
Non-steroidal anti-inflammatory drugs protect against chondrocyte apoptotic death

P. Mukherjee, C. Rachita, P.S. Aisen, G.M. Pasinetti

Neuroinflammation Research Center
of the Department of Psychiatry,
Mount Sinai School of Medicine,
New York, New York, USA.

Please address correspondence to:
Giulio Maria Pasinetti, MD, PhD,
Neuroinflammation Research Center,
Mount Sinai School of Medicine,
Department of Psychiatry, Box 1229,
One Gustave L. Levy Place, New York, N.Y.
10029, USA.

E-mail: gp2@doc.mssm.edu

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ABSTRACT

Recent evidence suggests that the degradation of cartilage in osteoarthritis is characterized by chondrocyte apoptosis, but little is known about the molecular mechanisms involved or potential protective measures. In the present study, we used an immortalized chondrocyte cell line to explore the mechanisms of apoptotic chondrocyte cell death. We found that staurosporine-mediated chondrocyte death depended on the concentration and time of incubation, and coincided with increased Bax:Bcl-X mRNA expression, cytochrome C release, and activation of caspase-3. Pre-treatment of the cultures with nimesulide, a preferential cyclooxygenase (COX)-2 inhibitor, or with ibuprofen, a non-selective COX-1/COX-2 inhibitor, protected the chondrocytes against the staurosporine-mediated nuclear damage and cell death in a concentration-dependent manner (10^{-12} to 10^{-6} M). Cell protection coincided with inhibition of the staurosporine-mediated induction of caspase-3 activation. Notably, the selective COX-2 inhibitor NS-398 (10^{-6} M, 24 hr pre-treatment) did not protect the cells against staurosporine-mediated apoptotic death. The data suggest that nimesulide and ibuprofen, in addition to their anti-inflammatory and analgesic benefits, may also have a protective effect in osteoarthritis through the inhibition of apoptosis in chondrocytes.

Introduction

Disintegration of articular cartilage is an early, invariable feature of osteoarthritis (OA), a primary cause of joint dysfunction and disability (1). Indeed, it is generally assumed that the primary instigating event in the aetiology of OA occurs in the cartilage. Elucidation of the molecular mechanisms involved in cartilage deterioration in OA may thus provide insights into potential protec-

tive interventions.

Age-related changes that have been observed in human articular cartilage include a reduction in tissue cellularity, and abnormal matrix calcification. It has been hypothesized that these changes increase susceptibility to OA. Recent evidence indicates that apoptotic mechanisms are involved in cartilage degradation related to both age and OA (2). For example, chondrocyte apoptosis may contribute to proteoglycan depletion (3) and pathological matrix calcification (4).

In general, activation of apoptotic cell death involves interactions between pro- and anti-apoptotic activities of members of the BCL-2 gene family, e.g. the ratio of Bax:Bcl-X expression, controlling the activation of caspases and subsequent apoptotic cell death. In particular, activation of caspase-3 has been linked to the execution of apoptotic cell death in various tissues, including cartilage (5, 6). There is evidence that altered BCL-2 expression in chondrocytes may be responsible for progressive programmed cell death in OA (6-8). Interestingly, prostaglandin E₂ may be involved in the induction of chondrocyte apoptosis (9), suggesting a possible role for non-steroidal anti-inflammatory drugs (NSAIDs) in chondroprotection.

In the present study with an immortalized chondrocytic cell line, we explored the mechanisms involved in staurosporine-mediated apoptotic chondrocyte death, and the potential protective role of NSAIDs commonly used to treat both inflammatory and degenerative arthropathies. The results indicate that chondrocyte apoptosis involves alterations of BCL-2 gene expression and activation of caspase-3. Nimesulide, a preferential COX-2 inhibitor, and ibuprofen, a non-selective COX-1/COX-2 inhibitor, blocked caspase-3 activation and apoptosis in the chondrocyte cultures, while surprisingly the

selective COX-2 inhibitor NS-398 was ineffective. These findings suggest that the some NSAIDs may have chondroprotective activity involving caspase-3 inhibition that is distinct from their anti-inflammatory COX-2 inhibition.

