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ENHANCEMENT OF PHOSPHORYLATION AND TRANSCRIPTIONAL ACTIVITY OF THE GLUCOCORTICOID RECEPTOR IN HUMAN SYNOVIAL FIBROBLASTS BY NIMESULIDE, A PREFERENTIAL CYCLOOXYGENASE 2 INHIBITOR

JOHN A. DI BATTISTA, MENGKUN ZHANG, JOHANNE MARTEL-PELLETIER, JULIO FERNANDES, NADA ALAAEDDINE, and JEAN-PIERRE PELLETIER

***Objective*. To examine the effect of 2 nonsteroidal antiinflammatory drugs (NSAIDs), nimesulide (NIM), a preferential cyclooxygenase 2 (COX-2) inhibitor, and naproxen (NAP), on the functional parameters and transcriptional activity of the glucocorticoid receptor (GR) system in cultured human synovial fibroblasts (HSF).**

***Methods*. HSF were incubated with NIM (0.3, 3, and 30 μg/ml), NAP (15, 30, and 90 μg/ml), and dexamethasone (DEX; 0.01, 0.1, and 1 μM) on a time- and dose-dependent basis. The numbers of GR binding sites per cell were determined by radioligand receptor assay. Total cellular, cytoplasmic, or nuclear GR protein was measured by Western analysis using a specific anti-human GR antibody. Phosphorylation of GR was determined by specific immunoprecipitation of protein extracts from ^{32}P-orthophosphate–labeled HSF. Mitogen-activated protein kinase p44/42 (MAPK) phosphorylation was followed by Western analysis using a specific anti-phosphoMAPK antibody. Levels of activated nuclear GR capable of binding specifically to a ^{32}P-labeled oligonucleotide harboring the glucocorticoid/hormone response element (GRE) were evaluated by gel electrophoretic mobility shift analysis. The effects of NIM and DEX on transcriptional activation of the mouse mammary tumor virus (MMTV) promoter was determined by transfecting HSF with MMTV-luciferase (reporter gene) constructs.**

***Results*. NIM had no effect on the number of GR binding sites, in contrast to NAP and DEX. NIM and NAP did not influence cellular GR protein levels or nucleocytoplasmic shuttling, although DEX lowered GR messenger RNA and protein levels after 48 hours. NIM, but not NAP, markedly increased MAPK phosphorylation (suggesting an increase in MAPK cascade activity), GR phosphorylation, GR binding to GRE, and transcriptional activation of MMTV promoter through the GRE site in the promoter.**

***Conclusion*. This study is the first to report that the antiinflammatory effects of NIM, an NSAID, may be partly related to its activation of the GR system.**

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John A. Di Battista, PhD, Mengkun Zhang, MD, MSc, Johanne Martel-Pelletier, PhD, Nada Alaaeddine, MSc, Jean-Pierre Pelletier, MD: University of Montreal, and Centre de Recherche L. C. Simard, Centre Hospitalier de l'Université de Montréal; Montreal, Quebec, Canada; Julio Fernandes, MD, MSc: Centre de Recherche L. C. Simard, Centre Hospitalier de l'Université de Montréal; Montreal, Quebec, Canada.

Address reprint requests to Jean-Pierre Pelletier, MD, Unité de Recherche en Arthrose, Centre de Recherche L. C. Simard, Room Y-2616, Hôpital Notre-Dame, 1560 rue Sherbrooke est, Montreal, Quebec H2L 4M1, Canada.

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Nimesulide (NIM; 4-nitro-2-phenoxymethanesulfonanilide) is a preferential cyclooxygenase 2 (COX-2) inhibitor (1–3) with marked biologic effects in several in vivo models of inflammation (4,5). Recent data suggest that in addition to the well-described inhibition of prostaglandin synthesis in a number of cell types, 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 (6–8). Many of these actions have been attributed to the ability of NIM to increase cellular levels of cAMP by inhibiting cAMP-dependent phosphodiesterase type IV (6,7).

Using human osteoarthritic (OA) synovial fibroblasts in culture, we have shown (9) that NIM and naproxen (NAP), at therapeutic doses, could reduce the synthesis of urokinase plasminogen activator and

interleukin-6 (IL-6), while increasing the production of plasminogen activator inhibitor 1. Furthermore, it was demonstrated that NIM could suppress matrix metalloprotease 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.

We demonstrated that prostaglandins of the E series (e.g., PGE_2) augment the number of glucocorticoid receptor (GR) binding sites in human connective tissue cells, with a concomitant increase in sensitivity of the cells to glucocorticoids such as dexamethasone (DEX) (11–13). These observations were supported by later studies that showed a reduction in GR binding sites in human synoviocytes/chondrocytes by NSAIDs such as indomethacin and NAP, both nonselective COX-1/COX-2 inhibitors; these latter reductions could be reversed by coincubating with PGE_2, PGE_1, or a PGE_1 analog, misoprostol (14). These latter data suggested a relationship between COX activity and GR binding sites. However, we could not define the contribution of each COX isoform to GR binding because of the overlapping COX specificities of the NSAIDs used.

Given the recent results concerning the effects of NIM on a number of metabolic pathways and, in particular, the cAMP signaling cascade, which is associated with GR function, we performed a more detailed study on the response of the GR system in human synovial fibroblasts (HSF) to NIM. Comparative companion experiments were conducted with NAP, a nonselective NSAID, and DEX, the prototypic antiinflammatory steroid and activator of the GR (15). We chose this cell model because synovial lining cells (type B fibroblasts) are known to play an important role in the pathophysiology of joint destruction in arthritic diseases and because the synovial membrane is a target of antiinflammatory drugs (16–21). We report that NIM induced an increased GR phosphorylation with a concomitant upregulation in binding of the GR to glucocorticoid/hormone response element (GRE) sequences as judged by electrophoretic mobility shift assay (EMSA), and increased transactivation of mouse mammary tumor virus–luciferase (MMTV-LUC), a transfected reporter gene construct through a GRE site. NAP suppressed GR steroid binding activity but did not mimic the actions of NIM in terms of GR system activation.

