAGGC3′ end-labeled with [γ-^{32}P] dCTP (underlying indicates a κB consensus sequence or a binding site for NF-κB/cRel homodimeric or heterodimeric complex) using DNA labeling system (Amersham Life Science). Binding reactions were performed at 37°C for 30 min in 30 μl of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly (dI-dC), and 1 mM DTT. The specificity of binding was examined by competition with the 80-fold unlabeled oligonucleotide. DNA-protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5 × Tris Boric acid EDTA (TBE) buffer. Gels were vacuum-dried for 1 h at 60°C and exposed to X-ray film at −70°C for 24 h.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

RAW 264.7 cells were co-transfected with NF-κB-Luc plasmid plus the phRL-TK plasmid (Promega) using Lipofectamine LTX™ (Invitrogen, CA) as instructed by the manufacturers. After 4 h of transfection, cells were pretreated with pelubiprofen for 1 h and then stimulated with LPS (1 μg/ml) for 18 h. Each well was washed with cold-PBS and cells were lysed and the luciferase activity was determined using the Promega luciferase assay system (Promega).

IKK KINASE ASSAY

The IKKβ kinase activity was measured with recombinant human IKKβ kinase using HTScan® IKKβ Kinase Assay Kit (Cell Signaling Technologies, Beverly, MA). This assay was performed in cell free system and was detected using colorimetric ELISA methods.

ANIMALS

All animal experiments were conducted under university guideline of ethical committee for Animal Care and the Use of laboratory animals, College of Pharmacy, Kyung Hee University according to an animal protocol (Approval number # KHP-2009-10-06). Male Sprague–Dawley (SD) rats weighing 160–200g were purchased from Orient Bio Inc. (Seongnam-si, Korea) and maintained under constant conditions (temperature: 20 ± 2°C, humidity: 40–60%, light/dark cycle: 12 h) for 2 weeks or more. At 12 h before the experiment, only water was provided.

MEASUREMENT OF PAW EDEMA

Paw edema was induced by subplantar injection of 100 μl of phosphate buffered saline (0.9%) containing 1% carrageenan into the rat right hind paw. The test agents used in this study were pelubiprofen (25 and 100 mg/kg), ibuprofen (100 mg/kg; Sigma Chemical Co.). Test samples were first dissolved in 5% EtOH and 5% Cremophor and diluted with saline (Vehicle). Animals were pretreated orally with saline (control group), vehicle, ibuprofen (used as positive control) and pelubiprofen for 1 h prior to the induction of edema, respectively. The paw volume was measured using LE Digital plethysmometer (Panlab S.I, Barcelona, Spain) at 1, 3, and 5 h after carrageenan injection. In some experiments, soft tissues from carrageenan-injected paws were recovered by scalpel and immediately processed to obtain tissue extracts.

TISSUE MPO ACTIVITY ASSAY AND PGE_2 LEVELS

MPO activity was measured in soft tissue from carrageenan-injected paws, as a marker of neutrophil influx into the tissue. The tissue was thawed and homogenized in lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM tris, 10% glycerol, 1 mM PMSF, 1 μg/ml leupeptide, and 28 μg/ml aprotonine (pH 7.4). The homogenate was then centrifuged at 1,500*g* for 15 min, and the resulting supernatant assayed for MPO assay using EIA kits (Hycult biotechnology, Netherlands). And PGE_2 level in tissue was measured from homogenate using EIA kits (R&D Systems).

STATISTICAL ANALYSIS

Results are expressed as the mean ± S.D. of triplicate experiments. Statistically significant values were compared using ANOVA and

Dunnett's post-hoc test, and *P*-values of less than 0.05 were considered statistically significant.

RESULTS

PELUBIPROFEN SUPPRESSED LPS-INDUCED PGE_2 PRODUCTION DUE TO BOTH THE INHIBITION OF COX ACTIVITY AND COX-2 EXPRESSIONS IN RAW 264.7 CELLS