Materials and methods

Chondrocyte cultures

The immortalized rat chondrogenic cell line RCJ3.1C5.18 (gift of Dr. Jane Aubin, University of Toronto, Toronto, Canada) was maintained in -MEM medium supplemented with 15% FBS, 50 g/ml penicillin-streptomycin solution, 10 mM -glycerophosphate, 50 g/ml ascorbic acid and 10⁻⁸ M dexamethasone at 37°C in a humidified atmosphere of 5% CO₂ in air as previously described (10). Staurosporine (Sigma) was solubilized as a 1 mM stock solution in DMSO and stored at -20°C.

Assessment of cytotoxicity

Chondrocyte cell death was assessed using the colorimetric assay with the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which primarily assesses impairment of mitochondrial redox activities (11). In parallel, the apoptotic cell damage was confirmed by counting cells with evident pyknotic condensed nuclei surrounded by cytoplasmic eosinophilia, as seen by haematoxylin and eosin (H&E) histochemistry in 8-10 randomly selected fields. Chondrogenic cultures were plated at 10,000 cells per well in 96-well plates for MTT assay or at 50,000 cells per well in 8-chamber slides for H&E quantification.

Bcl-2 gene family member: RNase protection assay and in situ hybridization

At each appropriate time, primary chondrocyte cultures in 35 mm dishes were collected, and the total RNA was extracted using the Ultraspec RNA isolation system (Biotechx). Briefly, after isopropanol precipitation of the RNA as indicated in the Biotechx protocol, the pellets were resuspended in a solution of TE buffer (10 mM Tris pH 7.2 and 1 mM EDTA pH 8.0) containing 0.1% SDS and an equal volume of 4M NH₄OAc. The RNA was then re-precipitated at -20°C with 2.5 volumes of

ethanol. Expression of Bcl-X, Bax was assayed using a RiboQuant™ Multiprobe RNase Protection Assay (RPA) system (Pharmingen). Briefly, the template was transcribed *in vitro* with ³²P-UTP (and diluted to 3.0 x 10⁵ cpm/ l). Total RNA was then hybridized with the probe set for 12-16 hr at 56°C, and then a mixture of RNase A/T1 was added to digest any unhybridized, single-stranded RNA. The resulting protected fragments were resolved electrophoretically on 4.75% denaturing

polyacrylamide gel containing 8 M urea. The radioactively labeled fragments were quantified using a Molecular Dynamics Phosphoimager SI with IPLab Gel H software package. Data are expressed as a ratio of the specific mRNA of interest normalized to the constitutively expressed glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA.

