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Nimesulide reduces interleukin-1β-induced cyclooxygenase-2 gene expression in human synovial fibroblasts

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Summary

Objective: To characterize the effects of nimesulide (NIM) on basal and induced cyclo-oxygenase-2 (COX-2) gene expression in human synovial fibroblasts (HSF) and to define the intracellular mechanisms that mediate the changes in COX-2 expression and synthesis in response to the drug.

Design: HSF were incubated with NIM and NS-398 (0, 0.03, 0.3, 3 μg/ml) in the absence or presence of the COX-2 inducers interleukin-1β (IL-1β) or endotoxin (LPS). Treated cells were analysed for COX-2 mRNA and protein by Northern and Western blotting analysis, respectively. Putative transcriptional, post-transcriptional, and signaling effects of NIM on basal and induced-COX-2 expression were investigated by human COX-2 promoter studies, calcium studies, reactive oxygen species (ROS) evaluations, electrophoretic mobility shift analysis (EMSA) and half-life studies of COX-2 mRNA.

Results: NIM inhibited IL-1β-induced COX-2 expression and protein at sub and therapeutic concentrations (0.03–0.3 μg/ml) while the non-specific NSAID, naproxen, did not. Both drugs suppressed PGE2 release by about 95%. NIM had no effect on (1) IL-1β-induced increases in NF-κB or c/EBP signaling, or (2) human COX-2 promoter activity. Stability of induced COX-2 mRNA was unaffected by NIM treatments. Pre-treatment of cells with O_2 radical scavengers (e.g. PDTC) or with Ca^{++} channel blockers (e.g. verapamil) had a modest effect on IL-1β-induced COX-2 expression. NIM blocked ionomycin+thapsigargin and H_2O_2-induced increases in COX-2 protein synthesis.

Conclusion: NIM inhibits cytokine-induced COX-2 expression and protein at sub and therapeutic concentrations. At least part of this activity may be the result of NIM inhibition of calcium and/or free radical generation induced by cytokines. © 2001 OsteoArthritis Research Society International

Key words: Cyclo-oxygenase-2, Gene expression, Interleukin-1β, Nimesulide, Synovial fibroblasts, Intracellular calcium.

Introduction

Nimesulide (NIM, 4-nitro-2-phenoxymethanesulfonanilide), is a preferential cyclo-oxygenase-2 (COX-2) inhibitor[1–3] with marked biological effects in several *in vivo* models of inflammation[4,5]. It is believed that the molecular basis for the therapeutic actions of NIM lies in the drug's ability to inhibit the release of inflammatory prostaglandins by specifically suppressing cyclo-oxygenase-2 (COX-2) activity[3]. However, a number of studies suggest that in addition to the well-described inhibition of prostaglandin synthesis, NIM has pleiotropic effects particularly in terms of neutrophil function. For example, the drug inhibits the neutrophil respiratory burst, integrin-mediated adherence and synthesis of platelet-activating factor (PAF)[6–8]. Many of these actions have been attributed to the ability of NIM to increase cellular levels of cyclic AMP (cAMP) by inhibiting cAMP-dependent phosphodiesterase type IV[6,7].

Using human osteoarthritic synovial fibroblasts in culture, we showed[9] that NIM and naproxen (NAP), at therapeutic doses, reduces the synthesis of urokinase (uPA) and interleukin-6 (IL-6), while increasing the production of plasminogen activator inhibitor-1 (PAI-1). Furthermore, it was demonstrated that NIM decreases matrix metalloprotease (MMP) synthesis by cartilage *in vitro*[10]. Taken together, these results suggest that the drug can inhibit cartilage catabolism through mechanisms not associated with the inhibition of COX-2 activity and eicosanoid release. Recently, we demonstrated that NIM stimulates hyperphosphorylation of the glucocorticoid receptor (GR) in human synovial fibroblasts resulting in an activation of the GR in terms of glucocorticoid response element (GRE) binding and transactivation of GR-sensitive promoters (e.g. mouse mammary tumor virus long terminal repeat, MMTV-LTR)[11].

The aim of the present study was to determine whether a separate class of NSAIDs, namely specific COX-2 inhibitors, could effect changes in COX-2 expression in connective tissue cells. We show that NIM down-regulates IL-1β and LPS (endotoxin)-induced COX-2 mRNA expression and protein synthesis in human osteoarthritis (OA)-affected synovial fibroblasts at therapeutically relevant doses in addition to its well-described suppression of PGE_2 release.

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This inhibitory effect may be manifested through NIM-induced changes in calcium flux and/or metabolism of reactive oxygen species (ROS).

Materials and methods

CHEMICALS

Nimesulide (NIM), [N-(4-nitro-2-phenoxyphenyl)-methanesulfonamide], was kindly provided by Helsinn Healthcare SA (Lugano, Switzerland). Crystalline dexamethasone (DEX) [9-fluoro-11β, 17, 21-trihydroxy-16-methylpregna-1,4, diene-3, 20-dione], sodium fluoride, sodium ortho-vanadate, okadaic acid, leupeptin, aprotinin, pepstatin, phenylmethylsulphonylflouride (PMSF), dithiothreitol (DTT), and bovine serum albumin (BSA) were from Sigma-Aldrich Canada (Oakville, Ontario, Canada). Naproxen and NS-398 were obtained from Cayman Chemical Co. (Ann Arbor, MI), while nifedipine, verapamil and 1-pyrrolidinecarbodithioic acid (PDTC) were purchased from Calbiochem (La Jolla, CA). Sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, ammonium persulfate, and Bio-Rad protein reagent originated from Bio-Rad Laboratories (Richmond, CA). Tris, EDTA, $MgCl_2$, $CaCl_2$, chloroform, dimethylsulphoxide (DMSO), formaldehyde and formamide are products of Fisher Scientific (Nepean, Ontario, Canada). Human recombinant IL-1β (rhIL-1β) and LPS were obtained from Genzyme Corporation (Cambridge, MA). Dulbecco's Modified Eagle Medium (DMEM), phosphate-free and phenol-red free DMEM, Trizol reagent, heat inactivated fetal calf serum (FCS), and an antibiotic mixture [10 000 units of penicillin (base), 10 000 μg of streptomycin (base)] were products of Gibco BRL-Life Technologies (Burlington, Ontario, Canada).

