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Nimesulide, a Preferential Cyclooxygenase 2 Inhibitor, Suppresses Peroxisome Proliferator–Activated Receptor Induction of Cyclooxygenase 2 Gene Expression in Human Synovial Fibroblasts

Evidence for Receptor Antagonism

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Objective. **To characterize the inhibitory effects of therapeutic concentrations of the nonsteroidal antiinflammatory drug nimesulide (NIM) on peroxisome proliferator–activated receptor (PPAR)–induced cyclooxygenase 2 (COX-2) gene expression in human synovial fibroblasts (HSFs) from patients with osteoarthritis (OA) and to define the intracellular mechanisms mediating the response.**

Methods. **PPARα and PPARγ messenger RNA (mRNA) expression and protein synthesis in OA HSFs were measured by reverse transcription–polymerase chain reaction and electrophoretic mobility shift assay, respectively. Experiments investigating endogenous and overexpressed PPARα and PPARγ activation of COX-2 mRNA and protein were conducted by incubating nontransfected and transfected cells with increasing concentrations of cognate ligands WY-14,643 (α agonist), ciglitasone (γ agonist), and 15-deoxy–$\Delta^{12,14}$–prostaglandin J_2 (15d-PGJ_2) in the absence or presence of NIM and NS-398 (1 μM). COX-2 mRNA and protein were measured by Northern and Western blotting procedures, respectively. Receptor activation studies were evaluated by cotransfecting pSG5–Gal 4 DNA binding domain (DBD)–PPARα ligand binding domain (LBD) or pSG5–Gal 4 DBD–PPARγ LBD chimeric constructs with a 5× Gal 4 enhancer site tk-tataa-luciferase reporter under ligand stimulation in the presence or absence of increasing concentrations of NIM. Gene transactivation analyses were conducted by treating cells overexpressing cytomegalovirus (CMV)–PPARα or CMV–PPARγ expression constructs with either a PPAR response element (PPRE)–luciferase construct containing 3 DR1 acyl–coenzyme A (acyl-CoA) oxidase gene response elements or human COX-2 promoter constructs with WY-14,643, ciglitasone, and 15d-PGJ_2 in the presence or absence of increasing concentrations of NIM.**

Results. **Human synovial cells expressed functional PPAR isoforms, PPARα and PPARγ. Neither receptor agonists nor antagonists modulated the intracellular protein levels of PPAR. PPARα and, especially, PPARγ mediated the induction of COX-2 gene expression by receptor agonists. Stimulation of COX-2 mRNA expression and protein synthesis by 15d-PGJ_2 appeared to occur through a receptor-independent process. NIM inhibited PPAR agonist stimulation of COX-2 expression and synthesis in a dose-dependent manner in both nontransfected cells and cells overexpressing both receptor isoforms. NIM potently abrogated basal and ligand-stimulated $PPRE_{3X}$ DR1 acyl-CoA oxidase–driven luciferase activity and also human PPRE–containing COX-2 promoter activity.**

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Conclusion. **PPAR-mediated induction of COX-2 expression and synthesis in human OA synovial fibroblasts is inhibited by therapeutic concentrations of NIM through the functional antagonism of ligand-dependent receptor activation, with the resultant suppression of PPAR-dependent transactivation of target genes (e.g., COX-2).**

The activation of target cells by proinflammatory stimuli results in, among other responses, increased phospholipid-derived eicosanoid synthesis and release. Oxidized derivatives of arachidonic acid (AA), eicosanoids are a diverse group of molecules widely acknowledged to play a cardinal role in the etiopathogenesis of many immune and inflammatory diseases (1,2). Furthermore, acting locally in an intracrine, autocrine, or paracrine manner, eicosanoids initiate and modulate cell and tissue responses involved in many physiologic processes affecting essentially all organ systems (3,4). Prostanoids are synthesized through the coordination of multiple enzyme systems, although the rate-limiting step is the conversion of AA to prostaglandin H_2 (PGH_2) by cyclooxygenase (COX) (5,6). A ubiquitous, constitutive COX-1 isoform that serves a homeostatic function in differentiated cells and an inducible COX-2 enzyme that is up-regulated by growth factors, cytokines, and mitogens have been identified (7–15).

Peroxisome proliferator–activated receptors (PPARs, isoforms α, β, γ) are ligand-inducible nuclear transacting factors belonging to the steroid/thyroid/retinoid receptor superfamily (for review, see ref. 16). These receptors, activated by antidiabetic drugs (thiazolidinediones), natural fatty acids, leukotrienes, and prostaglandins of the A and J series, are believed to control a variety of target genes (e.g., acyl–coenzyme A [acyl-CoA] oxidase, a P2 adipocyte lipid-binding protein) involved in lipid metabolism and energetics, primarily in liver cells, colonic cells, and adipocytes (16–20). Considerably lower levels of PPARs have been detected in cells of monocyte/macrophage origin, neutrophils, and rodent T lymphocytes as well as in rat and human chondrocytes (16,21–23). In this connection, PPARs may play a role in inflammatory responses, because natural and synthetic ligands have been shown to inhibit proinflammatory cytokine expression in macrophages as well as nitric oxide (NO) and matrix metalloproteinase 13 (MMP-13) production in human chondrocytes (22,24,25).

Nonsteroidal antiinflammatory drugs (NSAIDs) were recently defined as a novel group of PPARγ activators, providing an additional mechanistic rationale for their observed adipogenic and peroxisomal activities (16,26). In this same capacity, NSAIDs suppress production of monocyte-derived inflammation mediators including tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) (24). However, a panel of NSAIDs of diverse specificities and potencies also increased expression of COX-2 in colonic epithelial cells (20); the PPARγ system was shown to mediate the latter response. Importantly in this regard, recent work suggests that elevated COX-2 expression is tumorigenic in colonic cells (27,28). In support of this, inactivation of the COX-2 gene in mice decreases intestinal tumorigenesis (polyp formation) (29). Paradoxically, however, patients for whom NSAIDs are regularly prescribed have a significantly lower risk of developing colorectal cancer than do age-matched controls (30). In a similar vein, perhaps, elevated expression of COX-2 on rheumatoid arthritis–affected synovial membranes was associated with synovial fibroblast proliferation, angiogenesis, and inflammation (31), providing a basis for explaining the antiinflammatory effects of NSAIDs.

Nimesulide (NIM) is a preferential inhibitor of COX-2 activity with marked biologic effects in several in vivo models of inflammation (32–36). Results of a number of studies have suggested that, in addition to the well-described inhibition of prostaglandin synthesis, NIM exerts pleiotropic effects, particularly in terms of neutrophil function (e.g., respiratory burst) (37–39). Many of these effects were attributed to the ability of NIM to increase cellular levels of cAMP by inhibiting cAMP-dependent phosphodiesterase type IV (38). We previously demonstrated that NIM stimulates glucocorticoid receptor (GR) transactivational activity in human synovial fibroblasts through mitogen-activated protein (MAP) kinase–mediated GR hyperphosphorylation (40). We also reported that NIM suppresses not only cytokine-induced COX-2 activity but also its messenger RNA (mRNA) expression and protein synthesis (41). The latter effect was manifested through changes in calcium flux, oxygen radical formation, and prostaglandin release.

In this report, we describe a PPAR-dependent induction of COX-2 expression and synthesis in human osteoarthritis (OA) synovial fibroblasts that is inhibited by the addition of increasing concentrations of NIM and its structural analog NS-398. We show that NIM behaves as a functional antagonist of PPAR activation and PPAR-dependent transactivation of target genes (e.g., COX-2). Our observations suggest that at least part of the well-documented antiinflammatory and anti–COX-2 activity of NIM may be manifested through the latter pathway.

