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Effect of naproxen sodium on the net synthesis of glycosaminoglycans and protein by normal canine articular cartilage in-vitro

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Abstract—The effects of the non-steroidal anti-inflammatory drug, naproxen sodium, on the metabolism of normal canine articular cartilage has been examined. At a concentration approaching that achieved in synovial fluid of patients treated with the drug (i.e. 30 μg mL^{-1}) naproxen sodium had no significant effect on net synthesis of either glycosaminoglycans or protein in organ cultures of femoral condylar cartilage, nor did it increase the proportion of newly synthesized glycosaminoglycans recovered from the culture medium, suggesting that it had no direct effect on the integrity of the extracellular matrix.

The extracellular matrix of articular cartilage consists largely of collagens and proteoglycans, which afford the tissue its elasticity and stiffness on compression (Mankin & Brandt 1989). Most of the proteoglycans in cartilage exist in aggregates with hyaluronic acid (HA), in a non-covalent association stabilized by link glycoprotein. The enormous size of the aggregate (over 50×10^6 daltons) serves to constrain individual proteoglycans within the collagen meshwork of the cartilage (Mankin & Brandt 1989). In addition, proteoglycans within aggregates may be relatively protected from proteolytic degradation in the tissue (Heinegard & Hascall 1974).

The quantity of articular matrix within the normal joint reflects a balance between the ongoing processes of synthesis and degradation of proteoglycans, collagen, HA and other matrix molecules. In osteoarthritis (OA), a net loss of articular cartilage matrix is apparent (Mankin 1989). In the early stages, an increase in proteoglycan synthesis occurs, representing an attempt by the chondrocyte to repair the lesion. With progression of OA, however, the rate of synthesis ultimately falls, reflecting chondrocyte "failure" (Mankin & Brandt 1989).

Even in the early stages of OA, when proteoglycan synthesis is several times greater than normal, the proteoglycan concentration of OA cartilage may be subnormal, indicating that the augmented rate of matrix synthesis has been surpassed by an even greater increase in the rate of matrix catabolism. Increased activity of both proteoglycanases (Dean et al 1987) and collagenase (Pelletier et al 1983) in OA cartilage has been well documented.

In view of these pathogenetic mechanisms for cartilage loss in OA, it is reasonable to consider the possibility that agents that suppress proteoglycan metabolism or interfere with proteoglycan aggregation in-vivo might accelerate cartilage degeneration in OA. Indeed, if such agents had a similar effect on normal articular cartilage they could interfere with the physiological turnover of matrix proteoglycans that underlies normal matrix remodelling.

In-vitro and in-vivo studies have indicated that salicylates and several other NSAIDs used to treat symptoms of OA may inhibit synthesis of the glycosaminoglycan (GAG) constituents of proteoglycans in articular cartilage (Palmoski & Brandt 1979, 1980, 1982, 1983a). The present study examines the effect of the NSAID, naproxen sodium, a propionic acid derivative, on GAG and protein synthesis by chondrocytes in organ cultures of normal canine articular cartilage.

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Materials and methods

Tissues and culture conditions. Distal femurs were removed aseptically from normal adult mongrel dogs immediately after the animals were killed with an overdose of sodium pentobarbitone. A sample (approx. 30 mg) was taken from the central weight-bearing region of the medial femoral condyle for determination of cartilage water content (Slowman & Brandt 1986) and uronic acid concentration (Bitter & Muir 1962) and for histological study (Slowman & Brandt 1986). The remainder of the cartilage was shaved from the weight-bearing surfaces of both femoral condyles to provide slices $<0{\cdot}5$ mm thick. Shavings from each animal were pooled, but tissue from each animal was handled separately.