Cytochrome C assay

Redistribution of cytochrome C was

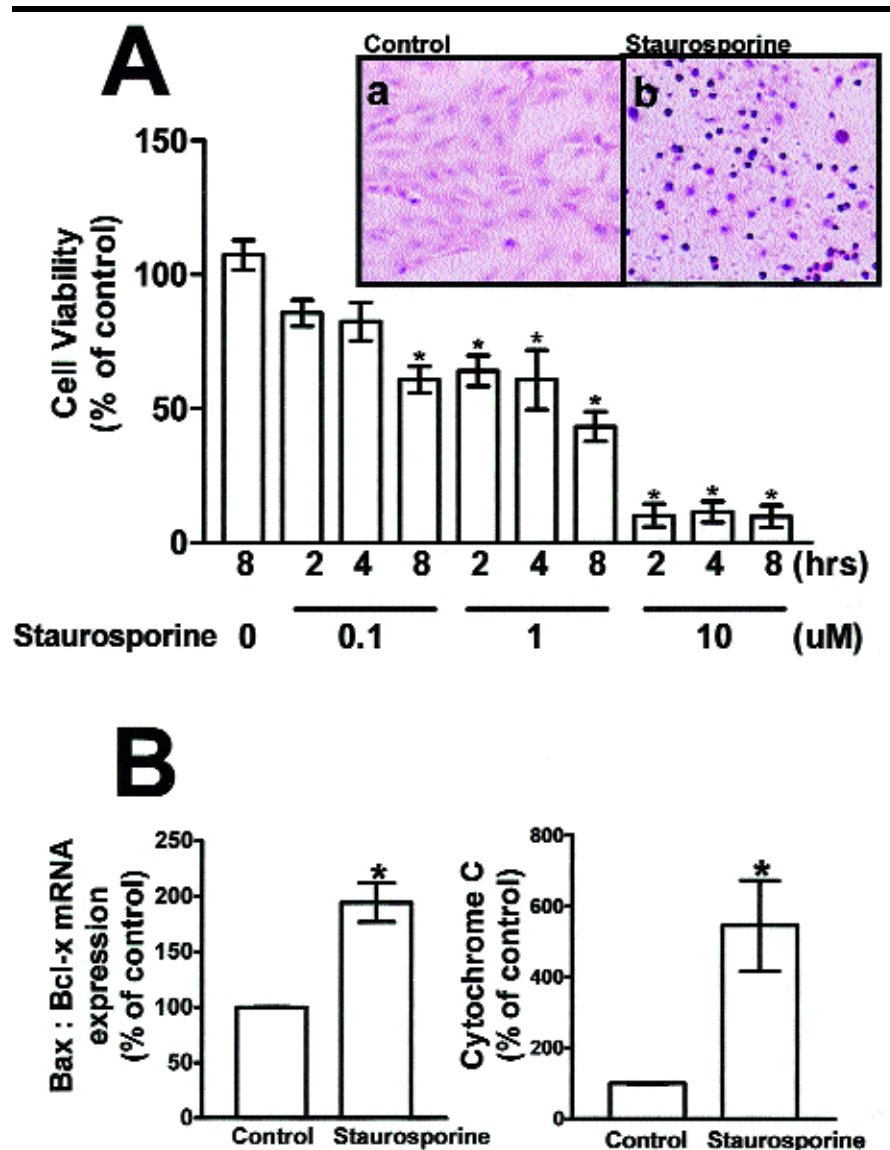


Fig. 1. Staurosporine-mediated apoptotic death in chondrocytes depends on the concentration and time. *P < 0.001 compared to the control value in cultures exposed to staurosporine alone. (A) The concentration and time courses of staurosporine-mediated chondrocyte death as assessed by MTT assay. The inset micrograph shows (a) control untreated chondrocyte cultures, and (b) staurosporine-treated chondrocytes (lower panel). Values represent means ± SEM of determinations made in 4-5 separate cultures. (B) Induction of caspase-3 and cytochrome C translocation to the cytoplasm assessed in chondrocyte cultures 4 hr after treatment with 1 M staurosporine.