MATERIALS AND METHODS

Chemicals. Nimesulide (*N*-[4-nitro-2-phenoxyphenyl]-methanesulfonamide) was kindly provided by Helsinn Healthcare SA (Lugano, Switzerland). Sodium vanadate, sodium fluoride, okadaic acid, leupeptin, aprotinin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), and bovine serum albumin were from Sigma (St. Louis, MO). Sodium dodecyl sulfate, acrylamide, bis-acrylamide, agarose, ammonium persulfate, and Bio-Rad protein reagent were from Bio-Rad (Richmond, CA). Tris, EDTA, $MgCl_2$, $CaCl_2$, chloroform, DMSO, formaldehyde, and formamide were from Fisher Scientific (Montreal, Quebec, Canada). WY-14,643, ciglitasone, and eicosatetraynoic acid were obtained from Alexis (San Diego, CA), and 15-deoxy–$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and NS-398 were from Cayman Chemical (Ann Arbor, MI). Dulbecco's modified Eagle's medium (DMEM), TRIzol reagent, heat-inactivated fetal calf serum (FCS), and an antibiotic mixture (10,000 units penicillin [base], 10,000 μg streptomycin [base]) were products of Gibco BRL Life Technologies (Burlington, Ontario, Canada).

Specimen selection and synovial fibroblast cultures. Synovial lining cells (human synovial fibroblasts; HSFs) were isolated from OA patients undergoing arthroplasty. The patients had been initially evaluated by a certified rheumatologist, and their conditions were diagnosed based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA (42). Normal HSFs were established as cell lines SN1–7, originating from organ donors, and procedures for procuring the tissues conformed to all guidelines of the institution's ethics committee. HSFs were released by sequential enzymatic digestion with 1 mg/ml pronase (Boehringer Mannheim, Indianapolis, IN) for 1 hour, followed by 6 hours 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 (40,43). Released HSFs were incubated for 1 hour at 37°C in tissue culture flasks (Primaria #3824; Falcon, Lincoln Park, NJ), allowing the adherence of nonfibroblastic cells possibly present in the synovial preparation. In addition, flow cytometric analysis (Epic II; Coulter, Miami, FL), using the anti-CD14 (fluorescein isothiocyanate) antibody, was conducted to confirm that no monocyte/macrophages were present in the synoviocyte preparations (43). 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 hours before the experiments, and only first- or second-passaged HSFs were used.

Preparation of cell extracts and Western blotting. Fifty to 150 μg of cellular extract (in radioimmunoprecipitation assay buffer; 50 m*M* Tris HCl, pH 7.4, 150 m*M* NaCl, 2 m*M* EDTA, 1 m*M* PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% Nonidet P40, 1 m*M* sodium orthovanadate, and 1 m*M* NaF) from control and treated HSFs were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis through 10% gels (final concentration of acrylamide) under reducing conditions and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada), as previously described (44). Following blocking (with 5% Blotto) and washing, the membranes were incubated for either 2 hours at room temperature or overnight at 4°C with primary antibodies (see below) in Tris buffered saline–Tween (TBST) containing 0.25% Blotto. Second anti-rabbit or anti-goat antibody–horseradish peroxidase conjugates (1:4,000 dilution) were subsequently incubated with membranes for 1 hour at room temperature, then washed extensively for 30–40 minutes with TBST, with a final rinsing with TBS at room temperature. Following incubation with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech and Pierce, Rockford, IL), membranes were prepared for autoradiography and exposed to X-Omat film (Eastman Kodak, Rochester, NY), then subjected to densitometric analysis with the ChemiImager 4000 imaging system (Alpha Innotech, San Leandro, CA) for semiquantitative measurements. Rabbit polyclonal anti-human COX-2 antibodies (Cayman Chemical) and goat polyclonal anti-PPARα and anti-PPARγ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used at dilutions of 1:7,500 and 1:2,000, respectively.

Northern blot analysis of mRNA. Total cellular RNA was isolated (1×10^6 cells = 20–30 μg RNA) using the TRIzol reagent, as previously described (44). Generally, 5–15 μg of total RNA was resolved on 1.2% agarose–formaldehyde gel and transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech) in Tris–acetate–EDTA buffer (20 m*M* Tris, 10 m*M* sodium acetate, and 0.5 m*M* EDTA; pH 7.8) by electroblotting overnight at 4°C. After prehybridization for 24 hours, hybridizations were carried out at 50–57°C for 24–36 hours, followed by high-stringency washing (44,45). The following probes, labeled with digoxigenin–dUTP by random priming, were used for hybridization: 1) a human COX-2 complementary DNA (cDNA) (1.8 kb, Cayman Chemical), initially cloned into a *Eco* RV site of pcDNA 1 vector (Invitrogen, San Diego, CA), was digested with *Pst* I and *Xho* I, resulting in the release of a 1.2-kb cDNA fragment; 2) a 780-bp *Pst* I/*Xba* I fragment released from human GAPDH cDNA (1.2 kb; American Type Culture Collection, Rockville, MD) that had been originally cloned into a *Pst* I site of a pBR322 vector. This latter probe served as a control of RNA loading, because GAPDH is constitutively expressed. All blots were subjected to densitometric analysis for semiquantitative measurements (see above).

Extraction of nuclear proteins and electrophoretic mobility shift assay (EMSA). Confluent HSFs in 4-well cluster plates ($3–5 \times 10^6$ cells/well) from control and treated cells were carefully scraped into 1.5 ml of ice-cold phosphate buffered saline and pelleted by brief centrifugation. Nuclear extracts were prepared as previously described (40).

Double-stranded oligonucleotides containing consensus sequences (Santa Cruz Biotechnology) were end-labeled with γ-^{32}P–ATP using T4 polynucleotide kinase (Promega, Madison, WI). The sense sequences of the oligos tested were as follows: PPAR DR1 acyl-CoA oxidase gene–like consensus 5′-CAA-AAC-TAG-GTC-AAA-GGT-CA-3′ or the mutant construct PPAR mut 5′-CAA-AAC-TAG-CAC-AAA-GCA-CA-3′. Binding buffer consisted of 10 m*M* Tris HCl, pH 7.5, 50 m*M* NaCl, 0.5 m*M* DTT, 0.5 m*M* EDTA, 1 m*M* $MgCl_2$, 4% glycerol, and 2.5 μg poly(dI-dC). Binding reactions were conducted with 15 μg of nuclear extract and 100,000 counts per minute of ^{32}P-labeled oligonucleotide probe at 22°C for 20 minutes in a final volume of 10 μl. Binding complexes were resolved by nondenaturing polyacrylamide gel electrophoresis