The property of NSAIDs to inhibit COX-mediated prostaglandin synthesis at the site of inflammation has been closely related to their anti-inflammatory efficacy. In order to better understand the potential of pelubiprofen as a NSAID, we investigated the effects of pelubiprofen on PGE_2 production and COX expressions in LPS-induced RAW 264.7 cells. Pelubiprofen had an IC_{50} of 0.13 ± 0.07 μM for inhibition of PGE_2 production in macrophages (Fig. 1B). NS398 (5 μM), selective COX-2 inhibitor, was used as positive control for inhibition of PGE_2 production. In addition, pelubiprofen exhibited the potency to COX activity with an IC_{50} of COX-1 and COX-2 of 10.66 ± 0.99 and 2.88 ± 1.01 μM, respectively (COX-1/COX-2 ratio for selectivity: 3.70) using enzyme-based COX catalytic activity. We further investigate whether the inhibitory effects of pelubiprofen on PGE_2 production is related to COX-1 and COX-2 expressions by Western blot and RT-PCR. COX-2 protein and mRNA levels were found to be markedly up-regulated in response to LPS, and pelubiprofen inhibited these responses in a concentration-dependent manner (Fig. 1C), although the concentration of pelubiprofen to inhibit COX-2 expression is higher than those to inhibit LPS-induced PGE_2 production or COX activity. However, it had no effect on the protein and mRNA expressions of COX-1 in RAW 264.7 cells (Fig. 1C). These results implied that pelubiprofen has the anti-inflammatory activity through inhibitions not only to the transcriptional regulation of inflammatory genes but also to the COX activity. The inhibitory action by pelubiprofen was not due to their cytotoxicity, because pelubiprofen did not affect cell viability as measured by MTT assay at the concentrations (15–60 μM) that showed the suppression of COX-2 protein and mRNA (Data not shown).

PELUBIPROFEN INHIBITED LPS-INDUCED EXPRESSIONS OF iNOS AND PRO-INFLAMMATORY CYTOKINES

To investigate the inhibitory effects of pelubiprofen on LPS-induced pro-inflammatory mediators in RAW 264.7 cells, production of NO and pro-inflammatory cytokines were measured after LPS exposure for 24 h using Griess reaction and EIAs, respectively. Pretreatment with pelubiprofen concentration-dependently inhibited LPS-induced NO production (by 76.03% inhibition at 60 μM) (Fig. 2A). As shown Figure 2B, expression of iNOS, which catalyzes the oxidative deamination of L-arginine to produce NO, were markedly inhibited by pelubiprofen at protein and mRNA levels. During the 24 h incubation in the resting state cells, TNF-α, IL-1β, and IL-6 were slightly produced. However, when exposed to LPS, production of these cytokine increased about 35-, 3-, and 40-fold over the basal level, respectively. Pretreatment of cells with pelubiprofen considerably reduced the production of TNF-α, IL-1β, and IL-6 in a concentration-dependent manner (by 49.62, 61.28, and 91.97% inhibition at 60 μM, respectively) (Fig. 2A). Moreover,

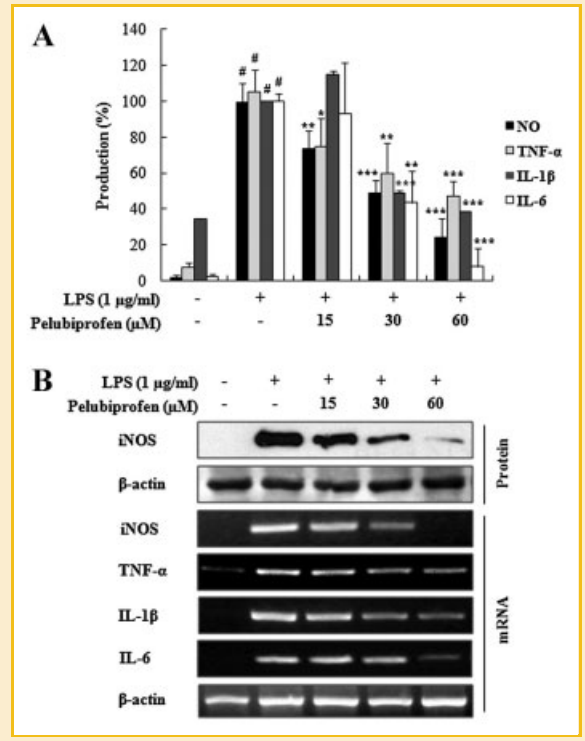

Fig. 2. The effects of pelubiprofen on LPS-induced NO and cytokines production and their protein and mRNA expressions in RAW 264.7 cells. Cells were treated with LPS 1 μg/ml alone or in the presence of pelubiprofen (15, 30, or 60 μM) for 24 h. Amount of NO was determined using the Griess reaction. The productions of TNF-α, IL-1β, IL-6 were determined using EIA kits. Values shown are means ± S.D. of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$ $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-induced group; significant differences between groups were determined using ANOVA and Dunnett's post-hoc test (A). Lysates or total RNA were prepared from control or LPS (1 μg/ml)-induced cells alone or LPS plus with pelubiprofen (15, 30, or 60 μM) for 24 or 4 h. iNOS proteins and iNOS (453 bp), TNF-α (276 bp), IL-1β (387 bp), IL-6 (142 bp) mRNAs were detected by Western blot and RT-PCR, respectively, as described in Materials and Methods section. β-actin was performed to verify that the protein or initial cDNA contents of samples were similar (B). The experiment was repeated three times and similar results were obtained.

the mRNA levels of TNF-α, IL-1β, and IL-6 were significantly up-regulated by LPS, and pelubiprofen pretreatments at 15, 30, or 60 μM markedly and dose-dependently inhibited these mRNA expressions.