SPECIMEN SELECTION AND SYNOVIAL FIBROBLAST CULTURES

Synovial lining cells (human synovial fibroblasts, HSF) were isolated from OA and rheumatoid arthritis (RA) patients undergoing arthroplasty who were diagnosed based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA/RA. Human synovial fibroblasts were released by sequential enzymatic digestion with 1 mg/ml pronase (Boehringer Mannheim Canada, Laval, Quebec, Canada) for 1 h, followed by 6 h with 2 mg/ml collagenase (type IA, Sigma) at 37°C in DMEM supplemented with 10% heat inactivated FCS, 100 units/ml penicillin and 100 μg/ml streptomycin[12,13]. Released HSF were incubated for 1 h at 37°C in tissue culture flasks (Primaria No. 3824, Falcon, Lincoln Park, NJ) allowing the adherence of non-fibroblastic cells possibly present in the synovial preparation. In addition, flow cytometric analysis (Epic II, Coulter, Miami, FL), using the anti-CD14 (fluorescein isothiocyanate, FITC) antibody, was conducted to confirm that no monocytes/macrophages were present in the synoviocyte preparations[12]. The cells were seeded in tissue culture flasks and cultured until confluence in DMEM supplemented with 10% FCS and antibiotics at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were incubated in fresh serum-free medium for 24 h before the experiments and only second or third passaged HSF were used.

PREPARATION OF CELL EXTRACTS AND WESTERN BLOTTING

Fifty to 100 μg of cellular extract (in RIPA buffer; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% NP-40, 1 mM sodium orthovanadate, and 1 mM NaF) from control and treated HSF were subjected to SDS-PAGE through 10% gels (final concentration of acrylamide) under reducing conditions, and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada). Following blocking (with 5% BLOTTO) and washing, the membranes were incubated for either 2 h at RT, or overnight at 4°C, with primary antibodies (see below) in TTBS containing 0.25% BLOTTO. Second anti-rabbit antibody-HRP conjugates (1:2000 dilution) were subsequently incubated with membranes for 1 h at RT, and then washed extensively for 30–40 min with TTBS, and a final rinsing with TBS at RT. Following incubation with an ECL chemiluminescence reagent (Amersham Pharmacia Biotech), membranes were prepared for autoradiography and exposed to Kodak X-Omat film (Kodak, Rochester, NY), then subjected to densitometric analysis for semi-quantitative measurements. The antibody used was the polyclonal antihuman COX-2 (Cayman Chemical Co., Ann Arbor, MI, 1:5000 dilution).

NORTHERN BLOT ANALYSIS OF MRNA

Total cellular RNA was isolated (1×10^6 cells=10–20 μg RNA) using the Trizol (Gibco BRL-Life Technologies) reagent. Generally, 10–15 μg of total RNA were resolved on 0.9% agarose-formaldehyde gel and transferred to Hybond-N$^{(TM)}$ nylon membranes (Amersham Pharmacia Biotech) in 20×SSC buffer, pH 7 by vacuum blotting. After pre-hybridization for 24 h, hybridizations were carried out at 50–57°C for 24–36 h, followed by high-stringency washing. The following probes, labeled with digoxigenin (DIG)-dUTP by random priming, were used for hybridization: human COX-2 cDNA (1.8 kb, Cayman Chemical Co.) was cloned into the EcoRV site of pcDNA 1 (Invitrogen, Carlsbad, CA) and released by PstI and XhoI digestion. A 780 bp PstI/XbaI fragment from GAPDH cDNA (1.2 kb; American Type Culture Collection, Rockville, MD) was subcloned into pGEM-3Z vector (Promega Corp., Madison, WI) and a cRNA probe were synthesized after linearization with PstI. This latter probe served as a control of RNA loading as GAPDH is constitutively expressed. All blots were subjected to densitometric analysis for semiquantitative measurements.