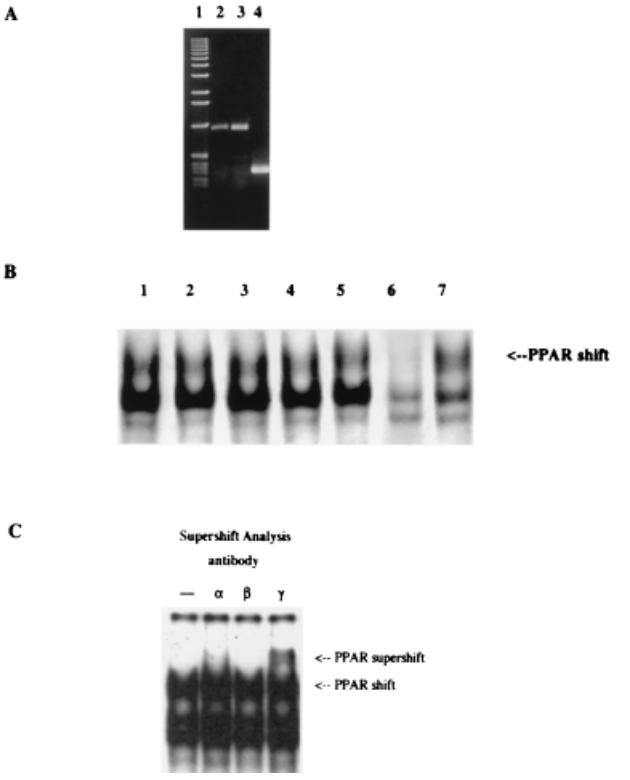

Figure 1. Expression of peroxisome proliferator–activated receptor α (PPARα) and PPARγ by human osteoarthritis (OA) fibroblasts. **A,** Confluent quiescent OA synovial fibroblasts (1.2×10^6 cells/well) in first-passage culture were extracted for RNA, and 1 μg was subjected to reverse transcriptase–polymerase chain reaction with specific sense and antisense primers for human PPARα, PPARγ, and GAPDH, as described in Materials and Methods. 1 = 1-kb ladder; 2 = human PPARα (hPPARα), 948 bp; 3 = hPPARγ, 900 bp; 4 = GAPDH, 318 bp. **B,** Nuclear extracts were prepared from confluent quiescent synovial cells incubated in the absence or presence of ciglitasone, WY-14,643, 15-deoxy–$\Delta^{12,14}$–prostaglandin J_2 (15d-PGJ_2), or nimesulide (NIM) for 6 hours. Fifteen micrograms of extract was incubated with a ^{32}P-labeled PPAR DR1 acyl–coenzyme A oxidase gene–like consensus 5′-CAA-AAC-TAG-GTC-AAA-GGT-CA-3′ or the mutant construct PPAR mut 5′-CAA-AAC-TAG-CAC-AAA-GCA-CA-3′ and subjected to electrophoretic mobility shift assay (EMSA) as described in Materials and Methods. 1 = control; 2 = ciglitasone, 50 μM; 3 = WY-14,643, 50 μM; 4 = 15d-PGJ_2, 10 μM; 5 = NIM, 1 μM; 6 = PPAR response element (PPRE) 100×; 7 = PPRE mut 100×. **C,** Fifteen micrograms of extract was incubated with a ^{32}P-labeled PPAR DR1 for 20 minutes at 22°C, and then 5 μg of goat polyclonal anti-PPARα, β, or γ was added for an additional 30 minutes before EMSA.

through 6% gels in a Tris–borate buffer system at 4°C, after which the gels were fixed, dried, and prepared for autoradiography (40,44). Supershift analysis was conducted by adding 2–5 μg of goat polyclonal anti-PPAR α, β, or γ for 30 minutes at 22°C to the preformed nuclear extract/^{32}P-labeled oligonucleotide probe reaction mixture prior to gel loading.

Plasmids and transfection experiments. Transient transfection experiments were conducted in 4-well cluster plates with 5×10^5 cells that were serum starved for 24 hours before experimentation, as previously described (22,40). Transfections were conducted with SN7 fibroblasts (HeLa cells were also used for confirmatory studies) at 40% confluence by the FuGENE 6 method (Boehringer Mannheim, Laval, Quebec, Canada) for 6–16 hours, according to the manufacturer's protocol. Cells were reexposed to complete culture medium for 16 hours and then were serum starved for 2–4 hours prior to drug treatments. Transfection efficiencies were monitored by cotransfection with 0.5 μg of pCMV β-gal, a β-galactosidase reporter vector under the control of a cytomegalovirus (CMV) promoter.

The PPAR response element (PPRE) luciferase construct, containing 3 DR1 acyl-CoA oxidase gene response elements cloned upstream from the herpes simplex virus thymidine kinase (tk) promoter, was a gift from Dr. C. K. Glass (University of California, San Diego) (25). The human COX-2 promoter construct used was a 2.072-kb fragment (−1870 [*Eco* RI] to +123 [*Msp* AII]). *Hind* III polylinkers were added, and the fragment was cloned into a *Hind* III site upstream of the firefly luciferase gene in a pGL3 basic vector (Promega). The construct was generously provided by Dr. Stephen Prescott (University of Utah, Salt Lake City) (20). Another COX-2 promoter construct used was based on the 7.270-kb COX-2 promoter (deposited by Meade et al., 1998, GenBank accession no. AF044206) containing a putative PPRE at −3900 bp. Primers were designed as follows: sense 5′-CTG-CCT-GTG-CAT-TTC-TGC-TCC-3′ (−3479 bp); antisense 5′-CTG-GCT-GTG-GAG-CTG-AAG-GAG-G-3′ (+90 bp). An ~4-kb fragment was amplified from an *Eco* RI digest of a human placenta genomic library (Gene Walker; Clontech, Palo Alto, CA) to which *Hind* III polylinkers were added, with subsequent subcloning into a *Hind* III site in a pGL3 basic vector (20).

The receptor expression chimeras used for signaling studies were pSG5–galactose 4 (Gal 4) DNA binding domain (DBD)–PPARα ligand binding domain (LBD) and pSG5–Gal 4 DBD–PPARγ LBD, together with a 5× Gal 4 enhancer site tk-tataa-luciferase reporter system, plus the CMV–PPARα and CMV–PPARγ receptor expression constructs (provided by Dr. Stephen Kliewer, Glaxo Wellcome, Research Triangle Park, NC) (17). Luciferase values, expressed as enhanced relative light units, were measured in a Lumat LB 9507 luminometer (EG & G, Stuttgart, Germany) and normalized to the level of β-galactosidase activity (optical density at 450 nm [OD_{450}] after 24-hour incubation) and cellular protein (bicinchoninic acid procedure; Pierce).

Oligonucleotide primers for PPARs. The oligonucleotide primers for the polymerase chain reactions (PCR) were prepared with the aid of a Cyclone DNA synthesizer (Biosearch, Montreal, Quebec, Canada) and used at a final concentration of 200 nmoles/liter. The sequences for the PPAR primers were as follows: 5′-GAC-GAA-TGC-CAA-GAT-CTG-AGA-AAG-C-3′ and 5′-CGT-CTC-CTT-TGT-AGT-GCT-GTC-AGC-3′ (antisense) for the human PPARα fragment of 948 bp and 5′-GGC-AAT-TGA-ATG-TCG-TGT-CTG-TGG-AGA-TAA-3′ and 5′-AGC-TCC-AGG-GCT-TGT-AGC-AGG-TTG-TCT-TGA-3′ (antisense) for the PPARγ fragment of 900 bp (46). The sequences for the GAPDH (which served as a standard of quantitation) primers were 5′-CAG-AAC-ATC-ATC-CCT-GCC-TCT-3′, which