MATERIALS AND METHODS

Steroids and chemicals. NIM was kindly provided by Helsinn Healthcare (Lugano-Pazallo, Switzerland). 1,2,4,6,7-^{3}H-(*N*)-DEX (specific activity 84.1–94.1 Ci/mmole) was purchased from the Radiochemical Centre (Amersham, UK) and purified by thin layer chromatography (dichloromethane: acetone 8:2), as required, prior to use. Radioinert DEX (9α-fluoro-11β,17α,21α-trihydroxy-16α-methylpregna-1,4,diene-3,20-dione) originated from Ikapharm (Ramat Gan, Israel) and was recrystallized 3 times (from methanol or acetone/benzene) prior to use. CHAPS, NAP (*d*-2-[6-methoxy-2-naphthyl]propionic acid), sodium orthovanadate, iodoacetamide, leupeptin, aprotinin, pepstatin, dithiothreitol (DTT), bovine serum albumin, collagenase, pronase, PGE_2, and gamma globulin were from Sigma (St. Louis, MO). Sodium dodecyl sulfate (SDS) and Bio-Rad protein reagent originated from Bio-Rad (Richmond, CA). Tris, EDTA, $MgCl_2$, $CaCl_2$, chloroform, 8-hydroxyquinoline, formamide, and Scinti-Verse II are products of Fisher Scientific (Montreal, Quebec, Canada). Recombinant human IL-1β was obtained from Genzyme (Boston, MA). Dulbecco's modified Eagle's medium (DMEM), phosphate-free and phenol red–free DMEM, TRIzol reagent, heat-inactivated fetal calf serum (FCS), antibiotic–antimycotic mixture (10,000 units of penicillin [base], 10,000 μg of streptomycin [base], and amphotericin B [Fungizone]) were products of Gibco (New York, NY).

Specimen selection and synovial fibroblast cultures. Synovial membranes were obtained from donors (mean ± SD age 65 ± 2 years) at necropsy, within 12 hours of death. All donors were free of arthritic diseases before death, and findings of macro- and microscopic examination showed the tissue to be normal (14,22).

HSF were released by sequential enzymatic digestion with 1 mg/ml of pronase (Boehringer Mannheim, Indianapolis, IN) for 1 hour, followed by 6 hours with 2 mg/ml of collagenase (type IA; Sigma) at 37°C in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin (14,22). HSF were incubated for 1 hour at 37°C in tissue culture flasks (Primaria no. 3824; Falcon, Lincoln Park, NJ) allowing for the adherence of nonfibroblast cells possibly present in the synovial preparation. In addition, flow cytometric analysis (Epics II; Coulter, Miami, FL) using the Mo2 (fluorescein isothiocyanate) antibody (recognizes CD14) was conducted to confirm that no monocyte/macrophages were present in the fibroblast preparations (22). HSF were then 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 experiment; only primary or first passaged cells were used.

Radioligand binding assay. These studies were conducted as previously described (23). Prior to treatment with the test compounds (NIM 0.3, 3, and 30 μg/ml; NAP 30 μg/ml; DEX 0.01, 0.1, and 1 μM), synovial fibroblasts were preincubated in phenol red–free DMEM supplemented with 0.5% charcoal-treated, heat-inactivated FCS plus antibiotics for 24 hours at 37°C. Following experimentation, cells were rinsed with phosphate buffered saline (PBS), and single-point assays were conducted in which a saturating concentration of ^{3}H-DEX (50 nmoles/liter) was added in the presence or absence of a 200-fold molar excess of radioinert DEX for 30 minutes at 37°C. Kinetic parameters of the DEX binding were previously established (with human chondrocyte cultures [13,23]) and

indicated that 50 nmoles/liter is twice the concentration necessary to saturate binding sites under our experimental conditions. Receptor-bound radioactivity was measured as previously described (23).

Preparation of cytosolic and nuclear protein extracts by Western blotting and immunoprecipitation. One hundred micrograms of cytosolic extract (in buffer; 10 m*M* Tris HCl, pH 7.4, 150 m*M* NaCl, 2 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% Nonidet P40 [NP40], 1 m*M* sodium orthovanadate, and 1 m*M* NaF) from control and treated cells (NIM 0.3, 3, and 30 μg/ml; NAP 15, 30, and 90 μg/ml; DEX 0.01, 0.1, and 1 μM) were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) through 7.5% gels (final concentration of acrylamide) under reducing conditions, followed by Western analysis as previously described (23). The antibodies used were a rabbit polyclonal anti-human GR epitope corresponding to amino acids 750–769 of the carboxy terminus of the GRα of human origin (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal antibodies to phospho-p44/42 MAPK (detects doubly phosphorylated Thr^{202}/Tyr^{204}; 1:1,000 dilution) and to total MAPK (1:1,000 dilution) (both from New England Biolabs, Beverly, MA).

For nuclear extracts, cells were first lysed in ice-cold hypotonic lysis buffer containing 10 m*M* HEPES–KOH, pH 7.9, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* DTT, 1 m*M* Pefabloc, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 m*M* sodium orthovanadate, 1 m*M* NaF, and 1% NP40. Nuclei were recovered by brief centrifugation, washed once, and subjected to extraction with high salt buffer containing 20 m*M* HEPES–KOH, pH 7.9, 0.42*M* NaCl, 1.2 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.3 m*M* EDTA, 25% glycerol, 0.5 m*M* Pefabloc, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 m*M* sodium orthovanadate, and 1 m*M* NaF as previously described (24). The nuclear extracts were recovered by centrifugation at 16,000*g* for 30 minutes at 4°C, and 100 μg was used for Western analysis as described above.