PELUBIPROFEN INHIBITED LPS-INDUCED NF-κB ACTIVATION IN RAW 264.7 CELLS

Since NF-κB is a key factor that regulates the expressions of pro-inflammatory proteins (e.g., iNOS and COX-2) and of pro-inflammatory cytokines (e.g., TNF-α and IL-1β) induced by LPS [Kang et al., 2007; Okamoto et al., 2008], EMSA and luciferase reporter gene assays were performed to determine whether pelubiprofen regulates LPS-induced NF-κB activity in LPS-induced RAW 264.7 cells. Analysis of reporter gene expression using pNF-κB-luc demonstrated that pelubiprofen concentration-dependently inhibited NF-κB-dependent luciferase activity (Fig. 3A). To further

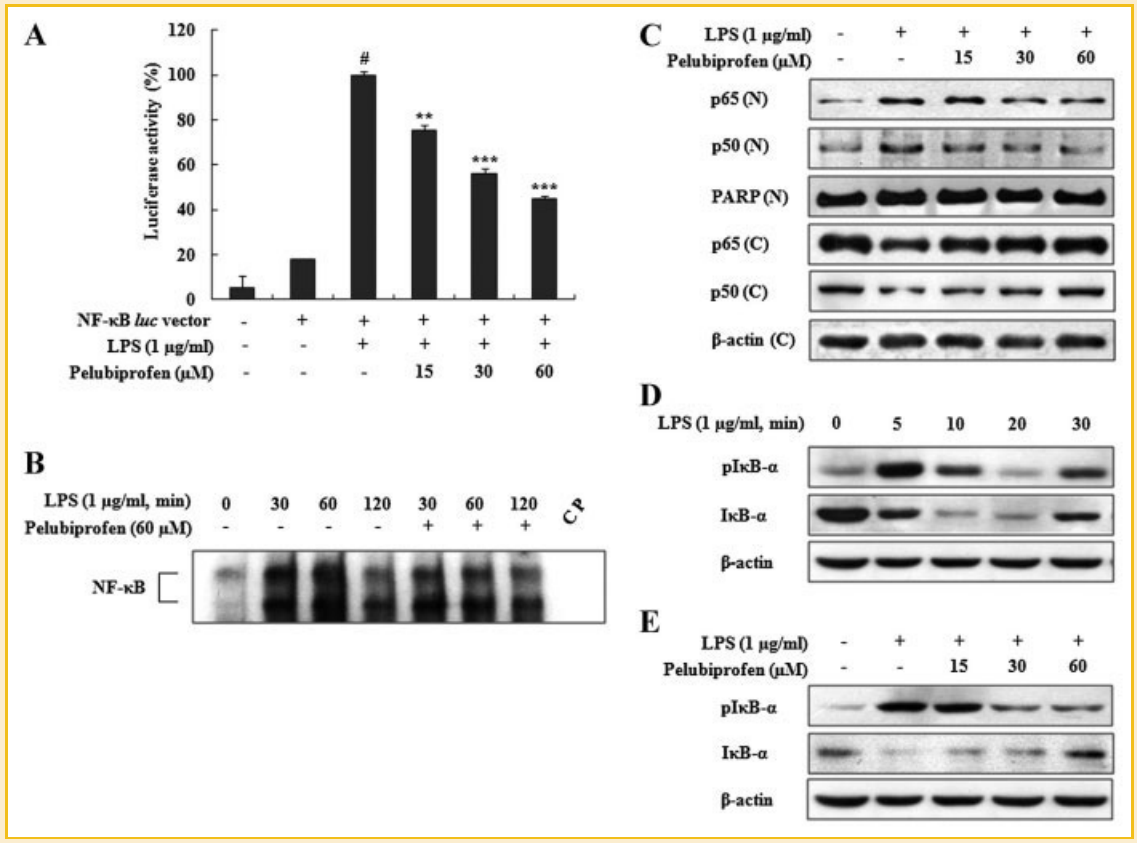

Fig. 3. The effects of pelubiprofen on LPS-induced NF-κB activation in RAW 264.7 cells. Cells were transiently transfected with pNF-κB-luc reporter construct with the phRL-TK vector as an internal control and then treated with LPS for 18 h in absence or presence of pelubiprofen (15, 30, or 60 μM). The level of luciferase activities were determined using a Promega luciferase assay system and a luminometer. Values shown are means ± S.D. of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{**}P < 0.01$, $^{***}P < 0.001$ versus LPS-induced group; significant differences between groups were determined using ANOVA and Dunnett's post-hoc test (A). Nuclear extracts from cells treated with LPS 1 μg/ml alone or in the presence of pelubiprofen (15, 30, or 60 μM) for indicated time were prepared and used for analysis of NF-κB-DNA binding using EMSA. The arrow indicates the position of the NF-κB-DNA binding. Specificity of binding was examined by competition with 80-fold excess of unlabeled NF-κB oligonucleotide (CP) (B). Nuclear (N) and cytosolic (C) extracts were isolated from cells treated with LPS 1 μg/ml alone or in the presence of pelubiprofen (15, 30, or 60 μM) for 1 h. Nuclear and cytosolic extracts were prepared for the Western blot analysis of NF-κB protein using specific anti-p65 and anti-p50 monoclonal antibodies, respectively, and PARP and β-actin were used as internal control for nuclear and cytosolic fraction, respectively (C). Cells were treated with LPS for indicated time (0–30 min). Whole cell lysates were analyzed by Western blot using specific antibodies (D). Cells were treated with LPS alone or in the presence of pelubiprofen (15, 30, or 60 μM) for 5 min (p-IκB-α) or 10 min (IκB-α). Whole cell lysates were analyzed by Western blot using specific antibodies. PARP and β-actin were used as internal controls (E). The experiment was repeated three times and similar results were obtained.

