Effect of nimesulide on glucocorticoid receptor activity in human synovial fibroblasts


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Abstract

Fibroblasts from human synovial membranes were cultured with nimesulide, naproxen or dexamethasone. Nimesulide, but not naproxen, showed effects on the glucocorticoid system that may contribute importantly to its anti-inflammatory activity. Nimesulide at therapeutically relevant concentrations induced the intracellular phosphorylation and activation of glucocorticoid receptors, and activated their binding to the target genes. Naproxen or dexamethasone markedly reduced the number of glucocorticoid receptor binding sites, in contrast to nimesulide, which had no significant effect.

KEY WORDS: Glucocorticoid receptor, Synovial fibroblast, Nimesulide.

Nimesulide (NIM; 4-nitro-2-phenoxyethanesulphonanilide) is a preferential cyclooxygenase-2 (COX-2) inhibitor [1–3]. Recent data suggest that NIM has many other effects in addition to the well-described inhibition of prostaglandin synthesis in various cell types. Using human osteoarthritic synovial fibroblasts in culture, we showed that therapeutic concentrations of NIM or naproxen (NAP) in vitro could reduce the synthesis of urokinase (uPA) and IL-6 while increasing the production of plasminogen activator inhibitor-1 (PAI-1) [4]. Furthermore, NIM could suppress matrix metalloprotease synthesis by cartilage explants in vitro [5]. 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 synthesis.

Studies have shown a reduction in glucocorticoid receptor (GR) binding sites in human synoviocytes/chondrocytes by non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and NAP, both non-selective COX-1/COX-2 inhibitors. These reductions could be counteracted by co-incubating with prostaglandin E2 (PGE2), PGE1 or the PGE1 analogue misoprostol [6], suggesting a relationship between COX activity and GR binding sites. However, we could not define the contribution of each COX isofrom to GR binding because indomethacin and NAP inhibit both COX-1 and COX-2. In view of recent results that NIM can affect several metabolic pathways, we performed a more detailed study on the response of the GR system in human synovial fibroblasts (HSF) to NIM. Comparative experiments were conducted with the NSAID NAP, and the anti-inflammatory steroid/GR activator dexamethasone (DEX). We chose this human fibroblast model because synovial lining cells are known to play an important role in the pathophysiology of arthritic joint destruction [7–9].

Materials and methods

Specimens of synovial membranes were obtained at necropsy from donors free of arthritic disease. HSF were released by sequential enzymatic digestion, and cultured until confluence in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics at 37°C in a humidified atmosphere of 5% CO2 in air [6]. The cells were incubated in fresh serum-free medium for 24 h before the experiment; only these primary or first-passage cells were used in the experiments.

Radioligand binding assays were conducted as described previously [10, 11] after using the test compounds (0.3, 3, 30 µg/ml NIM; 30 µg/ml NAP; 0.01, 0.1, 1 µM DEX). Receptor-bound radioactivity was measured as reported previously [10].

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phoresis (SDS–PAGE) under reducing conditions, followed by Western analysis as previously described [10, 11]. The antibodies used were a rabbit polyclonal anti-human GR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and monoclonal antibodies to phospho-p44/42 MAPK and to total MAPK (both from New England Biolabs, Beverly, MA, USA).

For nuclear extracts, cells were first lysed in ice-cold hypotonic lysis buffer containing 10 mM HEPES–KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 1 mM Pefabloc™, 10 μg/ml each of aprotinin, leupeptin and pepstatin, 1 mM sodium orthovanadate, 1 mM NaF and 1% Nonidet P-40. The nuclear extracts were recovered by centrifugation and used for Western analysis according to Miller et al. [12]. For [³²P]orthophosphate labelling, HSF (3–5 × 10⁶ cells/well) previously stimulated with DEX (1 h), NIM or NAP, were pre-incubated in phosphate- and phenol-red-free DMEM containing 0.5% FCS and 100 μCi/ml of [³²P]orthophosphate for 48 h, and incubated for 4 h.

For nuclear extracts, cells were incubated for 16 h with the anti-human GR antibody. The immune complexes were precipitated with Protein A–agarose slurry, eluted with hot SDS–PAGE buffer, and subjected to gel electrophoresis and autoradiography.

The sequence of the oligonucleotide primers for the polymerase chain reactions (PCR) was for the GR primers 5′-AGCAGTGTGCTTGCTCAGGAGAGGG-3′, which corresponds to position 46–70 bp of the N-terminal sequence, and 5′-GAGAGGCTTGCAGTCCTCATTCGAG-3′ (anti-sense) from position 720–744 bp. Total RNA was extracted with the Trizol reagent, and 2 μg was reverse-transcribed and then subjected to PCR as previously described [13].

Nuclear extracts were prepared from control and treated cells as in the previous section. Double-stranded oligonucleotides containing consensus and mutant glucocorticoid response element (GRE) sequences (Santa Cruz Biotechnology) were end-labelled with [³²P]ATP using T4 polynucleotide kinase. Binding reactions were

**Fig. 1.** Nimesulide induced the intracellular phosphorylation and activation of the glucocorticoid receptors and activated their binding to the target genes.
conducted with 30 μg of nuclear extract and 32P-labelled oligonucleotide probe. Binding complexes were resolved by non-denaturing polyacrylamide gel electrophoresis and prepared for autoradiography.

The effect of NIM and DEX on transcriptional activation of the mouse mammary tumour virus (MMTV) promoter was determined by transfecting HSF with MMTV-Luciferase (LUC, reporter gene) constructs. Luciferase values, expressed as light units, were normalized to the level of β-galactosidase activity.

Results

NIM had no effect on the number of GR binding sites, in contrast to NAP and DEX, which caused marked reductions (75 and 85% respectively). NIM or NAP did not influence cellular GR protein levels or nucleocytoplasmic shuttling, although DEX lowered GR mRNA and protein levels. NIM, but not NAP, markedly induced MAPK phosphorylation (suggesting an increase in MAPK cascade activity), GR phosphorylation, GR binding to GRE and transcriptional activation of the MMTV promoter through the GRE site in the promoter (5.5-fold, 30 μg/ml).

Discussion

Clinical studies indicate that NIM has several favourable characteristics as an anti-inflammatory drug [14]. New molecular targets have been identified that help to explain some of the therapeutic effects of this drug. The present results indicate modulation of the GR system by NIM, so that some of the anti-inflammatory effects of NIM in vivo might be due to phosphorylation and activation of the GR with the resultant changes in the expression of glucocorticoid target genes (Fig. 1). To our knowledge, ours is the first report of this type of drug acting on the GR system. The effects of NIM, in terms of GR binding, phosphorylation and DNA binding, contrast markedly with those of NAP. This is probably not unexpected as the two NSAIDs differ markedly in their chemical composition, relative specificities for the COX isoenzymes, mechanisms of action and pharmacokinetcs [15].

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