EXTRACTION OF NUCLEAR PROTEINS AND EMSA EXPERIMENTS

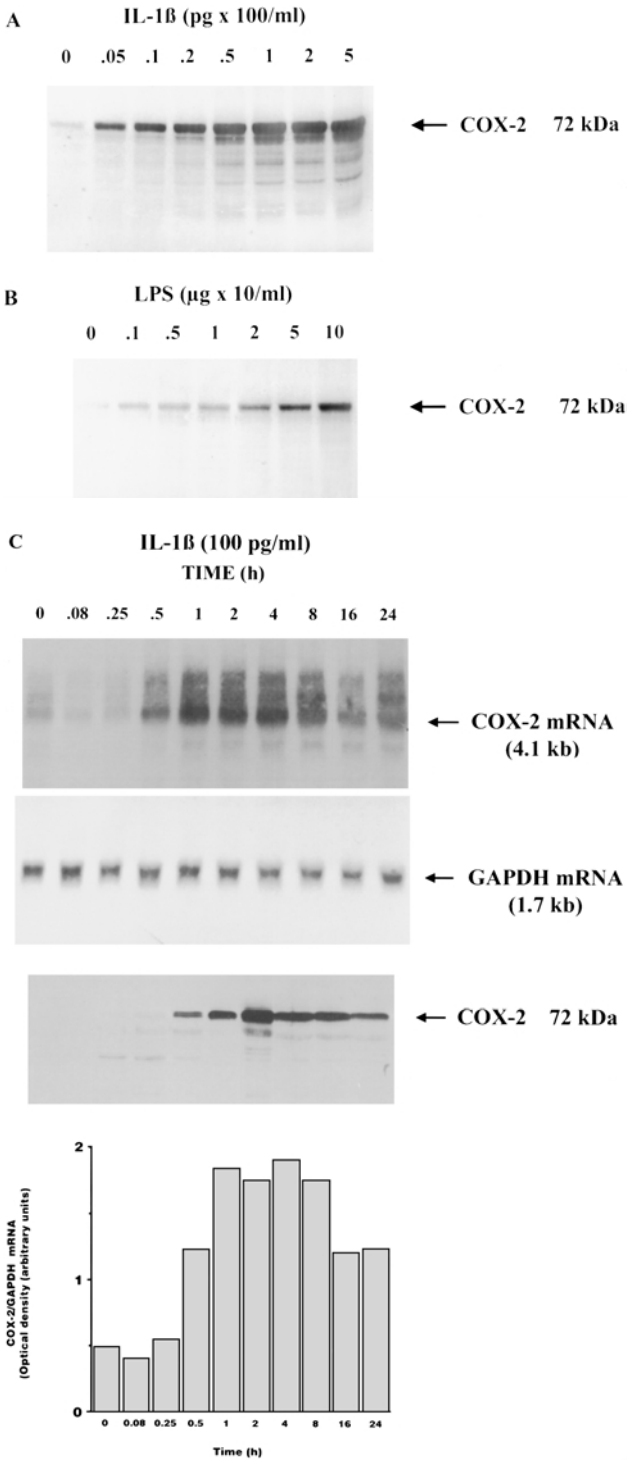
Confluent HSF in four-well cluster plates ($3–5\times10^6$ cells/well) from control and treated cells were carefully scraped into 1.5 ml of ice-cold PBS and pelleted by brief centrifugation. Nuclear extracts were prepared as previously described[11].

Double-stranded oligonucleotides containing consensus sequences (Santa Cruz Biotechnology, Santa Cruz, CA) were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). The sense sequences of the oligos tested were as follows: NF-κB; 5′-AGT TGA GGG GAC TTT CCC AGG C-3′: c/EBP-COX-2; 5′-CAC CGG GCT TAC GCA ATT TTT TTA A-3′. Binding buffer consisted of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol and 2.5 μg poly (dI-dC). Binding reactions were conducted with 15 μg of nuclear extract and 100 000 cpm of ^{32}P-labeled oligonucleotide probe at 22°C for 20 min in a final volume of 10 μl.

Binding complexes were resolved by non-denaturing polyacrylamide gel electrophoresis through 6% gels in a Tris-borate buffer system, after which the gels were fixed, dried and prepared for autoradiography.

MEASUREMENT OF PGE_2

Prostaglandin E_2 was measured in conditioned medium by ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The detection limit and sensitivity was 39 pg/ml.



Results

TIME-COURSE AND DOSE–RESPONSE ACTIVATION OF COX-2 mRNA AND PROTEIN SYNTHESIS BY IL-1β AND LPS IN HUMAN SYNOVIAL FIBROBLASTS

To establish the appropriate experimental conditions to study the effects of NIM on induced COX-2 expression, we determined temporal and concentration optimums for the inductive process. Western analysis revealed that the EC_{50} value for the induction of COX-2 protein synthesis was as follows: IL-1β (98 pg/ml or 9.8 U/ml, CV=4.1%) [Fig. 1(A)]. LPS (endotoxin) weakly stimulated COX-2 protein synthesis and the effect was not saturable being directly proportional to the concentration of LPS up to 100 µg/ml [Fig. 1(B)]. As such in subsequent experiments with NIM, IL-1β was used at 98 pg/ml and, where indicated, we added LPS at 20 µg/ml since it gave a 50% response albeit under nonsaturating conditions.

Time course studies indicated that maximum stimulation of COX-2 mRNA by IL-1β occurred at between 1–8 h followed by a gradual decline. The COX-2 protein levels peaked somewhat later than mRNA at 4–8 h with the same slow cellular depletion that characterized COX-2 mRNA [Fig. 1(C)].

EFFECT OF NIM ON BASAL AND INDUCED COX-2 mRNA EXPRESSION AND PROTEIN

NIM inhibited IL-1β-induced COX-2 expression and protein at all concentrations (0.03, 0.3, 3.0 µg/ml) (see Fig. 2) and similar results were obtained with the analog NS-398. Temporal analysis revealed that the inhibitory effect of NIM was at its most robust at 16–24 h (data not shown). Previous studies in our laboratory revealed that NIM and a prototypic non-specific COX inhibitor, naproxen, exhibited differential effects in terms of the transactivation of the glucocorticoid receptor[11]. In the present study, naproxen (NAP), at a pharmacologically relevant concentration, had no effect on IL-1β-induced COX-2 expression [Fig. 3(A)]. A profile of PGE_2 release in the presence of NIM and NAP is shown in Fig. 3(B). As expected, the prototypic antiinflammatory steroid dexamethasone (1 µmol/l) also inhibited