For ^{32}P-orthophosphate labeling, HSF (3–5 $\times$ 10^6 cells/well) previously stimulated with DEX for 1 hour or with NIM or NAP for 48 hours were preincubated for 45 minutes in phosphate- and phenol red–free DMEM containing 0.5% FCS. Fresh medium was then added containing 100 μCi/ml of ^{32}P-orthophosphate (10 mCi/mmole; Amersham Canada, Oakville, Ontario, Canada) and incubated for 4 hours at 37°C, after which time nuclear extracts were prepared and incubated for 16 hours at 2°C with 10 μg/ml of anti-human GR antibody. The immune complexes were precipitated for 16 hours at 2°C with an equal volume of a protein A–agarose slurry and eluted with hot SDS-PAGE buffer. Following resolution by 7.5% gel electrophoresis, the gels were dried and subjected to autoradiography.

Oligonucleotide primers. The oligonucleotide primers for the polymerase chain reactions (PCR) were prepared with the aid of a DNA synthesizer (Cyclone model; Biosearch, Montreal, Quebec, Canada) and used at a final concentration of 200 nmoles/liter. The sequences for the GR primers were 5′-AGC-AGT-GTG-CTT-GCT-CAG-GAG-AGG-G-3′, which corresponds to positions 46–70 bp of the published N-terminal sequence (25), and 5′-GAG-AGG-CTT-GCA-GTC-CTC-ATT-CGA-G-3′ (antisense) corresponding to positions 720–744 bp. The expected size of the amplified fragment was 698 bp. The sequences for the GAPDH (which served as a standard of quantitation) primers were 5′-CAG-AAC-ATC-ATC-CCT-GCC-TCT-3′, which corresponds to positions 604–624 bp of the published sequence (26), and 5′-GCT-TGA-CAA-AGT-GGT-CGT-TGA-G-3′, corresponding to positions 901–922 bp, for an amplified product of 318 bp.

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 (13). RT and PCR assays were carried out with the enzymes and reagents of the GeneAmp RNA PCR kit manufactured by Perkin-Elmer Cetus (Norwalk, CT). Both the RT and PCR reactions were done in a Gene ATAQ Controller (Pharmacia LKB Biotechnology, Uppsala, Sweden).

The amplification process was conducted over 25 cycles. The first cycle consisted of a denaturation step at 95°C for 2 minutes, followed by annealing and elongation at 60°C for 1 minute. All subsequent cycles at 95°C and 60°C were executed for 1 minute and 1.5 minutes, respectively, with the exception of the last cycle, where 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 buffer system as previously described (13). Semiquantitative measurements of the reaction products were made by taking optical density readings using a laser scanning densitometer (model GS-300; Hoefer Scientific Instruments, San Francisco, CA).

Extraction of nuclear proteins and EMSA. Confluent HSF 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 PBS and pelleted by brief centrifugation. Nuclear extracts were prepared as described in the previous section (see above).

Double-stranded oligonucleotides containing consensus and mutant GRE sequences (Santa Cruz Biotechnology) were end-labeled with α^{32}P-ATP using T4 polynucleotide kinase (Promega, Madison, WI). The sense sequences of the oligos tested were as follows: for GRE, 5′-AGA-GGA-TCT-GTA-CAG-GAT-GTT-CTA-GAT-3′. Other oligonucleotides used in competition studies were as follows: for activator protein 1 (AP-1), 5′-CGC-TTG-ATG-AGT-CAG-CCG-GAA-3′; for nuclear factor κB (NF-κB), 5′-AGT-TGA-GGG-GAC-TTT-CCC-AGG-C-3′; for cAMP responsive element (CRE), 5′-AGA-GAT-TGC-CTG-ACG-TCA-GAG-AGC-TAG-3′; for signaling protein 1 (Sp-1), 5′-ATT-CGA-TCG-GGG-CGG-GGC-GAG-C-3′; for AP-2, 5′-GAT-CGA-ACT-GAC-CGC-CCG-CGG-CCC-GT-3′; for CCAAT-enhancer binding protein (C/EBP), 5′-CAC-CGG-GCT-TAC-GCA-ATT-TTT-TTA-A-3′; for NF-1, 5′-CCT-TTG-GCA-TGC-TGC-CAA-TAT-G-3′; for octamer transcription factor 1 (OCT-1), 5′-TGT-CGA-ATG-CAA-ATC-ACT-AGA-A-3′.

The binding buffer consisted of 10 m*M* Tris HCl, pH 7.5, 50 m*M* NaCl, 0.5 m*M* DTT, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, and 2.5 μg poly(dI-dC). Binding reactions were conducted with 30 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. In super-shift experiments, the anti-GR antibody (2.5 μg or 5 μg) was preincubated with nuclear extract for 30 minutes at 22°C prior to addition of the radiolabeled probe. Binding complexes were resolved by non-

denaturing PAGE through 6% gels in a Tris–borate buffer system, after which the gels were fixed, dried, and prepared for autoradiography.

Cell transfections and reporter assays. Transient transfection experiments were conducted in 4-well cluster plates with 5×10^5 cells that were serum starved for 24 hours before use. Transfections (2.5 μg of plasmid) were conducted by the Lipofectamine (Gibco BRL) method for 6 hours, according to the manufacturer's protocol. Cells were reexposed to complete culture medium for 16 hours prior to the addition of increasing concentrations of NIM (0–30 μg/ml) or DEX (0–1 μM) for an additional 48 hours. Transfection efficiencies were controlled by cotransfection with 0.5 μg of pCMV-β-gal, a β-galactosidase reporter vector under the control of cytomegalovirus (CMV) promoter. The MMTV-LTR was cloned upstream from a luciferase reporter gene followed by an SV40 polyadenylation fragment that provides signals required for termination of transcription and polyadenylation. The long-terminal repeat (LTR) region contains 4 consensus GRE that demonstrate responsiveness to glucocorticoids and can be deleted by *Hind* III digestion (−88 to −190 bp) to generate a construct (-GRE-MMTV-LTR) that is no longer responsive to glucocorticoids. Luciferase values, expressed as light units, for all experiments were normalized to the level of β-galactosidase activity. Transfection experiments conducted under standardized conditions (see below) using 0.5 μg of a pCMV-β-gal plasmid revealed that HSF have a relative transfection efficiency of 16% compared with 3T3 or HeLa cells.