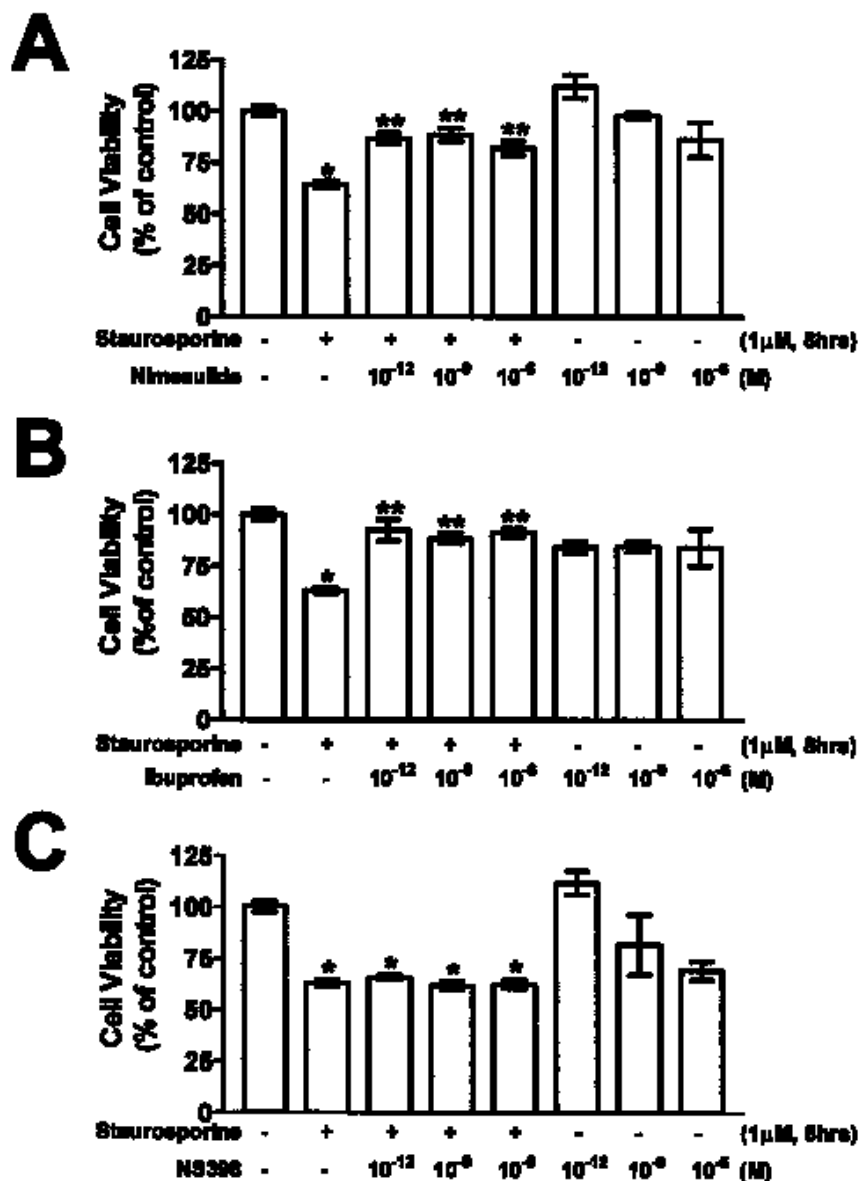


Fig. 2. Nimesulide or ibuprofen, but not NS398, protected against staurosporine-mediated apoptotic death in chondrocytes assessed by MTT assay. In A to C, values represent means \pm SEM of determinations made in 4-5 separate cultures. * $P < 0.001$ compared to the control value in cultures exposed to staurosporine alone, and ** $P < 0.01$ compared to corresponding values for cultures treated with staurosporine.

monitored by immunoblotting. Briefly chondrocyte cultures plated in 6-well plates were harvested in 500 μ l of ice-cold buffer containing 50mM Tris-HCl, 1 mM EDTA, 1mM DTT, complete protease inhibitor (Boehringer Mannheim) and 250 mM sucrose after washing once with ice cold PBS. Chondrocyte cultures were disrupted by douncing 10 times with a pestle in a 7 ml Wheaton douncer. After centrifugation in a minifuge (10 min, 4°C) the supernatants were further centrifuged at 12,000 xg for 40 min. The resulting supernatants were used for immuno-

blotting. Aliquots or cytosol were resolved electrophoretically by SDS-PAGE. The blots were then probed with a monoclonal antibody raised against the terminal carboxyl segment of cytochrome C (Pharmingen).

Assessment of caspase-3 activity

At each time indicated, chondrocyte cultures were washed with phosphate buffered saline (PBS) and harvested in 100 μ l cell lysis buffer (50 mM HEPES pH 7.4, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA, 0.5 mg/mL PEFA block-SC). Lysates were then incubat-

ed on ice for 15 min, centrifuged at 10,000 xg for 10 min at 4°C, and the protein concentrations were determined. Lysates (soluble supernatants) were either used immediately or stored at -80°C. Aliquots of protein (50 μ g) were incubated at 37°C with assay buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol and 200 mM Ac-DEVD-pNA (Biomol). Caspase-like activity was monitored colorimetrically (405 nm) by quantifying the release of p-nitroaniline from the appropriate cleaved caspase substrates at appropriate time intervals.

Statistics

Statistical analysis was made using ANOVA followed by Bonferroni's corrected post-hoc analysis, with p values < 0.05 taken as significant.

Results

Staurosporine-mediated apoptotic death in chondrocytes depends on the concentration and incubation time

Exposure of primary of chondrocytes to staurosporine resulted in concentration- and time-dependent loss of cellular viability as assessed by MTT assay (Fig. 1A).

Staurosporine-mediated toxicity in the chondrocyte cultures was further assessed by changes in nuclear morphology and cellular integrity. We found that following exposure to increasing concentrations of staurosporine (0.1 to 1 μ M, 4 hr) there was a proportional elevation in the number of chondrocytes with morphological features of apoptosis. Chondrocyte damage was characterized by condensed pyknotic nuclear morphology associated with cytoplasmic eosinophilia (Fig. 1, inset a) when compared to control untreated cultures (Fig. 1, inset b).

Staurosporine-mediated chondrocytic apoptosis coincided with an increased cytochrome C release and an increased ratio of Bax:Bcl-X mRNA expression, both of which are indices of apoptotic cell death (Fig. 1B).