Triplicate samples of the cartilage slices were cultured in Costar tissue culture dishes containing 3 mL of Ham's F-12 nutrient mixture, pH 7·4, supplemented with 10% newborn calf serum, L-ascorbic acid (50 mg L^{-1}), streptomycin (100 mg L^{-1}), penicillin (100 units mL^{-1}), amphotericin-B (250 μg L^{-1}; Sigma Chemical Co. St. Louis, MO), bovine serum albumin (5 mg L^{-1}) and various concentrations of naproxen sodium, encompassing the concentration achieved in synovial fluid of patients treated orally with therapeutic doses of the drug (30 μg mL^{-1}). Naproxen sodium dissolved freely in the culture medium; under the experimental condition employed it is soluble at 60 mg L^{-1}.

The cartilage slices were incubated with gentle rocking at 37 C under 5% CO_2:95% air for 20 h, following which the spent medium was removed and replaced with fresh medium containing $Na_2{}^{35}SO_4$ (20 μCi mL^{-1}) (New England Nuclear Corp., Boston, MA) and naproxen sodium in the concentration originally employed. Incubation was continued for an additional 4 h, following which the medium was removed and the cartilage was washed with cold F-12. Medium and washes were combined, placed in Spectrapor Number 3 membrane tubing (approximate molecular weight cut-off = 3500) (Spectrum Medical Industries, Inc., Los Angeles, CA) and dialysed at 4°C for 72 h against water until radioactivity in the retentate reached a basal level.

Determination of net GAG synthesis. The effect of various concentrations of naproxen sodium on net GAG synthesis was determined on triplicate samples (approximately 10 mg wet weight) of cartilage from each of 5 animals. After incubation of the tissue with $Na_2{}^{35}SO_4$ and recovery of the medium as described above, the washed cartilage was gently blotted, weighed, and suspended in 0·05 M Tris hydroxymethylaminomethane, pH 8·0. Pronase (1 mg mL^{-1}; Cal-Biochem, San Diego, CA) was added and the sample was digested for 24 h at 56°C. The digest was then dialysed at 4°C against water, following which the sacs were washed with distilled water and the retentate and washes were combined.

Net GAG synthesis was determined from the sum of the non-dialysable $^{35}SO_4$ counts min^{-1} in 1·0 mL samples of the medium and 0·5 mL samples of the pronase digest, which were mixed separately with 10 mL of Ready-Solve HP 60 (Beckman Instruments, Inc., Irvine, CA) and counted in a Model LS 230 Beckman liquid scintillation spectrometer. Results were adjusted for differences in wet weight of the cartilage.

Table 1. Effects of naproxen sodium on $^{35}SO_4$ glycosaminoglycan synthesis by normal canine articular cartilage.

Animal No.	Compositional analysis of cartilage: H_2O content, % of total wt	Compositional analysis of cartilage: Uronic acid % wet wt	Net $^{35}SO_4$ glycosaminoglycan synthesis, % of control* (naproxen concentration (μg mL^{-1})): 6	30	150
1	69·0	2·9	105·6	114·1	108·9
2	68·6	3·2	145·7	118·0	72·4
3	73·3	4·4	100·3	101·2	98·0
4	70·1	4·0	104·5	95·3	77·4
5	73·4	4·2	103·4	100·9	76·7
Mean ± s.e.	70·9 ± 1·0	3·7 ± 0·3	111·9 ± 8·5	105·9 ± 4·3	86·7 ± 7·1

* % of value obtained with cartilage from the same knee cultured in the absence of drug.

Table 2. Effects of naproxen sodium on protein synthesis by normal canine articular cartilage.

Animal No.	Compositional analysis of cartilage: H_2O content, % of total wt	Compositional analysis of cartilage: Uronic acid % wet wt	Net incorporation of [^{3}H]leucine into protein, % of control* (naproxen concentration (μg mL^{-1})): 6	30	150
6	76·6	5·6	110·2	100·7	85·4
7	71·3	3·7	106·5	101·9	73·3
8	72·9	3·7	104·1	91·5	95·3
9	73·7	5·3	104·5	103·6	86·6
10	76·0	4·9	99·5	97·4	85·8
Mean ± s.e.	74·1 ± 1·0	4·6 ± 0·4	105·0 ± 1·7	99·0 ± 2·1	85·3 ± 3·5

* % of value obtained with cartilage from the same knee cultured in the absence of drug.