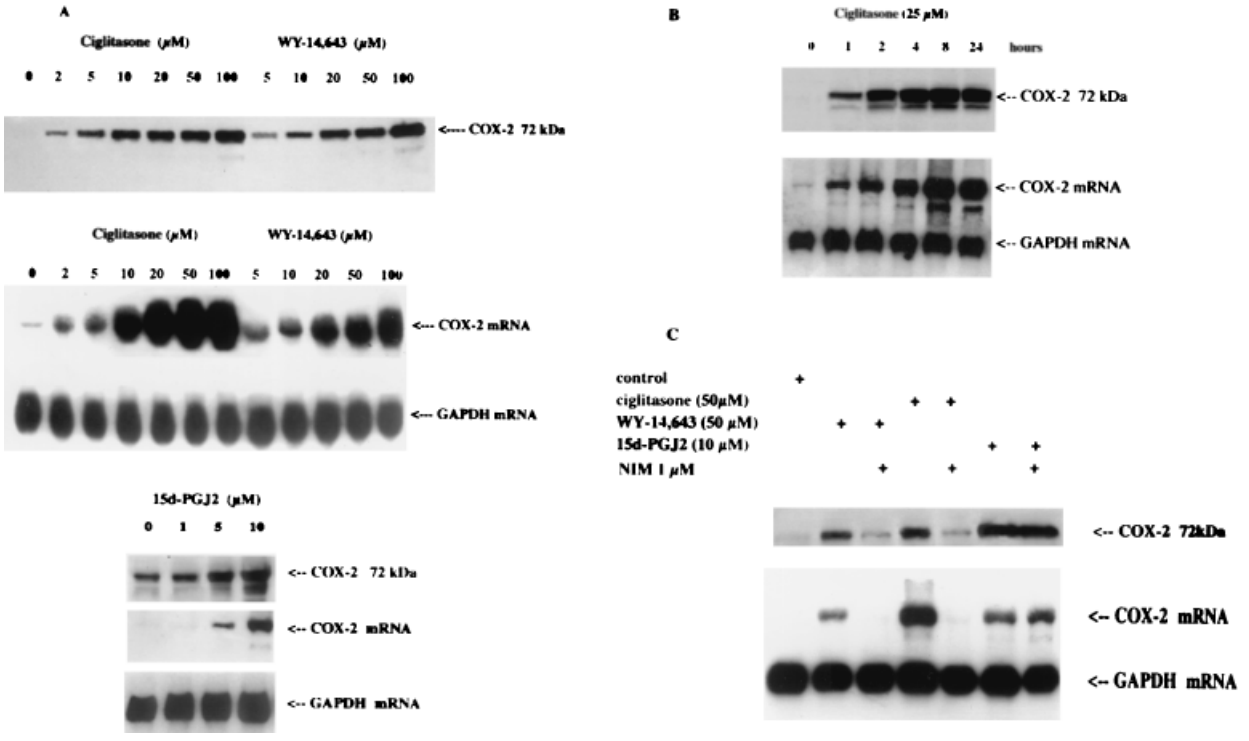

Figure 2. NIM inhibition of PPAR ligand induction of cyclooxygenase 2 (COX-2) mRNA and protein in OA human synovial fibroblasts. **A,** Confluent synovial fibroblasts were incubated with increasing concentrations of ciglitasone (0, 2, 5, 10, 20, 50, and 100 μM), WY-14,643 (5, 10, 20, 50, and 100 μM), or 15d-PGJ_2 (0, 1, 5, and 10 μM) for 6–8 hours at 37°C. Monolayers were either extracted for protein and 50–150 μg was analyzed for COX-2 protein by Western blot analysis using a specific anti–COX-2 antiserum, or RNA was extracted and 5–15 μg was analyzed for COX-2/GAPDH mRNA by Northern blot analysis. Complementary digoxigenin-labeled cDNA probes for COX-2 and GAPDH (as a control for loading and mRNA recovery) were used as described in Materials and Methods. **B,** Cells were incubated with 25 μM of ciglitasone for 0, 1, 2, 4, 8, or 24 hours, and cells were processed for COX-2 mRNA and protein analysis as described in Materials and Methods. **C,** Cells were incubated with WY-14,643, ciglitasone, or 15d-PGJ_2 for 6 hours at 37°C in the presence or absence of NIM as indicated. Protein was isolated for Western blot analysis of COX-2. See Figure 1 for other definitions.

corresponds to position 604–624 bp of the published sequence, and 5′-GCT-TGA-CAA-AGT-GGT-CGT-TGA-G-3′, position 901–922 bp, for an amplified product of 318 bp (40).

Reverse transcription (RT) and PCR. Two micrograms of total RNA, extracted with the TRIzol reagent, was reverse transcribed and then subjected to PCR, as previously described (40). RT and PCR were carried out with the enzymes and reagents of the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT). Both RT and PCR were performed in a Gene ATAQ Controller (Pharmacia LKB, Uppsala, Sweden).

The amplification process was conducted over 30 cycles: the first cycle consisted of a denaturation step at 95°C for 1 minute, followed by annealing and elongation at 60°C for 30 seconds and 72°C for 1.5 minutes, respectively. All subsequent cycles were executed under the same conditions, with the exception of the last cycle, during which the elongation step was extended to 7 minutes.

The PCR products were analyzed and verified by electrophoresis on 1.15% agarose gels in a Tris–borate–EDTA (TBE) buffer system, as previously described (40). Semiquantitative measurements of the reaction products were made by taking OD readings using densitometry (see above).

Statistical analysis. All results were expressed as the mean ± SD or mean and the coefficient of variation of 2–5 separate experiments, as indicated. Transfection experiments were performed in triplicate. Statistical analysis of the data was performed by nonparametric (Mann-Whitney) testing if Gaussian distribution of the data could not be confirmed. P values less than 0.05 were considered significant.

RESULTS

Human synoviocyte expression of PPARα and PPARγ: effect of cognate ligand on receptor levels. To investigate the mRNA expression profile of PPARs in human OA synoviocytes, we performed RT-PCR analysis with total RNA extracted from multiple donors and observed a 948-bp fragment for PPARα and a more abundant 900-bp fragment for PPARγ, as predicted by previous reports (45). The ratio of PPARγ:PPARα mRNA was 2.09 ± 0.33 (mean ± SD; n = 5) ($P < 0.001$). A representative PCR is shown in Figure 1A.

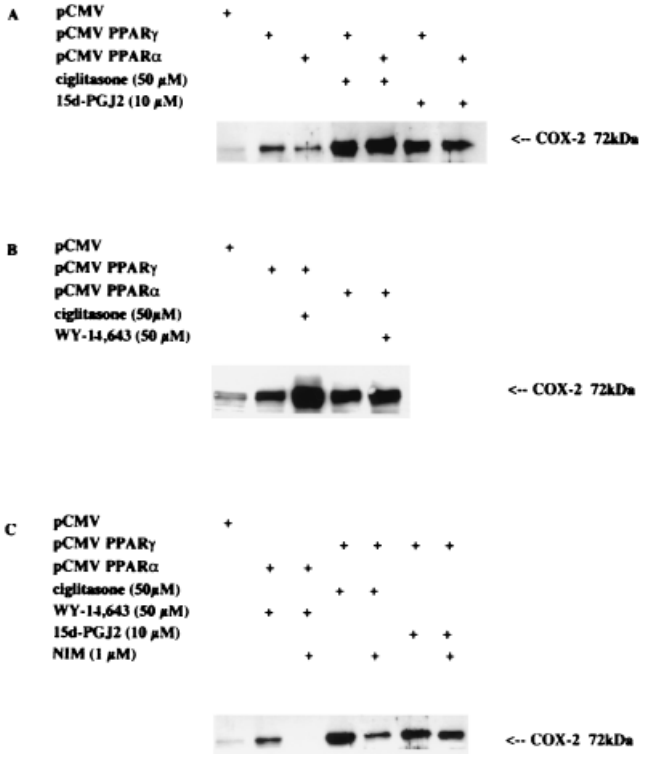

Figure 3. NIM inhibition of PPAR ligand induction of cyclooxygenase 2 (COX-2) protein in PPAR-transfected human synovial fibroblasts. Cells were transfected with 100 ng each of cytomegalovirus promoter (pCMV), pCMV–PPARα, or pCMV–PPARγ for 16 hours as described in Materials and Methods. Cells were then serum starved for 2 hours prior to incubation with or without **A,** ciglitasone or 15d-PGJ_2; **B,** ciglitasone or WY-14,643; or **C,** ciglitasone, WY-14,643, or 15d-PGJ_2 in the absence or presence of NIM for a further 6 hours as indicated. Monolayers were extracted for protein, and COX-2 was determined by Western blot analysis. See Figure 1 for other definitions.