examine the DNA-binding activity of NF-κB, nuclear extracts isolated from LPS-induced RAW 264.7 cells pretreated or not with pelubiprofen were reacted with NF-κB specific ^{32}P-labeled oligonucleotides. As shown in Figure 3B, the DNA-binding activity of NF-κB was markedly increased by LPS only treatment, whereas this binding was reduced by pelubiprofen. In resting cells, NF-κB is sequestered in the cytosol by its inhibitor IκB, and when induced by LPS, IκB is phosphorylated by IKKs, ubiquitinated, and rapidly degraded via 26 S proteosome, which results in the release and translocate to the nucleus of NF-κB [Karin and Ben-Neriah, 2000]. Accordingly, we investigated whether pelubiprofen prevents the translocations of the subunits of NF-κB (p65 and p50) to nucleus. We found that pretreatment with pelubiprofen concentration-dependently attenuated LPS-induced NF-κB translocations from the cytosol to the nucleus (Fig. 3C). We further examined whether pelubiprofen inhibits the LPS-induced degradation and phosphoylation of IκB-α in RAW 264.7 cells by Western blot. In resting cells, the phsophorylated forms of IκB-α were hardly detectable, but LPS-induced phosphorylation of IκB-α displayed a biphasic expression pattern during 30 min (Fig. 3D). LPS-induced IκB-α degradation was maximal within 10 min after exposure to LPS and then the level of IκB-α gradually recovered after 30 min (Fig. 3D). As shown in Figure 3E, it was found that pelubiprofen concentration-dependently reduced LPS-induced phosphorylation and degradation of IκB-α.

PELUBIPROFEN INHIBITED LPS-INDUCED PHOSPHORYLATIONS OF IKK AND TAK1 IN RAW 264.7 CELLS

The activation of IKK is a key step in the activation of NF-κB via the phosphorylations of IκBs [Ghosh and Karin, 2002; Shin et al., 2010]. To establish whether pelubiprofen directly affects IKK-β activity, we performed ELISA-based IKK-β activity assays. As shown in Figure 4A, pelubiprofen inhibited IKK-β activity in a concentration-dependent manner (by 37.84% inhibition at 60 μM). IKKs