Fig. 1. Dose-dependent activation of COX-2 protein synthesis by IL-1β (A) and LPS (B) and time course (C) of IL-1β stimulation of COX-2 mRNA and protein in human synovial fibroblasts. Confluent synovial fibroblasts (1.2×10^6 cells in six-well plates) were pre-incubated for 24 h in DMEM supplemented with 1% FCS plus antibiotics at 37°C in order to synchronize the cell population. Cells were then treated with increasing concentrations of (A) IL-1β (0–500 U/ml) or (B) LPS (0–100 µg/ml) for 16 h at 37°C or (C) in the presence of vehicle (control) or IL-1β (10 U/ml) for 5, 15, 30 min, 1, 2, 4, 8, 16, 24 h at 37°C. Monolayers were extracted for protein and 50 µg were analysed by Western analysis for COX-2 protein. Alternatively, RNA was extracted and 10 µg were analysed for COX-2 mRNA by Northern analysis using a complementary DIG labeled cDNA probe for COX-2 and cRNA probe GAPDH (as a control for loading and mRNA recovery) as described in the Materials and methods section. Representative blots are shown in A, B, C. Densitometric and mathematical analysis of the data (N=3) yielded the following equation: y=2617+9461 log (x), r=0.97, concentration of IL-1β, x-axis vs optical density, y-axis: y=1959+94.7x, r=0.999, concentration of LPS, x-axis vs optical density, y-axis. Densitometric ratio COX-2/GAPDH mRNA (mean of two experiments, CV<11.3%) is graphically illustrated.

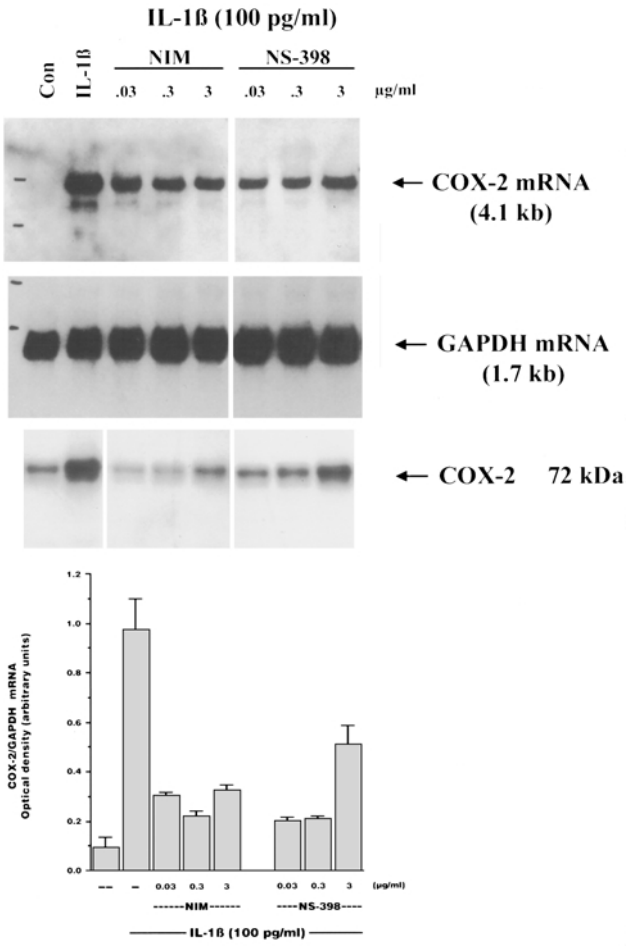


Fig. 2. Effect of NIM and NS-398 on IL-1β-induced COX-2 mRNA expression and protein synthesis in human synovial fibroblasts. Confluent synovial fibroblasts were incubated with vehicle or IL-1β (10 U/ml) in the absence or presence of 0.03, 0.3, 3 µg/ml of NIM and NS-398 for 16 h at 37°C. Monolayers were either extracted for protein and 50 µg were analysed by Western analysis for COX-2 protein or RNA was extracted and 10 µg were analysed for COX-2 mRNA by Northern analysis. A complementary DIG labeled cDNA probe for COX-2 and cRNA probe GAPDH (as a control for loading and mRNA recovery) were employed as described in the Materials and methods section. Ratio COX-2/GAPDH mRNA (mean of three experiments, CV<15.6%), determined by densitometric analysis, is shown graphically.

IL-1β/LPS-induced COX-2 expression and synthesis (data not shown). The inhibition of IL-1β induced COX-2 mRNA expression at lower doses of NIM was not the result of changes in the half-life of the COX-2 mRNA (Fig. 4).

EFFECT OF NIM ON COX-2 PROMOTER ACTIVITY

To test whether the effects of NIM on COX-2 expression were promoter based, we transfected HSF with a human COX-2 promoter construct containing at least 1.8 kb of the 5′ flanking promoter region of the COX-2 gene containing two NF-κB and AP-2 sites, a c/EBP, Ets-1 and CRE motif, and three Sp-1 sites upstream from the 5′-tataa-3′ box. NIM, at 0.03, 0.3, and 3 µg/ml, had no effect on either basal or IL-1β-induced COX-2 promoter activity in transfected HSF (Fig. 5).