Determination of net protein synthesis. Triplicate samples of cartilage (approximately 10 mg wet weight each) were incubated as described above in 3 mL of medium containing naproxen sodium and 5 μCi mL^{-1} of [^{3}H]leucine (60 Ci mmol^{-1}; New England Nuclear Corp.). At the end of the incubation period the medium was removed and the cartilage slices were washed with cold F-12, blotted gently and weighed. Unincorporated ^{3}H was removed from the tissue by washing at 2°C with 5 mL 0·6 M trichloroacetic acid/20 mM leucine until background activity was achieved (Hascall et al 1983). The cartilage was then digested overnight at 56°C with pronase, as described above, following which the small amount of undigested material that remained was removed by centrifugation. ^{3}H in the supernatant was determined by liquid scintillation counting and the results were adjusted for tissue weight.

Results

The articular cartilage from each animal was smooth, bluish-white and glistening, with no surface irregularities. Histological sections from each joint, stained with Safranin-O fast green, showed no abnormalities.

Net GAG synthesis. Based on the nondialysable $^{35}SO_4$ radioactivity after pronase digestion of the cartilage, at the lowest concentration of naproxen sodium tested (6 μg mL^{-1}) net GAG synthesis in cartilage from five animals averaged 111·9% of the control value. At naproxen sodium concentrations of 30 μg mL^{-1} and 150 μg mL^{-1}, GAG synthesis averaged 105·9% and 86·7%, respectively, of the control (Table 1). None of these differences was significant (paired t-test).

In each experiment only 3–7% of the total newly synthesized GAG was present in the culture medium, while the remainder was found in the pronase digest of the tissue. In no case did incubation with the drug increase the proportion of $^{35}SO_4$-GAG found in the medium.

Net protein synthesis. Naproxen sodium produced only a minimal concentration-dependent reduction in protein synthesis under the conditions employed. At a naproxen sodium concentration of 6 μg mL^{-1}, net protein synthesis was 105% of the control; at 30 μg mL^{-1} it was 99%, and at 150 μg mL^{-1} it was 85% of the control level (Table 2). The modest suppression of protein synthesis seen with the highest concentration of naproxen was not significantly different from the value obtained wth 30 μg mL^{-1} although, in comparison with the effect obtained with 6 μg mL^{-1}, the suppression produced by 150 μg mL^{-1} was statistically significant ($P < 0·01$, paired t-test). It should be noted, however, that a naproxen concentration of 150 μg mL^{-1} is not clinically reasonable, since it is some five times higher than the concentration achieved in synovial fluid of patients treated with this drug.

Discussion

In this study we have shown that, at a concentration identical to that achieved in synovial fluid of patients treated with an anti-inflammatory dose of the drug, naproxen sodium does not decrease GAG synthesis in this canine cartilage explant model. Although a five-fold greater concentration produced some suppression of GAG synthesis (85·7% of control), this concentration is clinically irrelevant; systemic toxicity would preclude attainment of synovial fluid levels of this magnitude.

Similar results were found when net protein, rather than GAG, synthesis was measured. No suppression of protein synthesis was seen at a clinically relevant concentration of naproxen.

Several NSAIDs (e.g., salicylate, fenoprofen, sodium tolmetin, ibuprofen) have been shown to inhibit net GAG synthesis by slices of normal cartilage in-vitro (Palmoski & Brandt 1979, 1980). The effect is concentration-dependent and reversible. In contrast, in the same experimental system, indomethacin, piroxicam, diclofenac (Brandt 1987), tiaprofenic acid (Brandt, unpublished data) and naproxen, as shown in the present study, appeared to have essentially no effect on GAG synthesis, while sulindac had a slight (Palmoski & Brandt 1980), and benoxaprofen a marked, stimulatory effect (Palmoski & Brandt 1983b).