The presence of the PPAR nuclear transcription factor (protein) was determined by EMSA, using wild-type and mutant forms of ^{32}P-PPRE oligonucleotides. A typical gel shift profile is shown in Figure 1B and always consisted of 3 shifted bands. The uppermost band was displaced by the addition of a 100-fold molar excess of radio-inert wild-type PPRE but was largely unaffected by the mutant PPRE, confirming the specificity of the binding reaction. PPAR ligands WY-14,643, ciglitasone, and 15d-PGJ_2, as well as NIM, had no discernible effect on the overall level of the nuclear receptors. Supershift analysis confirmed that PPARγ was the predominant isoform in human OA synovial fibroblasts (Figure 1C).

PPAR ligand induction of COX-2 expression and synthesis. To explore the functionality of endogenous PPARs, we treated the cells with increasing concentrations of natural and synthetic PPAR ligands and found that COX-2 mRNA and protein were up-regulated in a dose-dependent manner (Figure 2A). The prostanoid, 15d-PGJ_2, was potent in this regard, increasing COX-2 expression by a mean ± SD of 2.95 ± 1.4–fold (n = 3) ($P < 0.001$) at 10 μmoles/liter. The synthetic preferential PPARγ ligand ciglitasone also markedly increased COX-2 expression at concentrations starting at 2–5 μM, and robust expression was observed at 10–100 μM; the ligand showed some cellular toxicity at concentrations above 100 μmoles/liter. The preferential PPARα ligand WY-14,643 had a more moderate effect as compared with ciglitasone, but strong expression was observed at 20–100 μM (n = 5 determinations). Time-course experiments with 25 μmoles/liter of ciglitasone suggested maximum induction of COX-2 mRNA and protein at 4–8 hours (Figure 2B). NIM (1 μM, 0.3 μg/ml) inhibited COX-2 mRNA expression and protein synthesis induced by WY-14,643 and ciglitasone (Figure 2C). Interestingly, 15d-PGJ_2–stimulated COX-2 synthesis was unaffected by coincubations with NIM (Figure 2C). As for nonselective COX inhibitors, we previously demonstrated (22) that indomethacin and naproxen stimulate COX-2 expression and synthesis at concentrations that were shown to activate PPAR (26).

To confirm the receptor response specificity for the PPAR-dependent induction of COX-2 expression, CMV promoter–driven PPARα and PPARγ constructs were overexpressed in cells, followed by cognate ligand stimulation. Expression was confirmed by Western blot analysis; bands at 55 kd were obtained using either goat polyclonal anti-PPARα or anti-PPARγ antiserum (results not shown). In the absence of ligand, receptor expression had very little effect on COX-2 expression (Figure 3A). However, in PPARγ-transfected cells, COX-2 expression was dramatically up-regulated with the addition of 50 μmoles/liter of ciglitasone (5.1 ± 1.2–fold; n = 3) ($P < 0.0001$). The ligand could also activate PPARα, as judged by the robust induction of COX-2 protein. Interestingly, the stimulatory profile of COX-2 by 15d-PGJ_2 was more modest than that by ciglitasone in receptor-overexpressed cells. PPARα activation by WY-14,643 resulted in a muted effect (1.4 ± 0.2–fold; n = 3) ($P < 0.03$) when compared with the response in the presence of PPARγ and ciglitasone (Figure 3B). NIM (1 μM) inhibited both WY-14,643 and ciglitasone activation of COX-2 in receptor-overexpressed cells but did not inhibit 15d-PGJ_2–dependent induction (Figure 3C).

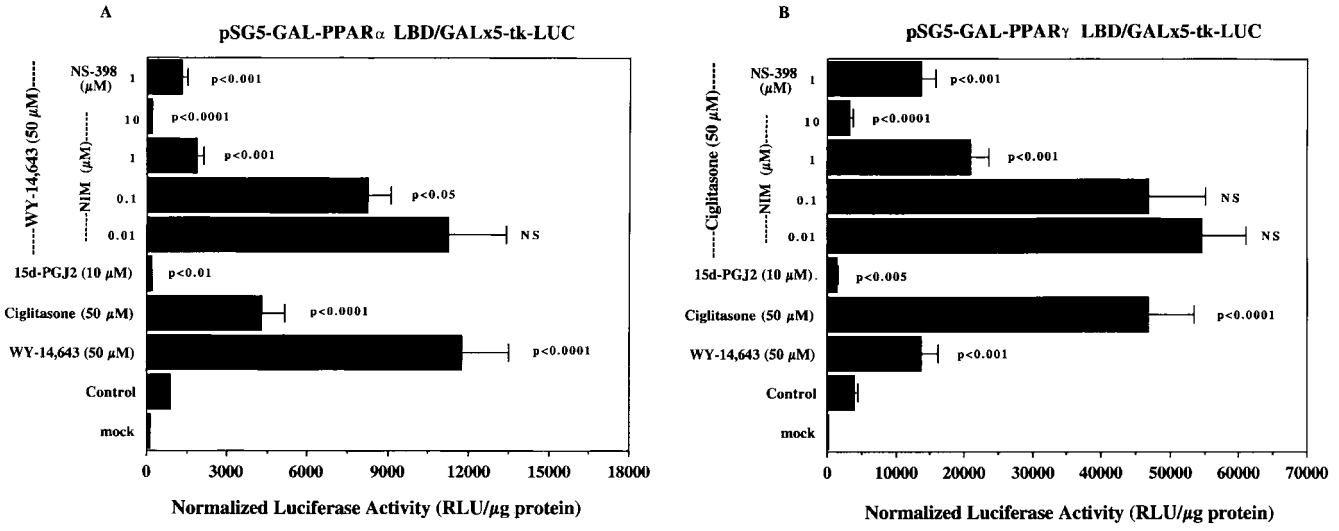

Figure 4. NIM inhibition of ligand activation of PPARα and PPARγ. Cells were cotransfected with 50 ng each of **A,** pSG5–galactose 4 (Gal 4) DNA binding domain (DBD)–PPARα ligand binding domain (LBD) or **B,** pSG5–Gal 4 DBD–PPARγ LBD and 1 μg of a Gal 4 5× enhancer site-tk-tataa-luc reporter. Transfection efficiency was monitored by cotransfection with 0.5 μg of cytomegalovirus promoter β-gal. Following serum deprivation for 2 hours, cells were incubated for 1 hour with increasing concentrations of NIM (0.01–10 μ*M* [0.0031–3.1 μg/ml]) and 1 μ*M* NS-398 prior to the addition of WY-14,643, ciglitasone, or 15d-PGJ_2 as indicated for 16 hours at 37°C. Cells were lysed and prepared for measuring luciferase activity, β-gal activity, and protein content as described in Materials and Methods. Values are expressed as the mean and SD of 3 experiments performed in triplicate. **A,** WY-14,643, $P < 0.0001$; ciglitasone, $P < 0.0001$; or 15d-PGJ_2, $P < 0.01$ versus control. WY-14,643 + NIM (0.01, 0.1, 1, 10 μ*M*), *P* not significant (NS), $P < 0.05$, $P < 0.001$, $P < 0.0001$, respectively, versus WY-14,643. WY-14,643 + NS-398, $P < 0.001$ versus WY-14,643. **B,** WY-14,643, $P < 0.001$; ciglitasone, $P < 0.0001$; or 15d-PGJ_2, $P < 0.005$ versus control. Ciglitasone + NIM (0.01, 0.1, 1, 10 μ*M*), *P* NS, *P* NS, $P < 0.001$, $P < 0.0001$, respectively, versus ciglitasone. Ciglitasone + NS-398, $P < 0.001$ versus ciglitasone. RLU = relative light units; LUC = luciferase (see Figure 1 for other definitions).