Statistical analysis. All results are expressed as the mean ± SD of 3–5 separate experiments. Statistical significance was assessed using the Student's *t*-test or analysis of variance and significant differences were confirmed only when the probability was less than or equal to 5%.

RESULTS

Radioligand binding studies. We previously reported that nonspecific COX inhibitors like NAP reduced the apparent number of GR binding sites in HSF (14). Although 12 hours was sufficient to have a significant effect in this regard, 48-hour periods resulted in the most statistically reproducible suppression. We therefore chose 48 hours to continue our experiments with NIM. Our results indicated that regardless of the dose, NIM did not influence GR binding, whereas under the same culture and experimental conditions, NAP reduced the number of GR sites to 25% of control levels (n = 4) at 30 μg/ml (Figure 1). As a positive control, we treated synovial fibroblasts with DEX, since it is known that the steroid induces a general suppression of the GR system at all levels (for review, see ref. 27). Indeed, DEX decreased binding in a dose-dependent manner, with the maximal effect (15% of control) being reached at 1 μM (n = 4) (Figure 1) after 48 hours.

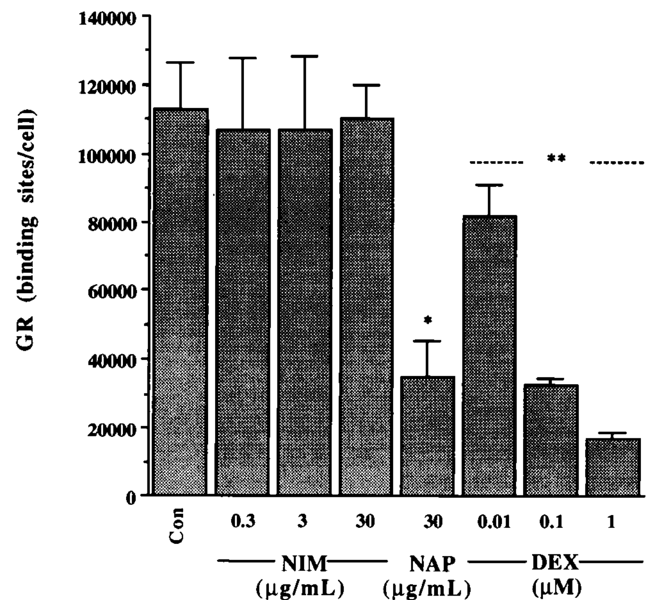

Figure 1. Effect of nimesulide (NIM), naproxen (NAP), and dexamethasone (DEX) on the number of glucocorticoid receptor (GR) binding sites in primary cultures of human synovial fibroblasts. Confluent synovial fibroblasts (1.2×10^6 cells in 6-well plates) were preincubated for 24 hours at 37°C in phenol red–free Dulbecco's modified Eagle's medium supplemented with 0.5% fetal calf serum plus antibiotics in order to synchronize the cell population. Cells were then treated with 0.3, 3, and 30 μg/ml of NIM, 30 μg/ml of NAP, and 0.01 μM, 0.1 μM, and 1 μM DEX for 48 hours at 37°C in the same medium. Cells were then rinsed with phosphate buffered saline, and a radioligand binding assay was performed as described in Materials and Methods. Values are the mean and SD (n = 4). * = $P = 0.006$ for 30 μg/ml of NAP versus control (Con), by Student's *t*-test; ** = F = 62.066, $P < 0.0001$ for all DEX concentrations versus controls, by analysis of variance. All other comparisons were not significant.

Western analysis of GR immunoreactive protein. Although NIM did not appear to influence total cell GR binding sites, it is conceivable that the drug could affect the cellular compartmentalization (e.g., cytoplasmic to nuclear) of the receptor, as does the natural ligand (27,28). Cell fractionation procedures revealed that GR-immunoreactive protein was preferentially detected in nuclear extracts in untreated synovial fibroblasts, although there was a significant amount in the cytosolic fraction (cytoplasmic-to-nuclear [C:N] ratio = 1:6.9; n = 3). We used these observations as a baseline to follow any possible redistribution of GR protein under NIM (or NAP) control. In fact, there was neither an apparent depletion of cytosolic GR protein nor a concomitant increase in nuclear GR protein that was dose-dependent (Figure 2A). Image analysis revealed that the C:N ratio remained constant in control and treated samples (see

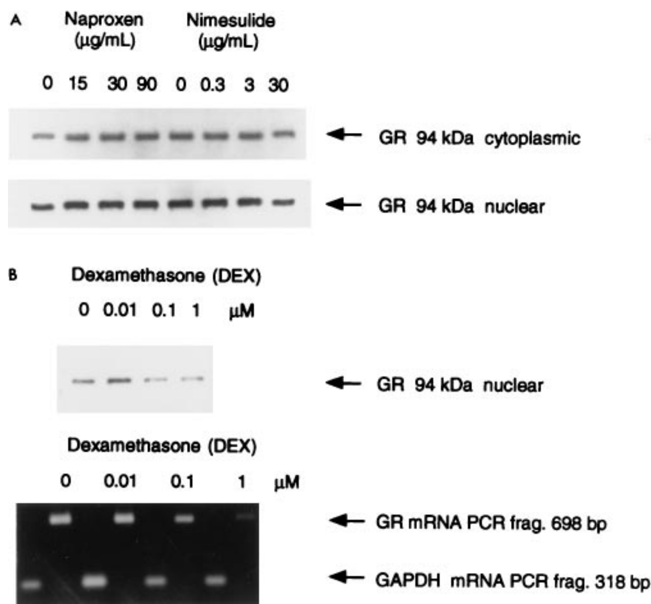

Figure 2. Effect of nimesulide (NIM) and naproxen (NAP) on the levels of glucocorticoid receptor (GR) protein (**A**) and of dexamethasone (DEX) on the levels of GR protein and GR mRNA (**B**) in human synovial fibroblasts. Cells were incubated in the presence of NIM or NAP at the concentrations shown for 48 hours, after which the cytoplasmic and nuclear fractions were recovered and 100 μg of protein was subjected to Western blotting (as described in Materials and Methods) using a specific anti-human GR antibody. Scanning data ratios of GR protein cytoplasmic:nuclear (C:N) were as follows: untreated 0.81, NIM-treated at 0.3 μg/ml 0.82, 3 μg/ml 0.86, and 30 μg/ml 0.81, and NAP-treated at 15 μg/ml 0.81, 30 μg/ml 0.86, and 90 μg/ml 0.80. The cytoplasmic blot was overexposed for clarity; the actual C:N ratio in untreated samples was 0.145. In companion experiments, synovial fibroblasts were treated with DEX at the concentrations shown for 48 hours, and total RNA and nuclear proteins were isolated. Western analysis was performed on the nuclear fraction, and reverse transcription–polymerase chain reaction was used to measure GR mRNA as described in Materials and Methods.