Protection of staurosporine-mediated cell death by NSAIDs

To assess the protective role of

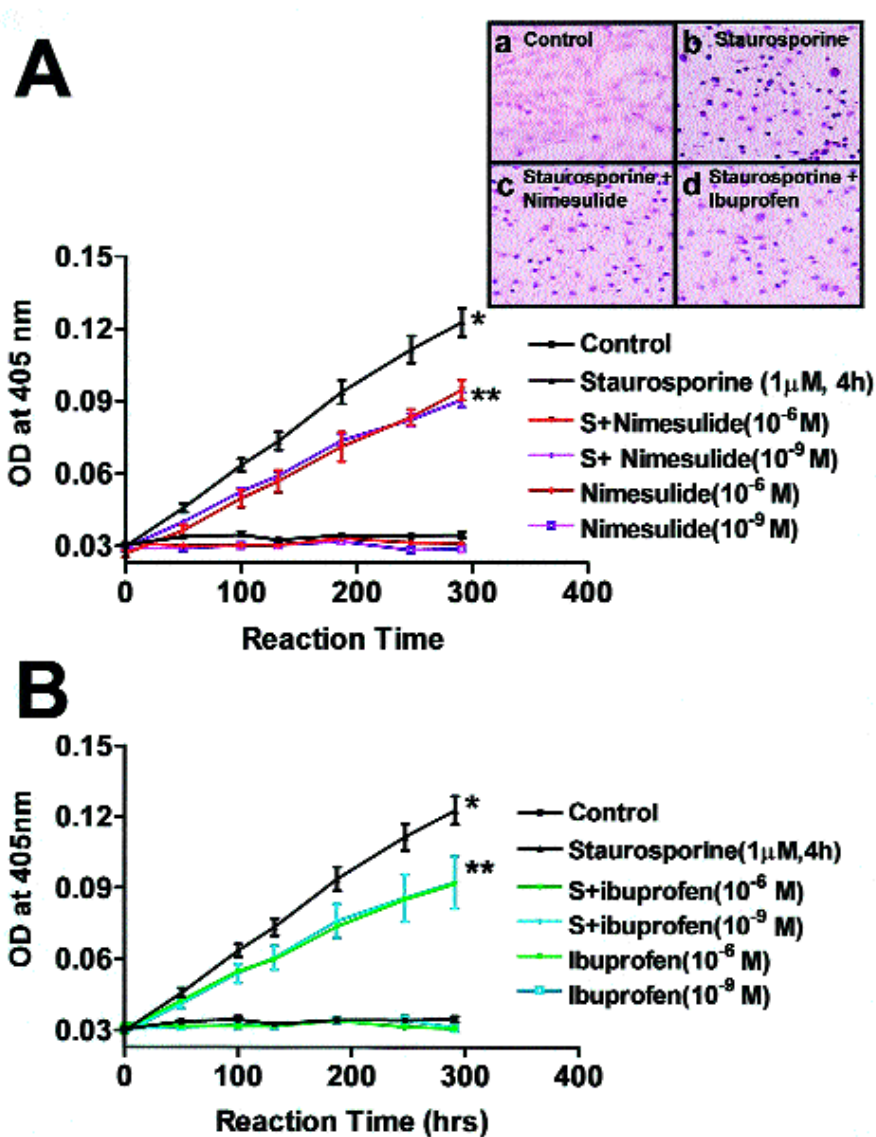


Fig. 3. Inhibition of staurosporine-mediated caspase-3 induction by nimesulide or ibuprofen coincided with a reduction in the number of chondrocytes with morphological features of apoptotic damage. Caspase-3-like protease activity in lysate extracts of chondrocyte cultures 4 hours after treatment with 1 μM staurosporine. Pre-treatment of chondrocyte cultures for 24 hours with 10⁻⁶ M of either nimesulide (A), or ibuprofen (B) revealed a marked reduction of caspase-3-like protease activity coincidental with a reduction in the number of chondrocytes with morphological features of apoptosis (inset). Panels 1-4 of the inset show micrographs of chondrocyte cultures visualized by H&E staining: **panel 1**, untreated control cultures; **panel 2**, cultures treated with staurosporine (1 μM, 4 hr); **panels 3 and 4**, cultures pre-treated for 24 hours with 10⁻⁶ of either nimesulide or ibuprofen respectively, showing a reduction of cell damage.