NIM inhibition of ligand-induced PPARα and PPARγ activation and DNA binding. Cognate ligand activation of nuclear receptors is a critical first step in regulating receptor-DNA binding and control of target gene expression (47). We examined the possibility that NIM inhibits the ligand activation of PPARs by employing a chimera system (26) in which the LBD of both PPARs is fused to the DBD of the yeast transcription factor Gal 4. The expression of these plasmids, together with that of a reporter construct harboring 5 copies of the Gal 4 response element controlling the expression of a luciferase reporter, provided a means for analyzing this possibility (26). WY-14,643 and ciglitasone, both at 50 μmoles/liter, activated PPARα signaling by 13.45 ± 2.01–fold and 4.93 ± 0.99–fold, respectively, while 50 μmoles/liter of ciglitasone and WY-14,643 stimulated PPARγ by 11.67 ± 1.69–fold and 3.39 ± 0.64–fold, respectively (n = 3) (Figures 4A and B). NIM (and its structural analog NS-398) inhibited basal (data not shown) and ligand activation of PPARα and PPARγ in a dose-dependent manner at therapeutically relevant concentration (50% inhibition concentration [IC_{50}] 0.602 ± 0.087 μ*M* for PPARα, 0.8 ± 0.11 μ*M* for PPARγ). Interestingly, the putative natural ligand 15d-PGJ_2 had no stimulatory effect in either of our assay systems and indeed caused a mild but significant down-regulation in terms of receptor activation.

NIM inhibition of PPAR-dependent transcriptional activation. PPAR target genes often have at least 1 copy of a PPRE in the 5′ flanking region, and transcriptional activation by PPAR ligands is mediated, at least in part, by these sequences (16). Therefore, we evaluated whether NIM could block transcriptional activation at a PPRE site. In the basic protocol, cells were transfected with a PPRE–luciferase construct (see above) in the absence or presence of PPARα and PPARγ expression constructs and with or without ligand stimulation. NIM and NS-398 were added in increasing concentrations where indicated. As shown in Figure 5A, the drugs inhibited basal and ligand-activated PPRE-driven reporter activity in a dose-dependent manner (91.9% inhibition at 10 μ*M* [NIM]). As expected, WY-14,643 and ciglitasone stimulated PPRE-driven luciferase activity by 7.72 ± 1.31–fold and 5.83 ± 1.52–fold, respectively (n = 4); 15d-PGJ_2 treatment had no stimulatory effect. In order to confirm receptor specificity in