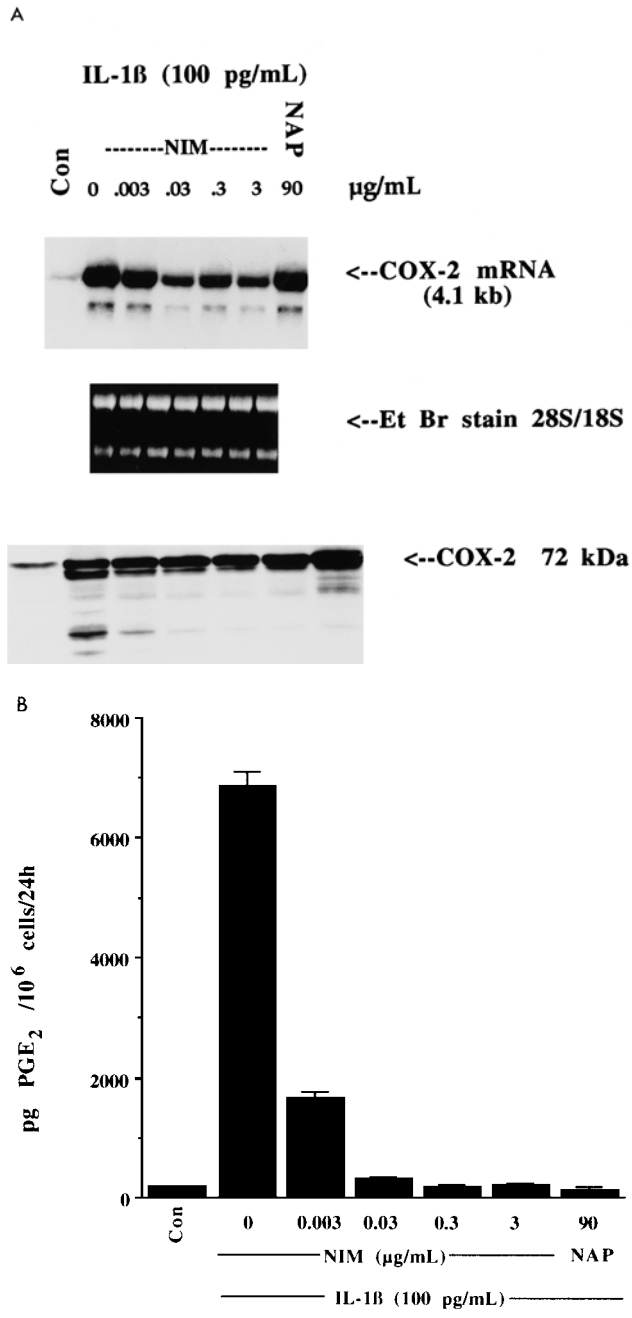


Fig. 3. Effect of NIM and naproxen on IL-1β-induced COX-2 mRNA expression, protein synthesis (A) and PGE_2 release (B) in human synovial fibroblasts. Confluent synovial fibroblasts were incubated with vehicle or IL-1β (10 U/ml) in the absence or presence of 0.003, 0.03, 0.3, 3 µg/ml of NIM and 90 µg/ml of naproxen (NAP) for 16 h at 37°C. Monolayers were either extracted for protein and 50 µg were analysed by Western analysis for COX-2 protein or RNA was extracted and 10 µg were analysed for COX-2 mRNA by Northern analysis as described in the Materials and methods section. The amount of PGE_2 released into the medium was measured by ELISA (*N*=3).

EFFECT OF NIM ON IL-1β INDUCTION OF C/EBP AND NF-κB OLIGONUCLEOTIDE NUCLEAR BINDING PROTEINS

Considerable evidence has accrued demonstrating that c/EBP and NF-κB are two important transacting factors that

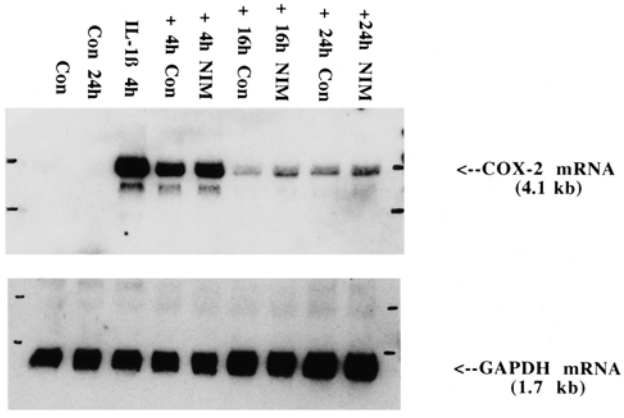


Fig. 4. Effect of NIM on IL-1β-induced COX-2 mRNA half-life in human synovial fibroblasts. Confluent synovial fibroblasts were incubated with vehicle or IL-1β (10 U/ml) for 4 h after which time the cells were transcriptionally arrested with actinomycin D (1 μg/ml). Arrested cells were further incubated with vehicle or NIM (0.3 μg/ml) for 4, 16 and 24 h. Monolayers were extracted for RNA and 10 μg were analysed for COX-2 mRNA by Northern analysis. A complementary DIG labeled cDNA probe for COX-2 and cRNA probe GAPDH (as a control for loading and mRNA recovery) were employed as described in Materials and methods. Densitometric ratio of COX-2/GAPDH mRNA (mean of two experiments, CV <3.9%) is illustrated graphically.