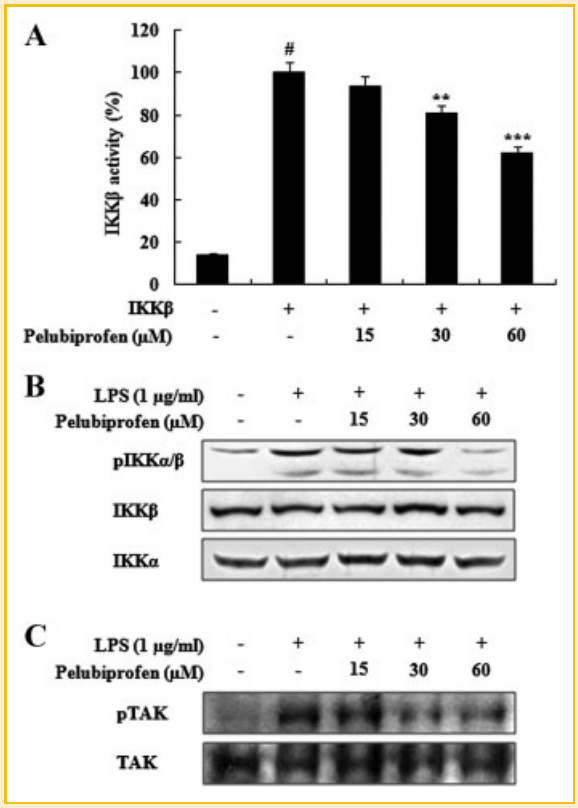

Fig. 4. The effects of pelubiprofen on IKK-β kinase activity and LPS-induced phosphorylation of IKK and TAK1 in RAW 264.7 cells. IKK-β kinase activity was measured by ELISA assay using recombinant human IKK-β kinase treated with different concentration (15, 30, or 60 μM) of pelubiprofen. IκBα biotinylated peptide was used by substrate of IKK-β kinase. Values shown are means ± S.D. of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$ $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-induced group; significant differences between groups were determined using ANOVA and Dunnett's post-hoc test (A). Cells were treated with LPS alone or in the presence of pelubiprofen (15, 30, or 60 μM) for 5 min. Whole cell lysates were analyzed by Western blot using specific antibodies. β-actin was used as internal controls (B, C). The experiment was repeated three times and similar results were obtained.

themselves are thought to be activated though phosphorylation and pretreatment with pelubiprofen (60 μM) markedly reduced the LPS-induced IKK-α/β phosphorylation, whereas pelubiprofen did not affect total amounts of IKK-α and -β (Fig. 4B). Since TAK1 has been implicated in the regulation of IKK-α/β phosphorylation by LPS [Wang et al., 2001], we further investigated the effect of pelubiprofen on the LPS-induced phosphorylation of TAK1. LPS-induced TAK1 phosphorylation was significantly blocked by pelubiprofen in a concentration-dependent manner (Fig. 4C).

THE ANTI-INFLAMMATORY EFFECTS OF PELUBIPROFEN VIA INHIBITION OF NF-κB IN CARRAGEENAN-INDUCED PAW EDEMA MODELS

Because pelubiprofen effectively inhibited PGE_2, NO, and pro-inflammatory cytokines productions in macrophages, studies were extended to determine whether pelubiprofen affected acute phase inflammation in animal models. In this study, we used carrageenan-induced edema because this model is widely employed for screening the effects of anti-inflammatory drugs. As shown Figure 5A, treatment with pelubiprofen inhibited carragenan-induced paw swelling and inhibitory effects of pelubiprofen started at 1 h and sustained themselves for 5 h. At 5 h, the inhibitory rates of pelubiprofen were 35.38% (25 mg/kg, p.o.) and 50.95% (100 mg/kg, p.o.) compared to the rate of vehicle group, whereas the positive control, ibuprofen (IBP, 100 mg/kg, p.o.) decreased the edema rate by 46.90% at 3 h. Neutrophil migration into inflamed paws was determined by MPO activity assay. Injection of carrageenan into rat paws resulted in an upregulation of MPO activity in tissue, whereas treatment with pelubiprofen (25 mg/kg and 100 mg/kg, p.o.) significantly reduced the carrageenan-induced MPO activity (19.60 and 44.86%, respectively) (Fig. 5B). Ibuprofen (100 mg/kg) also inhibited the MPO activity (62.83%). Moreover, reductions in PGE_2 production into inflamed paws after pelubiprofen treatment were similar to the observed reductions by ibuprofen (by 59.84 and 67.15% inhibition at 25 mg/kg and 100 mg/kg, respectively) (Fig. 5C). Furthermore, we investigated the effects of pelubiprofen on NF-κB activation in inflamed paw tissue using Western blot. As shown Figure 5D, treatment of carrageenan markedly induced the translocation of p65 to nuclear in paw tissue and that oral administration of pelubiprofen significantly suppressed the nuclear translocation of p65.