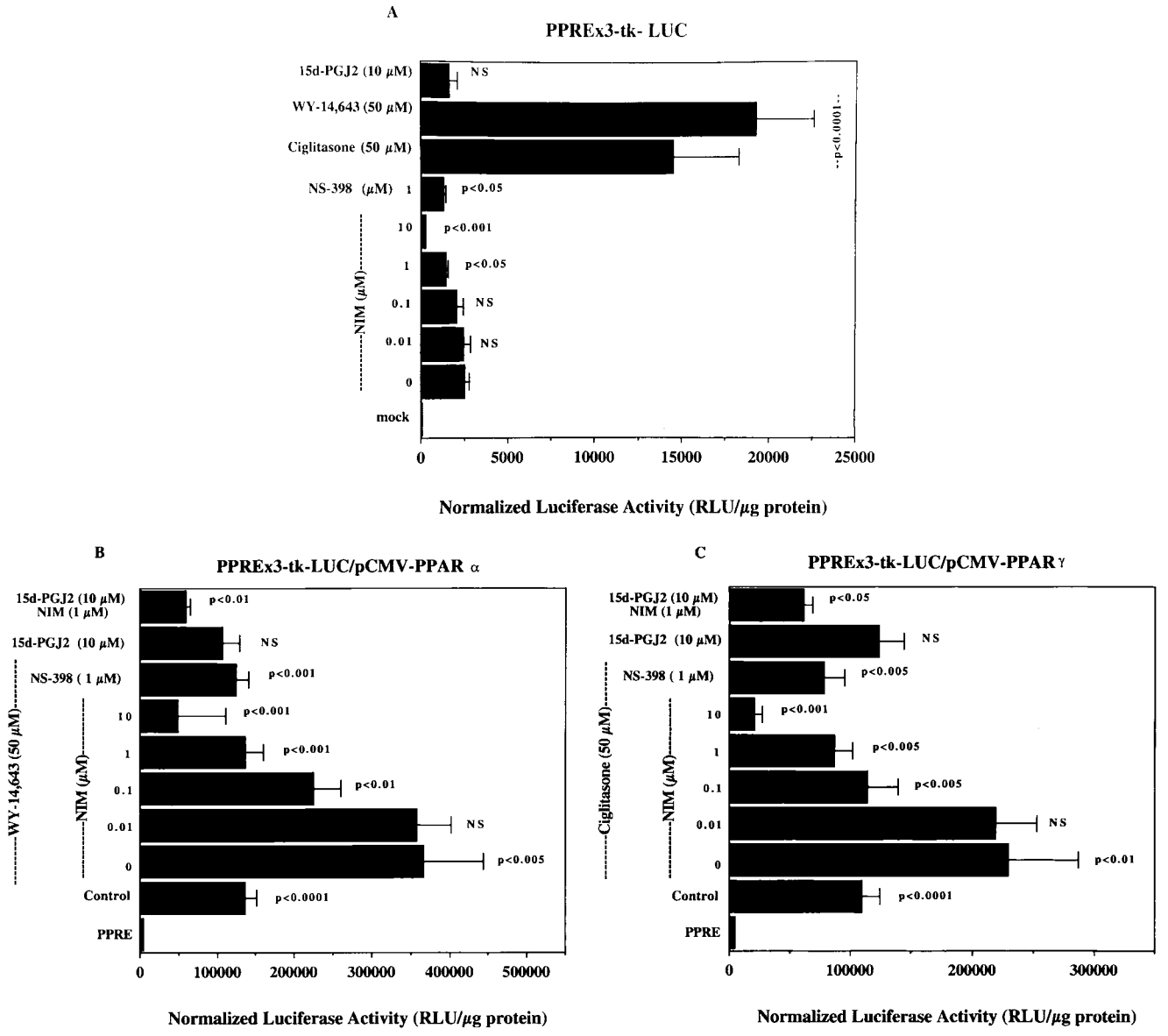

Figure 5. NIM inhibition of PPARα- and PPARγ-dependent transactivation. Cells were transfected with 1 μg of a PPRE 3$\times$ tk-luciferase reporter construct **(A)** or cotransfected with 50 ng each of cytomegalovirus promoter (pCMV)–PPARα **(B)** or pCMV–PPARγ **(C)** and 1 μg of a PPRE 3$\times$ tk-luciferase reporter. Transfection efficiency was monitored by adding 0.5 μg of pCMV β-gal to the plasmid mix. **A,** Following serum deprivation for 2 hours, cells were incubated for 6 hours with increasing concentrations of NIM (0, 0.01–10 μM [0.0031–3.1 μg/ml]), NS-398, WY-14,643, ciglitasone, or 15d-PGJ_2. Alternatively, cells were incubated with or without WY-14,643 **(B)** or ciglitasone **(C)** in the presence or absence of increasing concentrations of NIM (0.01–10 μM) or NS-398 for 16 hours at 37°C. Also in **B** and **C,** cells were incubated with 15d-PGJ_2 with or without NIM. Cells were lysed and prepared for measuring luciferase activity, β-gal activity, and protein content as described in Materials and Methods. Values are expressed as the mean and SD of 4 experiments performed in triplicate. **A,** NIM (0.01, 0.1, 1, 10 μM), P not significant (NS), P NS, $P < 0.05$, $P < 0.001$, respectively; NS-398, $P < 0.05$ versus control (0). Ciglitasone, $P < 0.0001$; WY-14,643, $P < 0.0001$; 15d-PGJ_2, P NS versus control (0). **B,** Control, $P < 0.0001$ versus PPRE. WY-14,643, $P < 0.005$ versus control. WY-14,643 + NIM (0.01, 0.1, 1, 10 μM), P NS, $P < 0.01$, $P < 0.001$, $P < 0.001$ versus WY-14,643. WY-14,643 + NS-398, $P < 0.001$ versus WY-14,643. 15d-PGJ_2, P NS versus control. 15d-PGJ_2 + NIM, $P < 0.01$ versus 15d-PGJ_2. **C,** Control, $P < 0.0001$ versus PPRE; ciglitasone, $P < 0.01$ versus control; ciglitasone + NIM (0.01, 0.1, 1, 10 μM), P NS, $P < 0.005$, $P < 0.005$, $P < 0.001$, respectively, versus ciglitasone; ciglitasone + NS-398, $P < 0.005$ versus ciglitasone; 15d-PGJ_2; P NS versus control; 15d-PGJ_2 + NIM, $P < 0.05$ versus 15d-PGJ_2. RLU = relative light units; LUC = luciferase (see Figure 1 for other definitions).

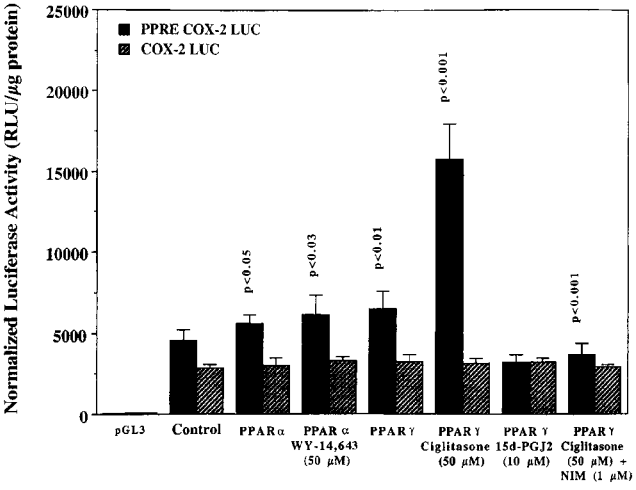

Figure 6. NIM inhibition of PPARα- and PPARγ-stimulated cyclooxygenase 2 (COX-2) promoter activity. Cells were transfected with 1 μg pGL3 basic, COX-2 promoter (2.07 kb) luciferase reporter, or PPRE–COX-2 promoter (4.05 kb) luciferase reporter, cotransfected with 50 ng each of cytomegalovirus promoter (pCMV)–PPARα or pCMV–PPARγ and in the absence or presence of WY-14,643, ciglitasone, 15d-PGJ_2, or ciglitasone + NIM as indicated. Transfection efficiency was monitored by adding 0.5 μg of pCMV β-gal to the plasmid mix. Luciferase activity, β-gal activity, and protein content were evaluated as described in Materials and Methods. Values are expressed as the mean and SD of 5 experiments performed in triplicate. $P < 0.05$, $P < 0.03$, $P < 0.01$ versus control. PPARγ versus PPARγ + ciglitasone, $P < 0.001$. PPARγ + ciglitasone versus PPARγ + ciglitasone + NIM, $P < 0.001$. RLU = relative light units; LUC = luciferase (see Figure 1 for other definitions).

terms of transactivational activity, CMV promoter–driven PPARα and PPARγ constructs were also overexpressed in cells in the presence of the PPRE–luciferase construct, followed by stimulation with WY-14,643 and ciglitasone. Here again, NIM (and NS-398) abrogated PPAR-dependent transactivation (IC_{50} 0.59 $\pm$ 0.092 μM for PPARα, 0.38 $\pm$ 0.21 μM for PPARγ) (Figures 5B and C).

PPARα and PPARγ stimulation of COX-2 promoter activity: inhibition by NIM. To determine whether PPARα- and PPARγ-induced increases in COX-2 expression were transcriptional and promoter-based, we cotransfected human COX-2 promoter luciferase reporter constructs in the absence or presence of CMV–PPARα and CMV–PPARγ expression constructs and with or without ligand stimulation. As shown in Figure 6, transfecting both receptor constructs had modest but significant effects on PPRE–COX-2 promoter activity. With the addition of WY-14,643 (50 μmoles/liter), however, we observed a 1.37 $\pm$ 0.19–fold PPARα-mediated increase, and ciglitasone (50 μmoles/liter) stimulated PPRE–COX-2 promoter activity by 2.43 $\pm$ 0.36–fold (n = 5) over PPARγ alone. NIM completely inhibited the PPARα- (data not shown) and PPARγ-dependent increases in promoter activity. We found that 15d-PGJ_2 did not induce COX-2 promoter activation in the presence of overexpressed PPARγ. In the absence of a functional PPRE in the COX-2 promoter construct, no induction under any experimental conditions was observed.

DISCUSSION

The present findings lend support to the growing number of reports (34–41) demonstrating that NSAIDs, particularly certain COX-2 inhibitors of the sulfonamide class (NIM, NS-398), exert specific biologic activities other than the simple inhibition of COX activity and release of prostaglandins. We believe that these latter effects, which we have defined as "allo-effects," could contribute to the overall therapeutic efficacy of the drugs.

While studying NSAID-induced adipogenesis and adipocyte differentiation, Lehmann et al (26) discovered that some NSAIDs were in fact PPAR activators. Using PPARα/PPARγ LBD and Gal 4 DBD chimeric constructs cotransfected with a Gal 4–luciferase reporter plasmid, they produced compelling evidence for PPAR activation. These observations were further supported by ligand binding assays using radiolabeled NSAIDs. Sufficient data were accrued to identify structural characteristics common to all PPAR ligands, and these included a lipophilic backbone and a carboxylate moiety (26).

However, the structural diversity of putative PPAR ligands is so large that identifying core features is somewhat difficult. Broadly speaking, NIM (and NS-398) share these characteristics, although the structural motifs responsible for their activity as PPAR antagonists in human synovial fibroblasts remain ill-defined. In this regard, sulindac sulfide and MF-tricyclic also antagonize PPAR by down-regulating the receptor's transcriptional activity through disruption of PPRE DNA binding in colorectal tumor cells (48). Although NIM did not affect DNA binding (as judged by gel-shift analysis in human synovial fibroblasts), NIM, sulindac sulfide, and MF-tricyclic are members of a class of NSAIDs referred to as methylsulfonanilides.