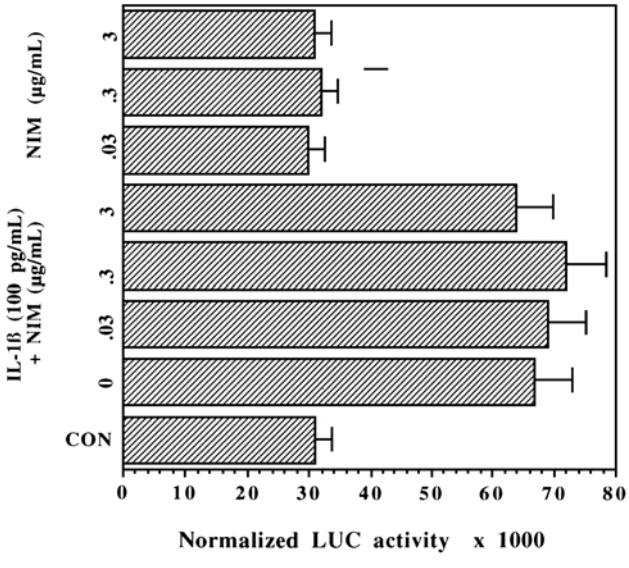


Fig. 5. Effect of NIM on basal and IL-1β induced human COX-2 promoter activity. Cells were co-transfected with 1 μg of the COX-2 promoter-LUC plasmid together with 0.5 μg of pCMV β-Gal (control for transfection efficiency) as described in the Materials and Methods section. The transfected cells were then incubated with IL-1β (10 U/ml) alone or with NIM (0.03, 0.3, 3 μg/ml) for 16 h at 37°C. Cells were then lysed and analysed for Luciferase (LUC) and β-galactosidase. LUC activity (N=2 determinations in duplicate).

mediate COX-2 promoter activation by IL-1β with the resultant increase in COX-2 gene transcription and mRNA accumulation[14–17]. As shown in Fig. 6, IL-1β (10 U/ml) strongly induced both c/EBP and NF-κB oligonucleotide binding but NIM, at a concentration (0.3 μg/ml) that inhibits COX-2 expression, had no effect on the level of nuclear protein binding to the ^{32}P-oligonucleotides tested.

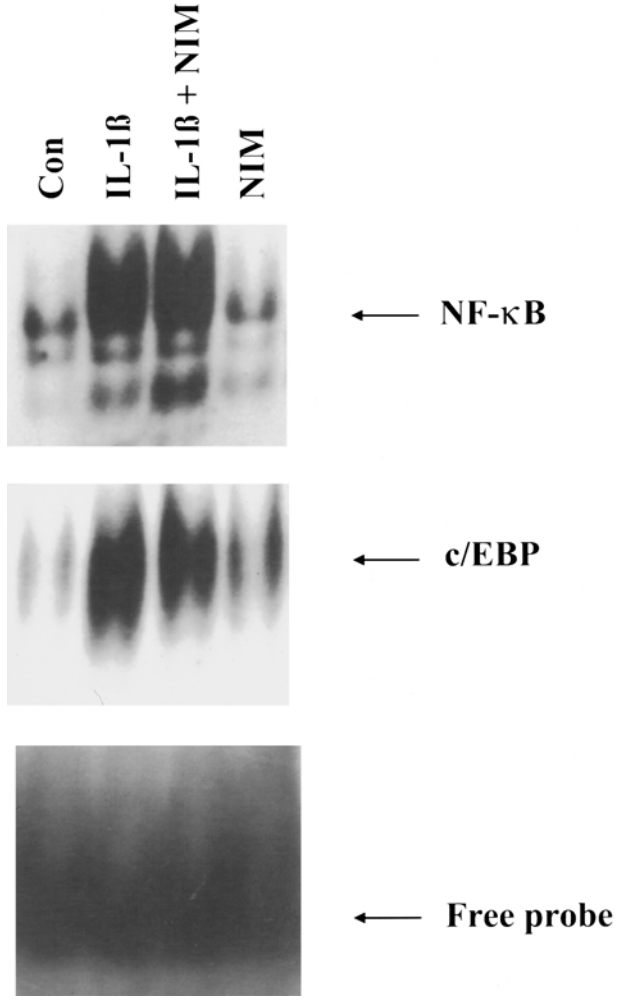


Fig. 6. Effect of NIM on IL-1β induced c/EBP and NF-κB oligonucleotide nuclear binding proteins. Confluent synovial fibroblasts were incubated with vehicle or Il-1β (10 U/ml) in the absence or presence of NIM (0.3 μg/ml), after which time nuclear extracts were prepared. Gel shift binding reactions were conducted as described in the Materials and methods section as follows: upper panel; lane 1, control nuclear protein extracts+^{32}P-labeled NF-κB oligonucleotide; lane 2, IL-1β stimulated nuclear extracts+^{32}P-labeled NF-κB oligonucleotide; lane 3, IL-1β+NIM treated nuclear extracts+^{32}P-labeled NF-κB oligonucleotide; lane 4, L-1β stimulated nuclear extracts+^{32}P-labeled NF-κB oligonucleotide with five-fold excess radioinert NF-κB oligonucleotide competitor. Middle panel; lanes 5–8 represent the same experiment as lanes 1–4 but ^{32}P-labeled c/EBP oligonucleotide was used.

REACTIVE OXYGEN SPECIES (ROS), CALCIUM AND IL-1β INDUCTION OF COX-2 PROTEIN SYNTHESIS

IL-1β induces a number of genes indirectly through short-lived reactive oxygen species (ROS) intermediaries that include NO, H_2O_2, and superoxide anion[18,19]. In human chondrocytes, IL-1β induced COX-2 expression occurs, in part, by changes in cellular calcium metabolism[20,21]. IL-1β-induced COX-2 protein was modestly suppressed (compared to NIM), by the L-type calcium channel blockers nifedipine and verapamil, with verapamil being more efficacious than nifedipine [Fig. 7(A)]. Increasing the intracellular levels of calcium through the use of the calcium ionophore ionomycin, in combination with endoplasmic