DISCUSSION

It has been demonstrated that NSAIDs, such as aspirin, ibuprofen, sulindac can inhibit activation of the NF-κB pathway [Grilli et al., 1996; Yin et al., 1998; Yamamoto et al., 1999]. In the current study, we addressed whether the anti-inflammatory agent pelubiprofen, which is structurally related to ibuprofen or flurbiprofen can inhibit NF-κB-mediated gene expression. To our knowledge, this is the first time that the pelubiprofen exhibited the anti-inflammatory effect through dual inhibitory effect on the COX-1 and COX-2 enzyme activities and the TAKl-IKK-NF-κB signaling pathway. The mechanism of action of this drug is due to inhibition of IKK-β kinase activity via TAK inactivation. These results suggest that pelubiprofen is one of the several non-steroidal anti-inflammatory agents that potently inhibited the NF-κB pathway by inhibiting IKK-β activity.

It is generally accepted that anti-inflammatory effects of NSAIDs arises mainly from non-selective inhibition on the enzymatic activity of COX, which convert arachidonic acid to PGs [Vane, 1971]. Most NSAIDs non-selectively inhibit COX enzymes, but also have stronger inhibitory effects on COX-1 activity [Takada et al., 2004; Gierse et al., 2005], providing the evidences for major gastrointestinal complications of NSAIDs. Pelubiprofen, one of the 2-arylpropionic acids family has relatively selective effects on COX-2 activity (COX-1/COX-2 ratio: 3.7), even if it is not comparable to COX-2 selective drug (Celecoxib: 375; Valdecoxib: 30,000) [Gierse et al., 2005]. Presumably, pelubiprofen could have the potential to delay or exclude the gastrointestinal ulcer than other NSAIDs because of the selective COX-2 inhibition. In addition, it has been