In the present study we did not assess the effect of naproxen sodium on the proportion of newly-synthesized proteoglycans that existed in aggregates, or on the molecular size of the proteoglycans. We showed previously that several NSAIDs had no effect on proteoglycan aggregation, but fenoprofen impaired the interaction of newly synthesized proteoglycans with hyaluronic acid (Palmoski & Brandt 1980).

Although cartilage GAG concentration was not measured at the end of the incubation period, no more than 7% of the total $^{35}SO_4$-GAG was recovered from the culture medium. Thus, naproxen sodium did not increase loss of newly synthesized GAG from the tissue, suggesting that it had no direct effect on the integrity of the extracellular matrix. Consistent with this suggestion, in previous experiments performed under experimental conditions similar to those employed herein, with a culture period of only 24 h, no significant change in uronic acid concentration occurred when normal canine articular cartilage was cultured in the presence of salicylate or several other NSAIDs (Brandt, unpublished data).

Pulse-chase studies have demonstrated that several other NSAIDs (e.g. salicylate, ibuprofen, sulindac sulphide, benoxaprofen) did not affect the rate of proteoglycan catabolism in normal canine knee cartilage (Palmoski & Brandt 1980); indeed, fenoprofen caused a slight decrease in the rate of GAG catabolism (Palmoski & Brandt 1980). While we did not directly assess the effect of naproxen sodium on GAG catabolism, it was recently shown that incubation of slices of normal canine articular cartilage in medium containing naproxen sodium in the concentrations used herein reduced GAG loss from the tissue during the culture period, presumably due to inhibition by the drug of matrix metalloproteinases, such as stromelysin and collagenase (Ratcliffe A, personal communication).

Although the present study assessed the effect only of naproxen, a propionic acid derivative, on normal articular cartilage, other in-vitro studies (Palmoski et al 1980) have examined the effect of salicylate on OA cartilage. The augmented proteoglycan synthesis in OA cartilage, reflecting repair activity by the chondrocyte, was abrogated by salicylate; in some cases proteoglycan synthesis in OA cartilage was inhibited by more than 90%. In contrast, salicylate-induced inhibition of proteoglycan synthesis in normal canine knee cartilage averaged only about 40% of that in control cartilage cultured in the absence of salicylate.

Salicylates, whose effects on joint cartilage have been studied much more extensively than those of other NSAIDs, affect OA cartilage in-vivo as well as in-vitro. In dogs that were fed aspirin daily for 9 weeks after transection of the anterior cruciate ligament, degeneration of articular cartilage in the unstable knee was more marked than that in the OA knees of dogs that did not receive aspirin (Palmoski & Brandt 1983a).

Similarly, atrophic femoral condylar cartilage from dogs whose hind limb had been immobilized for 6 weeks in an orthopaedic cast, and who were fed aspirin throughout the period of immobilization in amounts sufficient to maintain the serum salicylate concentration at 200–250 $\mu g\ mL^{-1}$, showed significantly greater decreases in matrix proteoglycan concentration and in the rate of proteoglycan synthesis than articular cartilage from the immobilized legs of dogs which did not receive aspirin (Palmoski & Brandt 1982).

Extrapolation of the data from in-vitro studies suggests that the effect of aspirin (and, presumably, of other NSAIDs) on articular cartilage may be related to the concentration of the drug to which the chondrocyte is exposed in-vivo (Brandt 1987). This, in the clinical setting, is determined largely by the mass of drug administered, which is determined by the antirheumatic "potency" of the compound and its toxicity. To the extent that the effective daily antirheumatic dose of naproxen (approximately 1 $g\ day^{-1}$) is lower than that of salicylate (approximately 3–4 $g\ day^{-1}$), naproxen may be expected to have less effect than salicylate on proteoglycan synthesis in degenerating cartilage.

This in-vitro study demonstrated that naproxen sodium has no significant effect on proteoglycan synthesis by normal canine articular cartilage in-vitro. Similar studies are needed of the effect of naproxen on degenerating cartilage, and of the in-vivo effects of naproxen on proteoglycan metabolism in animal models of cartilage degeneration and in patients with osteoarthritis.

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