Meade et al (20) identified a PPRE site in the distal promoter region (−3900 bp) of COX-2 and suggested that, in colonic epithelial cells, it may mediate the

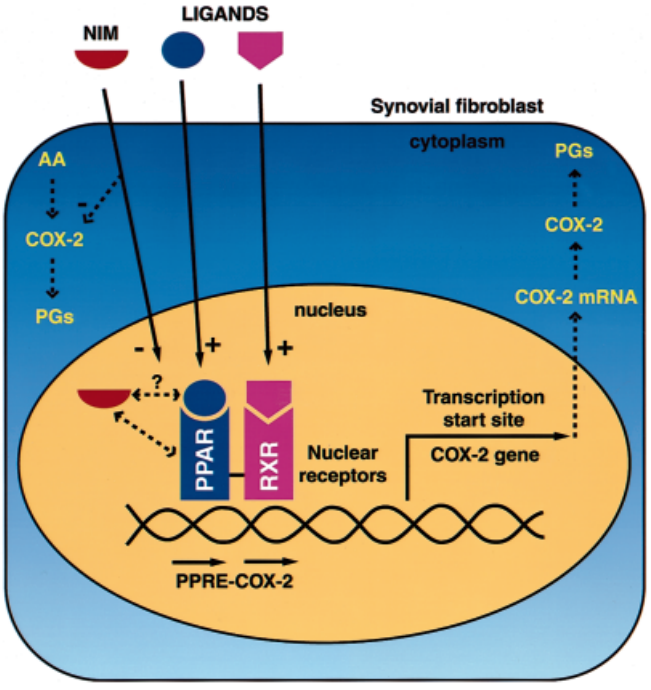

Figure 7. Proposed mechanism of action of NIM in human synovial fibroblasts. PPAR and retinoid X factor (RXR) ligands, alone or in tandem, can transactivate (+) the cyclooxygenase 2 (COX-2) gene through binding to the PPRE in the distal promoter region, resulting in increased levels of COX-2 mRNA, COX-2 protein, and prostanoid synthesis. NIM can permeate the cell and inhibit (−) COX-2 enzymatic activity and enter the nucleus to antagonize (−) PPAR ligand binding competitively or noncompetitively. AA = arachidonic acid (see Figure 1 for other definitions).

increased transcription of the gene in response to fatty acids and NSAIDs. Although the authors observed a multifold increase in the levels of COX-2 synthesis in response to peroxisome proliferator or *suprapharmacologic* doses of NSAIDs (including NS-398), the COX-2 promoter harboring the PPRE site at −3900 bp was induced by only 1.4-fold after identical treatments and was dependent on cotransfection with a PPAR expression plasmid (i.e., overexpression). Furthermore, nuclear protein extracts from untreated and agonist-treated (100 μM WY-14,643) colonic cells shifted a ^{32}P-PPRE–COX-2 oligonucleotide weakly and only when a PPAR expression plasmid was transfected prior to nuclear extraction.

The ~3–4-fold activation of the PPRE–COX-2 promoter in the presence of PPARγ and ciglitasone that we report here is clearly more robust than that observed in colonic cells but should be viewed within the context of the cells used. In the present culture system, human synovial fibroblasts (and human OA synovial membranes) produce microgram amounts (μg/10^6 cells) of PGE_2 following cytokine stimulation as a result of a 100-fold increase in COX-2 expression and synthesis (41,49); there is only a concomitant 2-fold induction of phospholipase A_2 (45). In our experience, no other cell or tissue type has this kind of very active COX-2 expression and COX-2–dependent prostanoid-producing capacity; these observations provide a plausible explanation for the differences between colonic cells and synovial fibroblasts in terms of COX-2 promoter activation. Furthermore, the discrepancy between the inhibitory effects of NIM (and NS-398) on COX-2 synthesis in the present study and the stimulatory effect of NS-398 in colonic cells (20) can be rationalized.

We also observed that suprapharmacologic (i.e., nontherapeutic, 100-μM) concentrations of NIM (or NS-398) increased steady-state COX-2 mRNA and COX-2 protein in the same cell culture system used here (Di Battista JA: unpublished observations). We associated this effect with cell toxicity, although results of additional preliminary experiments suggested augmented release of membrane-derived phospholipid mediators as a potential cause for this NIM-dependent up-regulation of COX-2 (Di Battista JA: unpublished observations).

Staels et al (46) reported that PPAR activation does not impact on basal or induced COX-2 expression directly (i.e., through the COX-2 PPRE). They used a protocol in which smooth muscle cells were treated first with PPAR agonists (e.g., WY-14,643), then with IL-1β to induce COX-2. The agonists had no effect on basal but inhibited IL-1β–induced COX-2 synthesis and promoter activity. The authors suggested that PPAR agonists inhibit IL-1β activation of COX-2 through blockade of the nuclear factor κB (NF-κB) signaling cascade. The one caveat with the study was that they used a human COX-2 promoter construct devoid of the putative PPRE site, which would be unresponsive to any effect by WY-14,643 mediated through PPAR.

One striking observation in the present study was the lack of correlation between the induction of COX-2 by 15d-PGJ_2 on the one hand and the absence of strong activation of PPAR and PPAR transactivational activity by the prostanoid in our transfection studies on the other. It would appear that the synthetic ligands WY-14,643 and ciglitasone induce COX-2 expression via PPAR/PPRE-dependent, promoter-based transcriptional activation in our cell culture model; 15d-PGJ_2 probably does so by a PPAR-independent mechanism.

The results were unexpected, since typically cy-

clopentenone prostaglandins, such as 15d-PGJ_2 and PGA_1, are considered natural PPAR agonists (16–19). Furthermore, they have been ascribed an antiinflammatory role to the extent that they inhibit, as previously alluded to, the production of proinflammatory cytokines, NO, and MMPs in a variety of cell types. To a large degree, notwithstanding effects on activator protein 1 and signal transducer and activator of transcription (for review, see ref. 16), the antiinflammatory activity was accounted for by their ability to inhibit inhibitor of NF-κB (IκB) kinases, NF-κB, and NF-κB transactivation of target genes via PPAR-dependent processes.

In a recent publication (49), we reported that the steady-state levels and stability of COX-2 mRNA and COX-2 protein translation were dependent on PGE_2 feedback activation of the p38 MAP kinase cascade in IL-1β–treated human synovial fibroblasts. The primary molecular target was the 1.5-kb 3′-untranslated region of COX-2 mRNA and, more specifically, distal adenylate-uridylate–rich (so-called Shaw-Kamen instability sequences) regions. Translational mechanisms controlling steady-state levels of COX-2 mRNA were also implicated. We provided preliminary evidence that leukotrienes, thromboxanes, and 15d-PGJ_2 could also function in this manner, and that the latter eicosanoids may directly activate p38 MAP kinase, resulting in a prolonged half-life and accumulation of COX-2 mRNA. Direct molecular interaction between a kinase and a prostanoid is not unprecedented, because Rossi et al (50) proposed a non–PPAR-dependent mechanism for NF-κB inhibition in which the reactive α,β-unsaturated carbonyl group in the cyclopentane ring forms "Michael adducts" with nucleophiles and covalently modifies specific proteins such as IκB kinase. In the latter case, the molecular interaction inhibited IκB kinase enzymatic activity, but this does not preclude the possibility that such interactions could activate a kinase such as p38.

In a rat model of acute inflammation (51), which involves carrageenan-induced pleurisy, 15d-PGJ_2 and its precursor (PGD_2) were detected at high concentrations in pleural exudates both in the early, neutrophil-dominant phase and again in the late, macrophage-dominant phase. This latter phase was associated with resolution of inflammation, because both exudate volumes and infiltrating cell numbers were decreased by several fold compared with the early inflammatory phase. Under conditions of COX-2 inhibition (NS-398), exogenously added 15d-PGJ_2 and PGD_2 mimicked all aspects of the resolution phase. Because COX-2 synthesis is under NF-κB control in certain cell types (52,53), it was suggested that the inhibition of IκB kinase by 15d-PGJ_2 and PGD_2 formed a negative autoregulatory loop that contributes to the resolution of inflammation (51). However, in the resolution phase, COX-2 protein levels were elevated concomitant with 15d-PGJ_2 and PGD_2 release, suggesting that maybe p38 MAP kinase was in fact being activated by ambient eicosanoids, with the resultant increase in COX-2 levels.

To our knowledge, this is the first report to describe functional antagonism of PPARs by NSAIDs in connective tissue cells (see Figure 7). This represents another example of the "allo-effects" of NIM and should promote further interest in finding new therapeutic applications for methylsulfonanilides.

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