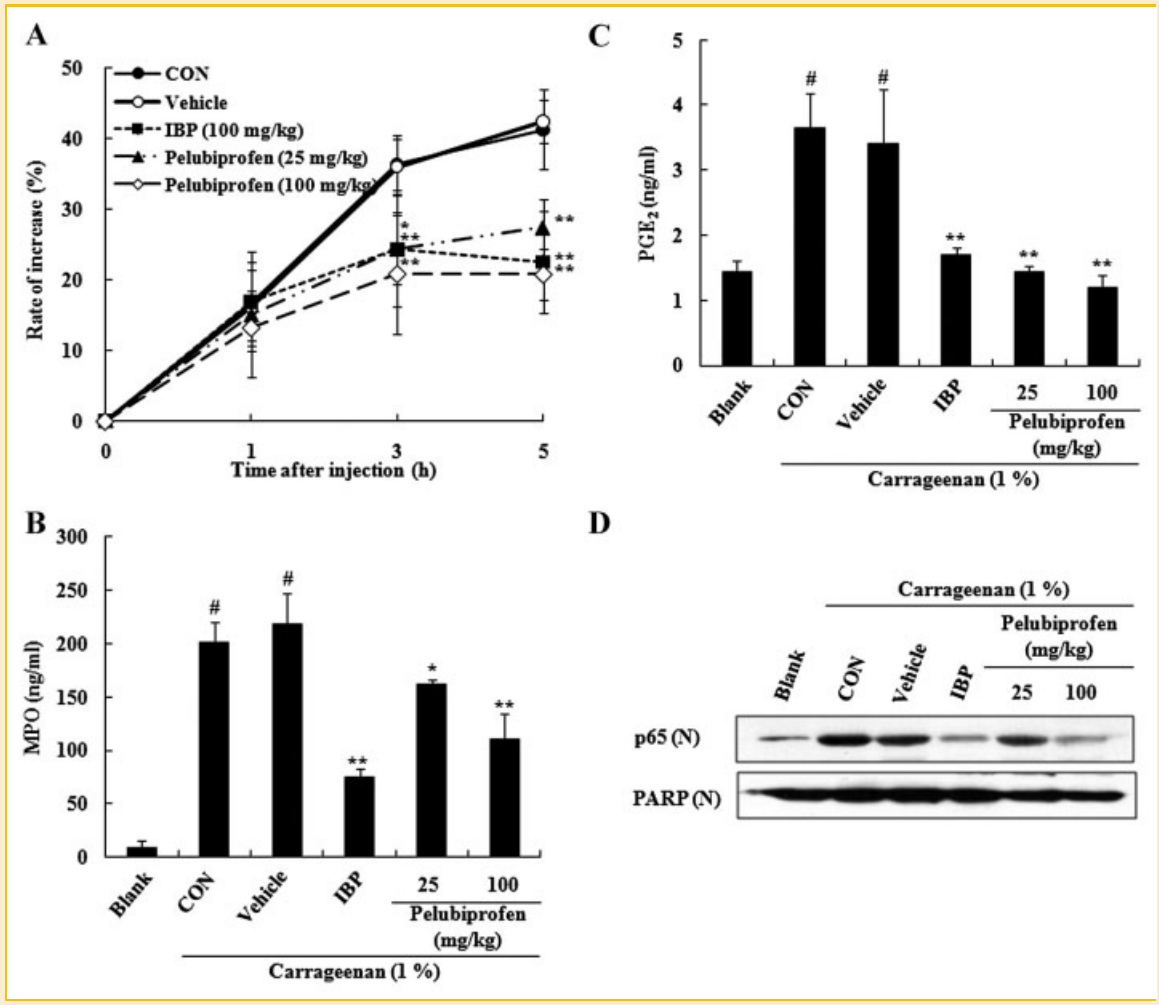

Fig. 5. The effects of pelubiprofen on carrageenan-induced inflammatory responses and NF-κB activation in rats. The initial hind paw volume of rats was determined volumetrically, a 1% solution of carrageenan in saline (0.1 ml per rat) was injected subcutaneously into right hind paws 1 h after the test sample (25 or 100 mg/kg, p.o.) had been administered. Paw volumes were measured for up to 5 h after injections and edema volumes were measured using a plethysmometer (A). Soft tissues from carrageenan-injected paws were collected at 5 h after carrageenan injection and homogenized. Amounts of MPO and PGE_2 in homogenates were measured using EIA kits. Ibuprofen (IBP, 100 mg/kg, p.o.) was used as positive control. Values shown are mean ± S.D. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus carrageenan-injected group ($n = 5$–6); the significances of differences were determined using ANOVA and Dunnett's post-hoc test (B, C). Nuclear (N) extracts were isolated from soft tissues from carrageenan-injected paws. The level of p65 was determined by Western blot using specific monoclonal antibody. PARP was used as internal control for nuclear fraction The experiment was repeated three times and similar results were obtained (D).

found that pelubiprofen concentration-dependently reduced the productions of NO, TNF-α, IL-1β, and IL-6 induced by LPS. Consistent with these findings, pelubiprofen significantly suppressed the LPS-induced expressions of iNOS, COX-2, TNF-α, IL-1β, and IL-6 at transcriptional level. Thus, we suggest that the inhibition of PGE_2 production by pelubiprofen underlies its inhibition of both the enzyme activity of COX-1 and COX-2 and expressions of COX-2. Thus, the inhibitory effects of pelubiprofen on COX activities and inflammatory mediators led to the hypothesis that pelubiprofen may also exhibit the COX-independent anti-inflammatory effects.

Several studies have demonstrated that certain NSAIDs cause anti-inflammatory effects by mediating inhibition of certain transcription factors such as NF-κB and activator protein (AP)-1 and other cellular kinases instead of COX activity and PGs synthesis inhibition [Tegeder et al., 2001b; Fratelli et al., 2003]. Interestingly, the inhibitory effect on NF-κB is common to flurbiprofen and both the isomers of ibuprofen. Since the R-isomer of both drugs do not inhibit COX activity, the effect of R-isomers on NF-κB is possibly not linked to their activity on PGE_2 production [Tegeder et al., 2001a]. These results are consistent with our finding that the transcriptional inhibition of pro-inflammatory mediators by pelubiprofen is associated with the NF-κB signal pathway. EMSA and NF-κB-dependent reporter gene assay revealed that pelubiprofen inhibited the DNA binding and transcriptional activity of NF-κB. To identify the mechanisms involved in the inhibition of NF-κB by pelubiprofen, we investigated the regulatory mechanism of NF-κB by pelubiprofen. Western blot analysis revealed that pelubiprofen inhibited LPS-induced phosphorylation and degradation of IκB-α, and reduced the amount of p65 and p50, subunits of NF-κB,

in nuclear fractions. These findings suggest that the inhibition of NF-κB activation by pelubiprofen is the result of the inhibition of phosphorylation and degradation of IκB-α, and thus, of the nuclear translocation of NF-κB in LPS-induced RAW 264.7 cells.