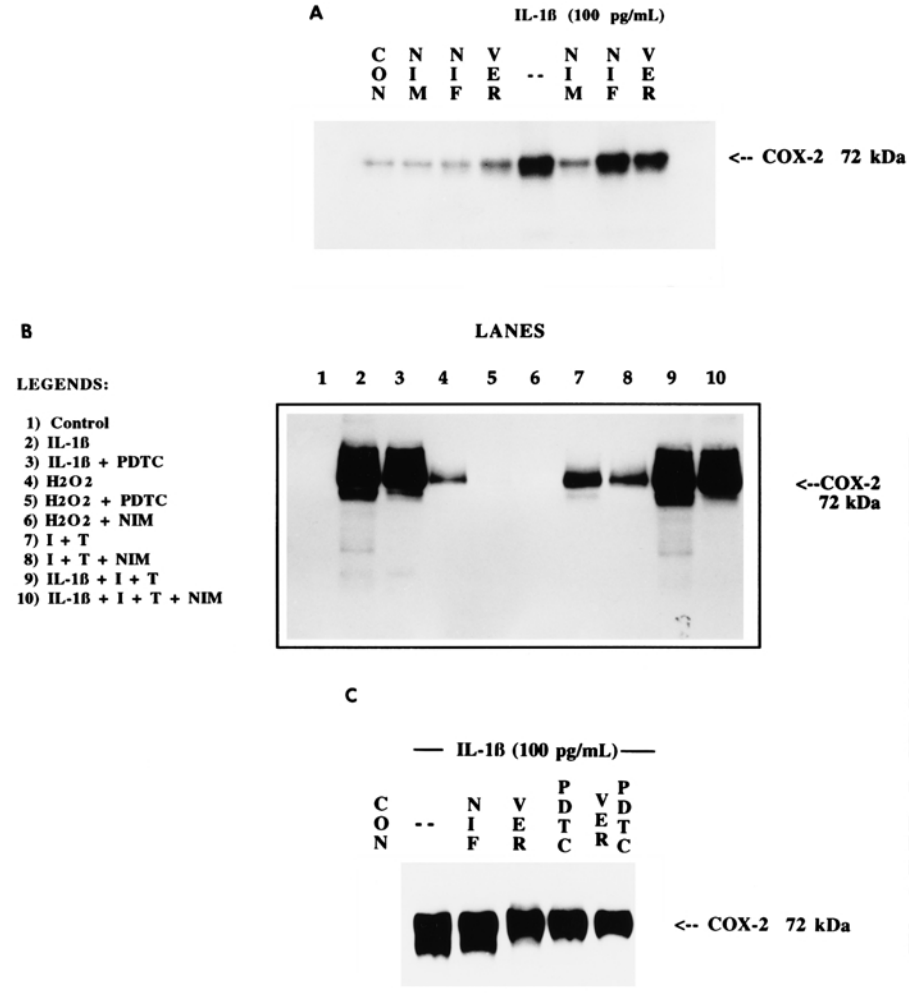


Fig. 7. Role of reactive oxygen species and calcium in IL-1β-induced COX-2 protein synthesis. Confluent synovial fibroblasts were incubated for 16 h at 37°C with vehicle, nimesulide (NIM, 0.3 μg/ml), nifedipine (NIF, 5 μmol/l), or verapamil (VER, 50 μmol/l) with or without 100 pg/ml of IL-1β(A). In (B), cells were incubated for 16 h at 37°C as described in the legend (PDTC, 100 μmol/l; H_2O_2, 100 nmol/l; NIM, 0.3 μg/ml; ionomycin (I), 10 nmol/l; thapsigargin (T), 20 nmol/l). In (C), cells were incubated for 16 h at 37°C without (Con) or with 100 pg/ml of IL-1β in the presence or absence of NIF (5 μmol/l), VER (50 μmol/l), PDTC (100 μmol/l), or PDTC+VER. In all cases, cells were processed for Western analysis (COX-2 protein) using 50 μg of cellular protein as described in the Materials and methods section.

reticular Ca^{2+}-ATPase inhibitor thapsigargin, resulted in an increase in COX-2 protein synthesis [compare lane 1 vs lane 7, Fig. 7(B)]. This induction was inhibited by NIM (0.3 μg/ml) (compare lane 7 vs lane 8). Combining IL-1β with ionomycin and thapsigargin slightly enhanced COX-2 synthesis over ionomycin and thapsigargin alone (lane 2 vs lane 9).

In an attempt to verify whether COX-2 induction by IL-1β is dependent on ROS in HSF, we treated cells with IL-1β in the presence of the ROS scavenger PDTC (100 μmol/l). As shown in Fig. 7(B) (lane 2 vs lane 3), PDTC had a modest effect on IL-1β induced COX-2 protein synthesis. Induction of COX-2 by H_2O_2 (100 nmol/l) was reversed by PDTC and NIM (lanes 4, 5 and 6). Combining PDTC with verapamil had a slight additive effect in terms of the suppression of induced COX-2 protein synthesis [Fig. 7(C), lanes 4, 5 and 6].

Discussion

This study was inspired by our rather novel findings that NSAIDs, in particular specific COX-2 inhibitors of the sulfonamide class (NIM, NS-398), owe their therapeutic efficacy in part to what we have defined as 'allo-effects'. The latter consist of activities other than the simple inhibition of cyclo-oxygenase activity and release of prostaglandins and can be either promoter-based, modulation of signaling cascades, suppression of free-radical generation or modulation of second messengers (e.g. calcium flux). Examples of 'allo-effects' would be changes in the expression level of target genes (e.g. COX-2), or the recently reported activation of ligand inducible nuclear receptor systems (e.g. glucocorticoid receptor) with the resultant induction of target gene promoters[11].