legend to Figure 2). DEX, on the other hand, reduced the nuclear (cellular) levels of GR-immunoreactive protein and GR mRNA after 48 hours in a dose-dependent manner, as has been shown in other cell culture systems (Figure 2B) (15).

Another important aspect of GR functionality is receptor hyperphosphorylation, a process necessary for GR binding to DNA, as well as target gene promoter activation. If NIM exerted an impact on GR phosphorylation, the drug must first increase, either directly or indirectly, the activity of a kinase(s) known to phosphorylate the GR protein. Indeed, NIM (30 μg/ml) was found to cause an increase in p44/42 MAPK phosphorylation, which became apparent only after 16 hours but remained elevated up to 48 hours (Figure 3A). This was preceded, however, by a mild decrease during the first 8 hours. The stimulation phase (16–48 hours) was abrogated by preincubation for 1 hour with the protein synthesis inhibitor cycloheximide (CHX; 1 μg/ml) prior to the addition of NIM. Total cellular MAPK protein, however, did not change over the course of the experiment (Figure 3A). NAP, used at a maximum tolerable dose (90 μg/ml), had either no effect or caused a mild decrease in the cellular levels of phosphorylated p44/42 MAPK (Figure 3A).

Furthermore, NIM (30 μg/ml) also stimulated GR hyperphosphorylation in a dose-dependent manner

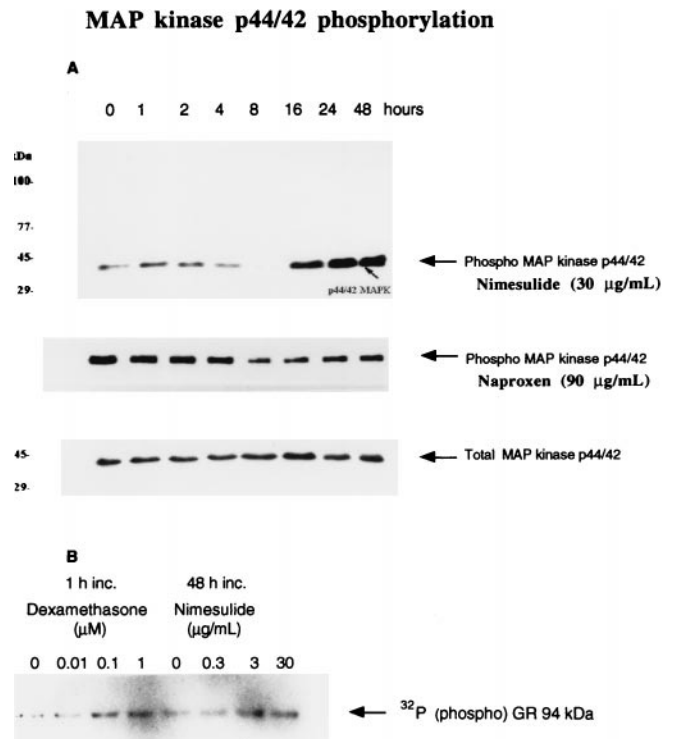

Figure 3. A, Effect of nimesulide (NIM) and naproxen (NAP) on the state of mitogen-activated protein (MAP) kinase p44/42 phosphorylation in human synovial fibroblasts. Cells were incubated with NIM (30 μg/ml) or NAP (90 μg/ml) at 37°C for the times shown. Whole cell extracts were isolated and subjected to Western analysis using specific antibodies to phosphoMAP kinase p44/42 and total MAP kinase p44/42 (representative results for both drugs) as described in Materials and Methods. **B,** Synovial fibroblasts were treated (inc.) with dexamethasone (DEX) for 1 hour or with NIM for 48 hours at the concentrations shown, after which the cells were transferred to phosphate-free Dulbecco's modified Eagle's medium plus 0.5% fetal calf serum containing ^{32}P-orthophosphate for 4 hours. Nuclear extracts were prepared and immunoprecipitated with the anti–glucocorticoid receptor antibody. Labeled proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the gels were dried and subjected to autoradiography as described in Materials and Methods.

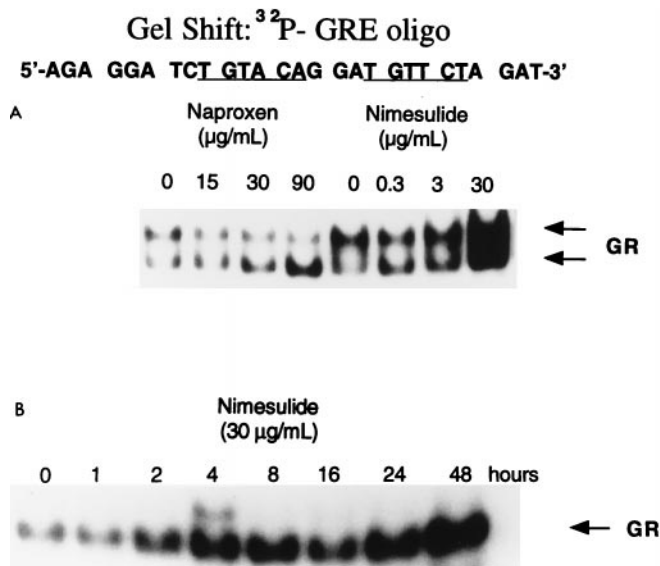

Figure 4. **A,** Effect of nimesulide (NIM) and naproxen (NAP) on the binding of nuclear extracts to ^{32}P-labeled glucocorticoid/hormone response element (GRE). Cells were incubated in the presence of NIM or NAP at the concentrations shown for 48 hours, after which the nuclear fractions were prepared and gel-shift analysis was performed as described in Materials and Methods. **B,** Time-course study of the binding of nuclear extracts to ^{32}P-labeled GRE from cells treated with NIM. Synovial fibroblasts were treated with NIM (30 μg/ml) for the times shown, after which the nuclear fractions were prepared and electrophoretic mobility shift assay was performed.