It has been suggested that NF-κB activation is mediated by two distinct signaling pathways, namely, the NF-κB translocation-dependent pathway, which involves the IKK-dependent phosphorylation and degradation of IκB-α [Pan et al., 2000], and by the phosphorylations of MAPKs, such as, ERK, Jun N-terminal kinase (JNK), and p38 [Craig et al., 2000]. Recent studies have shown that IKK complex is the critical step of NF-κB activation by regulating the phosphorylation of both IκB-α and p65. Within the IKK complex, IKK-α is largely responsible for p65 phosphorylation, whereas IKK-β is capable of phosphorylating both IκB-α and p65 [Perkins, 2007]. In previous study, it has been described that aspirin, salicylate, and other NSAIDs specifically inhibit IKK-β activity and this mechanism implicates the interaction of these compounds with ATP binding site of the enzyme [Manku and Horrobin, 1976; Yin et al., 1998; Yamamoto et al., 1999]. In the present study, we also found that pelubiprofen significantly inhibited activity and LPS-induced phosphorylation of IKK-β. These results provide a potential explanation for the ability of pelubiprofen to inhibit the activity of multiple kinases in cellular signaling.

Accumulated data showed that LPS is recognized by Toll-like receptor (TLR4), a member of the TLR family that is involved in innate immunity and inflammation response. Upon binding of TLR4 to LPS, the cytoplasmic region of TLR4 recruits myeloid differentiation protein 88 (MyD88), which links TLR4 to IL-1 receptor-associated protein kinase 1 (IRAK1) [Barton and Medzhitov, 2003]. IRAK1 binds TNF receptor-associated factor 6 (TRAF6), which in turn binds a pre-formed membrane bound complex of TAK1 binding protein 1 (TAB1)/TAK1/TAB2. Phosphorylation of TAK1 initiates the release of the complex from the membrane, and the active TAK1 phosphorylates the IKK complex, which in turn phosphorylates IκBs for degradation [Adhikari et al., 2007]. Our data showed that pelubiprofen suppressed TAK1 phosphorylation and subsequent downstream event, such as phosphorylation of IKK-α/β and IκB-α. Since TAK1 also phosphorylates members of the MAP kinase kinases (MKK) family, which in turn phosphorylates and activates JNK and p38 kinases, we investigated the effects of pelubiprofen on MAPKs (JNK, p38, and ERK). We found that pelubiprofen inhibited the phosphorylation of ERK, but no effect on the phosphorylation of JNK and p38 induced by LPS (Data not shown). These results are partially accorded with the little effects of pelubiprofen on the AP-1 activation using EMSA and reporter gene assay (Data not shown), because AP-1 is mainly activated by JNK and p38 pathway [Ono and Han, 2000; Chang and Karin, 2001]. Furthermore, previous findings concur with ours, that certain NSAIDs inhibit the angiogenesis mediating through direct effects on endothelial cells involving inhibition of ERK activity [Jones et al., 1999], and regulate the ERK-dependent cytoskeleton dynamics in Alzheimer's disease models [Lichtenstein et al., 2010]. These findings suggest that the inhibitory effect of pelubiprofen on various kinases is a unique observation and simultaneous inhibition of multiple signaling pathways might be attributed to anti-inflammatory effects of pelubiprofen. However, it remains to determined what is the upstream molecule if TAK1 targeted by pelubiprofen.

To confirm whether pelubiprofen also inhibits inflammatory responses via NF-κB inactivation in vivo, we turned our attention to carrageenan-induced paw edema test in animal model. Carrageenan has been used as a reagent to induce non-specific inflammatory reactions and is dependent on TLR4 and Myd88, which induce the activation of innate immunity and the peripheral release of NO and PGE_2 and TNF-α, which subsequently promotes IL-1β and IL-6 production in tissues [Tsuji et al., 2003]. In our study, pelubiprofen administration markedly inhibited the swelling of hind paw in carrageenan-induced rats and was also capable of reducing MPO (as an indicator of cellular infiltration of neutrophil) activity and PGE_2 production in inflamed paws. Moreover, the translocation of p65 to nucleus was inhibited by pelubiprofen in inflamed paw. However, it may be asked whether it is possible to achieve significant inhibition of NF-κB at clinical dosage in humans and more studies on that subject might have to be kept under consideration.

In summary, our results demonstrate that pelubiprofen exerts anti-inflammatory effects, which results from dual inhibitions of COX-1 and -2 enzyme activities and NF-κB-dependent pathways via TAK1 inactivation. More importantly, pelubiprofen was found to have an anti-edema effect in the carrageenan-induced paw edema model in rats, one of the well-established acute inflammatory models in vivo. Our findings showing inhibition by pelubiprofen of COX activity, inflammatory gene induction, and paw edema may help to understand the pharmacology and mechanisms of action of pelubiprofen.

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