Interleukin-1β induces COX-2 gene expression in human connective tissue cells by binding to a specific cell-surface receptor (IL-1RI) with the resultant activation of a signaling cascade(s) (e.g. IL-1RI/IL-1RacProtein/IRAK1/2/TRAF6/NIKor MEKK) which terminate(s) in the nucleus with increases in COX-2 promoter activity mediated by NF-κB[14,15]. In addition, c/EBP enhancer sequences are also believed to play a role and c/EBPβ/δ synergize transcriptionally with NF-κB for full activation of the human COX-2 promoter[15,16]. An alternative but less well described

mechanism is the mediation of IL-1β action by changes in cellular calcium levels[20,21] and cAMP[22]. Such changes can activate calmodulin-dependent protein kinases (e.g. CaMKII, KIV) and protein kinase A with the resultant increases in transcriptionally active CREB/ATF transcription factors[23]. The latter are known to strongly activate COX-2 expression via CRE sites in the proximal promoter region[24,25]. In addition, there is evidence that AP-1 complexes can activate COX-2 through transactivation via CRE site (there is no AP-1 site in the COX-2 promoter) although in the latter studies AP-1 complexes were not IL-1β generated[25,26]. Our data clearly demonstrate that the suppression of IL-1β induced-COX-2 expression by sub and therapeutic concentrations of NIM is not due to inhibitory effects on NF-κB or c/EBP-associated signaling cascades. It is also unlikely that NIM acts on IL-1β induced immediate–early genes like c-Jun/c-Fos (AP-1) since the action of NIM on COX-2 required more than 7–8 h and was most robust at 16–24 h.

IL-1β induces a number of genes (e.g. *c-fos*, MMP-1) through short-lived reactive oxygen species intermediaries which include NO, H_2O_2, and superoxide anion[18,19]. The IL-1β stimulated increase in COX-2 gene expression in human chondrocytes is significantly enhanced by co-incubations with agents that raise intracellular calcium concentrations[20,21]. In the present study, antioxidants which quench either H_2O_2 or O_2 modestly affected the IL-1β induction of COX-2 in HSF; NO is not produced by HSF so the issue is moot. Exogenously added H_2O_2 mildly induced COX-2 which was reversed with additions of PDTC (as expected) and NIM. The latter drug potently blocks superanion generation in neutrophils[6–8], confirming one of its 'allo-effects' as a quenching agent. However, it seems clear that IL-1β induction of COX-2 is only partially dependent on ROS generation and therefore the inhibitory profile exhibited by NIM, being more extensive than PDTC, suggests that the drug can impact on other cellular responses induced by cytokines. Important in this regard is the observation that NIM blocked ionomycin+thapsigargin-induced COX-2 synthesis, arguing that a role for NIM in calcium metabolism may be of some consequence. Supporting these findings are reports showing that the drug can inhibit calcium channel flux and intracellular accumulation of calcium in different cell types[27–28]. The present data also suggests that, as in chondrocytes, IL-1β stimulation of COX-2 required at least some change in cellular calcium metabolism. We used two inhibitors of L-type voltage-regulated Ca^{++} channels to confirm the observation, despite their different potencies. The concentrations chosen were based on our previous work and spectrofluorescent studies using Fura 2/AM loaded human connective tissue cells[29–31]. Nifedipine is slightly more specific than verapamil, but cannot be used at doses higher than the one we have chosen because it induces apoptosis. Verapamil is less toxic and therefore higher concentrations can be used for a fuller effect.

Figure 3 clearly illustrates a relationship between PGE_2 released and the level of COX-2 mRNA and protein. This relationship is not totally unexpected but one cannot immediately implicate a role for prostanoids/leukotrienes, particularly in light of the data obtained with naproxen. The COX-2 gene is considered an immediate–early response gene[26], and its induction by IL-1β occurs as early as 10–15 min in human connective tissue cells (MS in preparation). However, traces of PGE_2 are detectable after only 1–2 h, so the initial induction cannot be due to prostaglandin feedback. Nevertheless, products of arachidonic acid metabolism could conceivably impact on later COX-2 expression.

In planning this study we selected our cellular (tissular) model and biological effectors to be relevant to arthritic diseases (e.g. OA). Indeed IL-1β is produced by resident cells (e.g. macrophages, type A synoviocytes) of the inflamed synovium and functions in an autocrine/paracrine fashion, stimulating the release of a plethora of pro-inflammatory mediators[32]. Lipopolysaccharide (LPS), or endotoxin, is the major component of the outer surface of Gram-negative bacteria and is a potent activator of cells (macrophages/monocytes, endothelial cells, types A and B synoviocytes) of the inflammatory response[33]. A minor but potentially interesting observation in this present study was the lack of sensitivity of HSF to LPS action at least in terms of COX-2 synthesis and mRNA expression. Chondrocytes are quite sensitive to LPS in this regard[21], as are endothelial cells and macrophages[34]. Since the most recent data suggest that LPS signaling cascade uses an analogous molecular framework for signaling as IL-1β[34], subtle differences in IL-1β and/or LPS activated signaling pathways must exist between HSF and human chondrocytes and would be an interesting subject for further exploration.

In summary, at therapeutically relevant concentrations, NIM was shown to inhibit not only COX-2 activity but also COX-2 expression and synthesis. At least part of this inhibitory profile may be due to NIM effects on calcium and ROS metabolism. Thus, our work has demonstrated additional 'allo-effects' of this distinct sulfonamide class of COX-2 specific inhibitors and points strongly to the possibility that we may greatly enlarge their therapeutic usefulness.

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