after 48 hours, with increases being observed at 3 μg/ml (Figure 3B). As expected, DEX also stimulated GR phosphorylation in a dose-dependent manner, but the time-course was much shorter (within 1 hour) than that of NIM (Figure 3B); therefore, DEX is far more potent in this regard. As anticipated, in the absence of any important effect on a protein kinase system, NAP had no effect on GR hyperphosphorylation (results not shown).

Nuclear protein binding to GRE by gel-shift analysis. NIM caused a dose- and time-dependent increase in binding of nuclear protein extracts to a ^{32}P-labeled GRE consensus sequence oligonucleotide, as judged by gel-shift analysis (Figures 4A and B, respectively). NAP had little effect in this regard at concentrations up to 90 μg/ml (Figure 4A). The GR binds to the GRE as a dimer (Figure 4A, lower band) and/or can form multimers that appear as slower moving bands (Figure 4A, top band). Maximal induction (4.3-fold, n = 4) was observed at 30 μg/ml of NIM, although significant increases (2-fold) occurred at 3 μg/ml. Time-course studies revealed that increases in GRE consensus oligonucleotide binding following treatment with 30 μg/ml of NIM were biphasic, with peaks observed between 2 hours and 8 hours, and again at 48 hours.

To test the specificity of this binding, radioinert oligonucleotide competitors were added, and the GRE consensus sequence competed most avidly (Figure 5A). However, the CRE, C/EBP, and AP-1 oligos displaced binding to the GRE to a minimal extent. Consensus NF-κB, AP-2, and Sp-1 oligos also did not compete avidly (results not shown). As an additional proof, gel-shift analysis was performed using a specific anti-GR antibody, and the latter was shown to inhibit association of nuclear binding proteins to GRE (Figure 5B). The

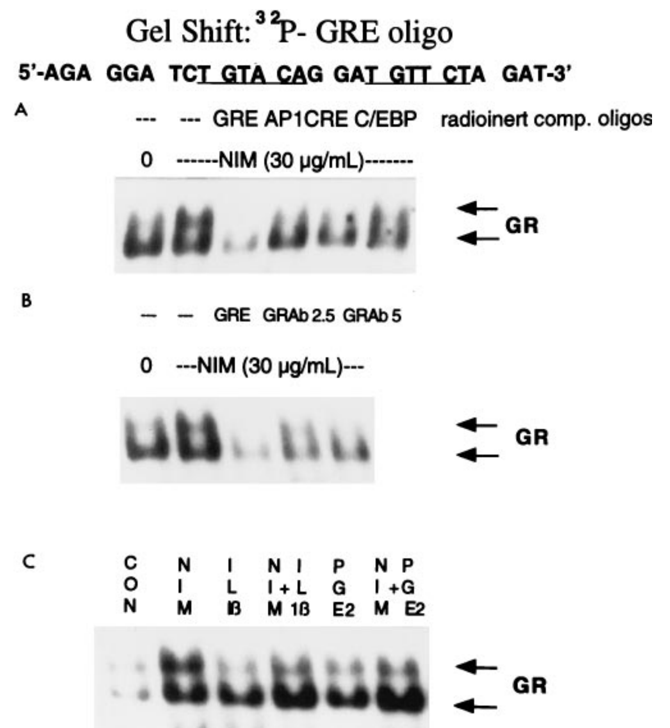

Figure 5. Gel-shift ^{32}P-labeled glucocorticoid/hormone response element (GRE) binding specificity by electrophoretic mobility shift assay. **A,** Cells were incubated with nimesulide (NIM) (30 μg/ml) for 48 hours, after which the nuclear extracts were prepared. Gel-shift binding reactions were conducted with ^{32}P-labeled GRE in the absence or presence of radioinert GRE (1-fold excess) or activator protein 1 (AP1), cAMP responsive element (CRE), and C/EBP (10-fold excess of each). **B,** Cells were treated as in **A**, and gel-shift reactions were performed with ^{32}P-labeled GRE in the absence or presence of radioinert GRE (1-fold excess) or anti-human GR antibody (GRAb; 2.5 or 5 μg/ml) as described in Materials and Methods. **C,** Synovial fibroblasts were incubated for 48 hours in the absence (CON) or presence of NIM (30 μg/ml), interleukin-1β (IL-1β; 100 units/ml), NIM + IL-1β, prostaglandin E_2 (PGE_2; 1 μM), or NIM + PGE_2. Gel-shift analysis was performed as described in Materials and Methods. comp. = competitor.

transcription factors NF-1 and OCT-1 are believed to function in an obligatory and concentrated manner with the GR to stimulate MMTV promoter activity (29,30). We therefore examined the effect of NIM on NF-1 and OCT-1 synthesis by gel-shift analysis and found that the drug had no effect on the synthesis or nuclear accumulation of either factor (results not shown).

Since NIM is a preferential inhibitor of IL-1β–induced COX-2 activity and PGE_2 production, we tested whether this pathway might mediate the effects of NIM on GRE consensus oligonucleotide binding. This was not the case; in fact, IL-1β and PGE_2 alone caused small-to-moderate increases in nuclear binding to the GRE consensus sequence (Figure 5C).