NSAIDs on chondrocytes against staurosporine-mediated apoptotic cell death, cell cultures were pre-treated for 24 hr with either the non-selective COX inhibitor ibuprofen, the preferential COX-2 inhibitor nimesulide, or the COX-2 selective inhibitor NS398, and then exposed to staurosporine (1 μM for 24 hr). Toxicity induced by staurosporine at 4 hr was significantly reduced by pre-treatment with nimesu-

lide (Fig. 2A) or ibuprofen (Fig. 2B) in concentrations ranging from 10⁻¹² to 10⁻⁶ M. Pre-treatment with NS398 did not protect the chondrocytes (Fig. 2C). Cultures treated with nimesulide, ibuprofen or NS398 alone for 24 hr did not differ from control cultures (Fig. 2A-C).

Staurosporine-mediated activation of caspase-3 in chondrocyte cell cultures

was inhibited by pre-treatment with nimesulide or ibuprofen

The protective effect of nimesulide or ibuprofen against staurosporine-induced apoptotic cell death was further assessed by monitoring both caspase-3 activation and nuclear morphology by H&E histochemistry. We found that by 4 hr after exposure with staurosporine (1 μM), the induction of caspase-3-like activity (see above) was significantly reduced by nimesulide or ibuprofen (Fig. 3 A,B). Inhibition of staurosporine-mediated caspase-3 induction in chondrocyte cultures treated with nimesulide or ibuprofen coincided with a reduction in the number of chondrocytes with morphological features of apoptotic damage (Fig. 3, inset). Cultures treated with nimesulide or ibuprofen alone did not differ from control cultures (not shown).

Discussion

In this study we found that the preferential COX-2 inhibitor nimesulide and the non-selective COX inhibitor ibuprofen, inhibited staurosporine-mediated apoptotic cell death in chondrocytes. The standard therapy of OA, which is among the most important causes of age-related pain and disability, includes analgesics, physical therapy, and surgery (12, 13). It has remained a matter of debate whether anti-inflammatory therapy, specifically NSAIDs, offers advantages or disadvantages over pure analgesics such as paracetamol in OA management. In addition to uncertainty regarding the relative analgesic potency of NSAIDs compared to paracetamol, results regarding NSAID effects on cartilage preservation have been conflicting (14, 15). The recent availability of selective COX-2 inhibitors, offering anti-inflammatory activity with reduced gastrointestinal toxicity, has further complicated the debate over optimal OA management. The goal of the present studies was to elucidate mechanisms of cartilage degradation in OA, particularly regarding chondrocyte apoptosis, and to evaluate the efficacy of anti-inflammatory drugs with regard to these mechanisms. Cartilage degradation in OA is characterized by chondrocyte apoptosis (16).

We hypothesize that loss of chondrocytes via this mechanism contributes to cartilage degradation. The observation that aging, a major risk factor for OA, is associated with increasing chondrocyte apoptosis (17-19), is consistent with this hypothesis. Apoptotic mechanisms may thus represent a target for chondroprotective therapy. Results from our *in vitro* studies raise the possibility that the NSAIDs nimesulide and ibuprofen reduce chondrocyte apoptotic cell death through inhibition of caspase-3. Thus, nimesulide and ibuprofen may represent a new preventive option for OA to block events in the apoptosis pathway.

A recent report that prostaglandin E2 induces chondrocyte apoptosis *in vitro* (9) is consistent with the possibility that ibuprofen and nimesulide acted in our experiments by inhibiting prostaglandin E2 production. However, the COX-2 inhibitor NS398 did not protect against staurosporine-mediated apoptotic cell death, so that the NSAIDs may have acted either by COX-1 inhibition or by mechanisms independent of COX. Investigation of the expression and activity of COX-1 and COX-2 in chondrocytes is currently under way in our laboratory.

Previous studies found that impairment of mitochondrial activities associated with oxidative stress leads to induction of caspase-3 coincidental with apoptotic cell death (20). Nimesulide and ibuprofen have anti-oxidant activity, and thus might control caspase activation through inhibition of mitochondrial swelling and release of cytochrome C. We are presently investigating the effect of nimesulide and ibuprofen on cytochrome C release as a potential protective mechanism.

In conclusion, our studies provide evidence of specific molecular mecha-

nisms involved in chondrocyte apoptotic cell loss, and suggest that the NSAIDs nimesulide and ibuprofen are useful in protecting against this degeneration. If the hypothesis that chondrocyte apoptosis contributes to cartilage damage in OA proves correct, our findings will indicate that these drugs, in addition to their analgesic effects, may confer chondroprotection. Further elucidation of the pathways leading to chondrocyte loss and degradation of extracellular matrix will improve the likelihood of developing effective strategies to preserve joint function.

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