Transfection studies of MMTV promoter activity. If NIM can increase GR phosphorylation and GRE nuclear consensus sequence binding, the productive consequence should be increased transactivation of target genes harboring GRE sites in their promoter regions. Hormone-responsive elements (4× GRE) have been reported in the MMTV-LTR (29,30), which requires the GR (at least) as a transcriptional transacting factor. When cells were transfected with a pMMTV-LUC (LUC reporter gene) construct in the presence of NIM, there was a significant increase (5.5-fold; n = 5) in LUC activity at 30 μg/ml, although increases (2.7-fold) were seen at 3 μg/ml (Figure 6). As expected, DEX proved a more potent inducer of promoter activity than NIM, with a 46-fold increase in promoter activity at 1 μM (Figure 6). The removal of a *Hind* III fragment containing 4 GRE elements resulted in a dramatic loss of the promoter response to both DEX and NIM (Figure 6).

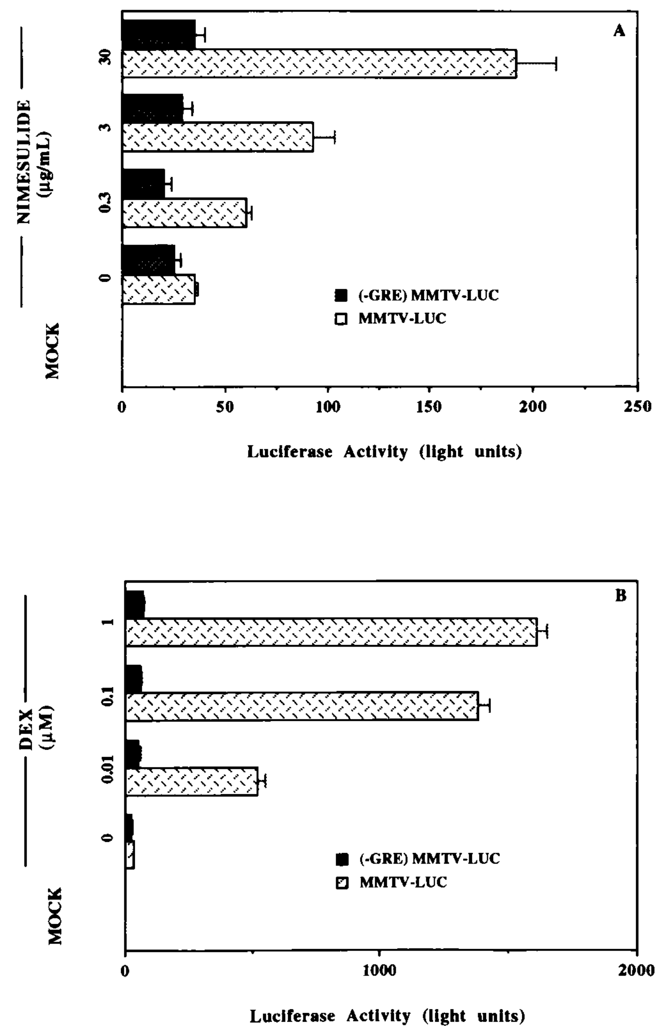

Figure 6. **A,** Nimesulide (NIM) and **B,** dexamethasone (DEX) increase mouse mammary tumor virus (MMTV) promoter activity via the glucocorticoid response element (GRE). Cells were cotransfected with 2.5 μg of MMTV-LUC or (-GRE) MMTV-LUC, together with 0.5 μg of pCMV-β-gal (control for transfection efficiency) as described in Materials and Methods, and then incubated with NIM or DEX at the concentrations shown for an additional 48 hours. Following this, cells were lysed and analyzed for luciferase (LUC) activity. Values are the mean and SD (n = 5 determinations). For NIM at 0.3, 3, and 30 μg/ml, F = 197.85, $P < 0.0001$ versus absence of NIM and for DEX, at 0.01, 0.1, and 1 μM, F = 2675.35, $P < 0.0001$ versus absence of DEX (by analysis of variance).

DISCUSSION

Clinical studies indicate that NIM has a number of favorable characteristics as an antiinflammatory drug, and a low incidence of gastrointestinal side effects (31). This latter property of the drug has been attributed to the fact that the drug preferentially inhibits COX-2 activity with little effect on COX-1 activity (1–3), the COX-1 isoform being considered essential to the maintenance of cellular homeostasis in the intestinal tract. In addition, NIM can limit neutrophil function and inhibit the synthesis of inflammatory mediators derived from connective tissue cells, all of which can have a beneficial effect on the pathophysiology of arthritic diseases, such as OA and rheumatoid arthritis (RA). New molecular targets have been identified (e.g., phosphodiesterase type IV) that help to explain some of the "allo effects" of this drug, and the present results indicate that the functionality of the GR system can be modulated by the NSAID. Some of the antiinflammatory effects in vivo of NIM might therefore be attributed to the phosphorylation and activation of the GR, with the resultant changes

in the expression of glucocorticoid target genes (e.g., matrix metalloproteases [30]) (23).

To our knowledge, this is the first report of a drug of this kind acting on the GR system. The effects of NIM, in terms of GR binding, phosphorylation, and DNA binding, markedly contrast those of NAP, which is probably not unexpected because the chemical composition of the two NSAIDs is not the same. Indeed, not only are their relative specificities (affinities) different for the COX isoenzymes, but they also function via completely different inhibitory mechanisms and kinetics (32).

The GR is a ligand-inducible transacting factor that, when complexed with the cognate ligand (hormone), becomes "activated" and takes on a form capable of binding to specific cognate regulatory elements (e.g., GRE) in the nucleus (27). A critical aspect of this activation process is hormone-induced receptor hyperphosphorylation (serine and threonine residues), which is essential for tight DNA binding and for modification of promoter activity (27,28,33). Following the activation or repression of transcriptional activity, the fate of the receptor–hormone complex is unknown, although there are models that describe receptor recycling (28,34). However, one observation that is consistent in almost all target cell types is that glucocorticoids induce a time-dependent (long-term, e.g., 48 hours) down-regulation of GR mRNA and protein (15). Indeed, in the present study, DEX induced the down-regulation of GR protein (Western blot), GR mRNA, and GRE binding. DEX, a prototypic antiinflammatory steroid, was used in all experiments to compare, on a mechanistic basis, the action of NIM.

Our results show that DEX affects neither total cellular GR protein nor nucleocytoplasmic shuttling, but does influence the state of GR phosphorylation by activating protein kinases (p44/42), which in turn may alter the phosphorylation state of the GR. This would be consistent with the marked increase in binding to GRE that was both time and dose dependent. Furthermore, this binding, which was presumably mostly due to the GR, was displaced by excess (1-fold) cold GRE and a specific anti-human GR antibody. However, the addition of a 10-fold excess of AP-1 and CRE consensus oligonucleotides resulted in some minimal displacement. This is not totally unexpected since AP-1 monomers (e.g., c-Jun/c-Fos) can heterodimerize with the GR, and these complexes have some affinity for AP-1 regulatory elements (29,30). The same could be said for CREB/activating transcription factor family members, which are known to also dimerize with the GR. Furthermore, and of considerable interest, is the recent revelation that the CREB binding protein (CBP), a coactivator essential for the transcriptional activity of nonreceptor transcription factors (e.g., CREB, AP-1), also functions as a nuclear receptor (e.g., GR) coactivator that is necessary for efficient modulation of target gene expression (35,36). Therefore, the 265-kd CBP is a coactivator that has the capacity to integrate and control gene expression from diverse signaling pathways. It would therefore be of some interest to study the effects of NIM on CBP synthesis, given the number of genes under the umbrella of CBP control.

We demonstrated previously that nonpreferential COX-1/COX-2–inhibiting NSAIDs, such as indomethacin or NAP, down-regulate the number of GR binding sites in human synoviocytes/chondrocytes by a mechanism that is dependent on the synthesis of prostaglandins (14). Since these cells were in a stationary, quiescent phase, it was assumed that constitutive COX-1 was responsible for the basal production of PGE_2. Our present study employed essentially the same cell culture system, and we could not detect any COX-2 protein in these cells. There is, however, considerable induction of COX-2 synthesis by cytokines such as IL-1β, and human OA and RA synoviocytes/chondrocytes express COX-2 at elevated levels (37,38). Therefore, a priori, the increased binding of NIM-induced nuclear proteins to GRE oligonucleotides could not be a COX-2–related effect. However, we attempted to verify this by activating endogenous PGE_2 synthesis via inducible COX-2 by treatment with IL-1β or by adding exogenous PGE_2. In neither case was there a significant reversal of the NIM-induced increase in GRE binding, and in fact, IL-1β and PGE_2 alone had a positive effect in this regard. The latter observation is not unexpected; we and others have shown that the eicosanoid up-regulated GR in human connective tissue cells (11,39). These data argue strongly for a COX-2–independent effect of NIM on GR binding to cognate regulatory elements.

The MMTV promoter is considered complex because hormone-mediated promoter activation is governed by the coordinated interaction of GR and nonreceptor transcription factors such as NF-1 and OCT-1 (30). In unstimulated transfected mammalian cells, the MMTV promoter is silent and organized into positioned nucleosomes, one of which encompasses the binding site for GR and NF-1 (40). Glucocorticoid (DEX) induction involves a functional synergism between GR, OCT-1, and NF-1, and simultaneous occupancy of the cognate promoter elements for all proteins (40,41). In human synoviocytes, there is a considerable dependence on the GRE for promoter activation because when the 4 GREs

(-GRE-MMTV-LUC) were removed, complete abrogation of LUC activity was observed. Although NIM induced promoter activity quite significantly, the results were consistently lower than with DEX.

These results could indicate that NIM increases MMTV promoter activity via other essential nonreceptor transcription factors such as NF-1 and OCT-1, in addition to the GR. However, we could detect no increased NF-1 or OCT-1 binding activity following treatment of synoviocytes with NIM. Therefore, considering the induction of a MAPK cascade (and perhaps protein kinase A [PKA] [6]) by NIM-treated synoviocytes, we favor the notion that the drug stimulates hyperphosphorylation of nuclear GRs that are weakly bound to DNA, and not yet transcriptionally active. This covalent modification renders the receptors fully competent to stimulate transcription. The number of transcriptionally competent GRs is likely to be much less than can be activated with DEX treatment, since the steroid can induce cytoplasmic-to-nuclear shuttling, and it is now well accepted that the target gene responsiveness to glucocorticoids is proportional to the number of GR sites in the nucleus (15,27,42). Hence, the higher LUC values with DEX probably reflect the number of GR molecules recruited to the promoter site.

The influence of MAPK and cyclin-dependent protein kinase (CDK) on GR transcriptional activity is likely to be complex and dependent on the cellular context (43). In certain yeast strains that harbor defective kinases, transfection of MAPK and receptor plasmids yielded negative results, to the extent that GR phosphorylation by MAPK inhibited GR transcriptional activity (43). To our knowledge, such studies have not been done in mammalian cells, but we obtained long-term increases in MAPK activity (as judged by phosphorylation patterns) in the presence of NIM. This coincided with GR hyperphosphorylation, although we do not have direct proof that mammalian p44/42 can phosphorylate mammalian GR in vitro or in human synovial fibroblasts. Clearly, NIM is not a classic mitogenic stimulus (e.g., growth factors, insulin), and further studies are required to elucidate the mechanism, particularly in light of recent reports of NIM stimulation of PKA, which is inhibitory of the MAPK cascade. As for CDK, we chose not to measure this cascade because our cells were in cell-cycle arrest (confluent, stationary phase), and detection would be unlikely.

In conclusion, the therapeutic efficacy of NIM as an antiinflammatory agent in the treatment of arthritic diseases can be ascribed, in large part, to its inhibition of COX-2. However, the present study, along with others, shows that the latter NSAID, in contrast to NAP, has an ever growing number of "allo effects" that can positively affect joint tissue metabolism. To our knowledge, this report is the first indication that the GR system, which mediates the effects of prototypic antiinflammatory steroids, may also be subject to NIM action.

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