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n-3 Fatty Acids Specifically Modulate Catabolic Factors Involved in Articular Cartilage Degradation*

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This study describes specific molecular mechanisms by which supplementation with *n*-3 fatty acids (*i.e.* those present in fish oils) can modulate the expression and activity of degradative and inflammatory factors that cause cartilage destruction during arthritis. Our data show that incorporation of *n*-3 fatty acids (but not other polyunsaturated or saturated fatty acids) into articular cartilage chondrocyte membranes results in a dose-dependent reduction in: (i) the expression and activity of proteoglycan degrading enzymes (aggrecanases) and (ii) the expression of inflammation-inducible cytokines (interleukin (IL)-1α and tumor necrosis factor (TNF)-α) and cyclooxygenase (COX-2), but not the constitutively expressed cyclooxygenase COX-1. These findings provide evidence that *n*-3 fatty acid supplementation can specifically affect regulatory mechanisms involved in chondrocyte gene transcription and thus further advocate a beneficial role for dietary fish oil supplementation in alleviation of several of the physiological parameters that cause and propogate arthritic disease.

Noninvasive, pharmaceutical-based therapies for the treatment of arthritic diseases are primarily limited to oral administration of nonsteroidal antiinflammatory drugs, which inhibit cyclooxygenase (COX)[1]-mediated production of inflammatory eicosanoids such as prostaglandins (1, 2). Parenthetically, clinical studies on dietary supplementation with *n*-3 (omega-3) fatty acids (the principle long chain polyunsaturated fatty acids found in fish oils) have also demonstrated modulation of inflammatory symptoms involved in the pathogenesis of arthritis (3–5). Such epidemiological observations have been largely anecdotal, because they did not investigate the molecular mechanisms whereby dietary *n*-3 fatty acid supplementation might affect the metabolism of cells within articular joint tissues and thereby provide relief to arthritic symptoms. Significantly, however, dietary supplementation with *n*-3 fatty acids elicits antiinflammatory effects in neutrophils and monocytes by inhibiting the 5-lipoxygenase pathway responsible for metabolism of arachidonic acid to leukotrienes (6). Furthermore, *n*-3 fatty acid supplementation can also suppress phospholipase C-mediated signal transduction (7), thus demonstrating additional molecular mechanisms whereby *n*-3 fatty acids can specifically affect cell metabolism.

One of the key pathological features common to degenerative joint diseases (arthritis) is the loss of cartilage proteoglycan (aggrecan), which precedes subsequent cartilage erosion. Catabolism of aggrecan is mediated by the proteolytic activity of aggrecanases (8–11), two isoforms of which have recently been purified and cloned (12, 13). Aggrecanase activity is up-regulated by cartilage exposure to pro-inflammatory cytokines such as IL-1 and TNF-α, and model cartilage explant and chondrocyte culture systems stimulated with IL-1 or TNF-α mimic the degradative processes involving aggrecan catabolism which occur during arthritis (14–18). In addition, exposure to these inflammatory mediators propogates the autocrine synthesis of cartilage cytokines, which contribute to the chronic progression of arthritis. Furthermore, cytokine-induced degradative activities in synovial joint tissues can be potentiated via the biosynthesis of inflammatory eicosanoids by the cyclooxygenases COX-1 and COX-2 (1, 2). COX-1, which is constitutively expressed in most tissues, is responsible for key aspects of eicosanoid biosynthesis, which are important in maintaining homeostasis during normal cellular metabolism (19). Conversely, COX-2 expression and activity is induced during inflammation, and it is this enzyme that is selectively involved in inflammatory aspects of arthritic disease (20, 21). Consequently, modulation of COX-2 activity has been a major target of pharmaceutical companies for intervention in the pathogenesis of arthritis (22).

To determine a molecular basis for potential therapeutic properties associated with dietary intake of fish oils, we investigated the effects of different classes of fatty acids on the expression and activity of cartilage aggrecanases, cytokines (IL-1α and TNF-α) and cyclooxygenases (COX-1 and COX-2). The results of these *in vitro* studies reveal that exposure of articular chondrocytes to *n*-3 fatty acids can specifically modulate, at the level of gene transcription, key factors involved in articular cartilage degradation.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Bovine Chondrocytes—Bovine articular cartilage was obtained from the metacarpo- and metatarsophalangeal joints of 7-day-old calves. Cartilage tissue slices were dissected under sterile conditions and subjected to standard Pronase and collagenase digestion to isolate the chondrocytes as described previously (9). Monolayer cultures were established in 60-mm diameter culture dishes by plating 1 ml/dish of a suspension of 6×10^6 chondrocytes/ml of DMEM ($\sim 2 \times 10^5$ cells/cm^2). Cultures were maintained for 8 h in the absence or presence (10–100 μg/ml) of polyunsaturated *n*-3 fatty acids (α18:3 linolenate,[2] 20:5 eicosapentaenoate or 22:6 docosahexaenoate), a poly-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF192770 and AF192771.

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[1] The abbreviations used are: COX, cyclooxygenase; IL, interleukin; TNF, tumor necrosis factor; RT-PCR, reverse transcription-polymerase chain reaction.

[2] Approved nomenclature (trivial names) for fatty acids is as recommended by the IUPAC-IUB.

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unsaturated *n*-6 fatty acid (18:2 linoleate), a saturated fatty acid (16:0 palmitate), or a monounsaturated fatty acid (18:1 oleate). All fatty acids were minimum 99% purity from Sigma-Aldrich Co., Poole, United Kingdom. Prior to their addition to culture media, fatty acids were incubated as described (23) for 16 h at 37 °C in Tyrode-HEPES buffer (20 mM HEPES, 140 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM glucose, pH 7.4) containing 3.5 mg/ml fatty acid-free bovine albumin (Fraction V, Sigma-Aldrich Co.) at a ratio of 3:1 fatty acid:albumin. The culture medium was removed and then replaced with fresh medium (without fatty acid) supplemented with or without 10 ng/ml IL-1α.

Fatty Acid Analysis of Chondrocyte Membranes—Chondrocyte cell layers were harvested using a rubber policeman and washed three times in phosphate-buffered saline by centrifugation for 10 min at 1000 × *g*. The cells were resuspended in 1 ml deionized water and sonicated for 30 min in an ultrasonic water bath. Once complete lysis of the cells had been achieved, extraction of the lipids was performed (24). Briefly, 1 ml of cell lysate was mixed with 3.75 ml of chloroform/methanol (1:2 v/v) and incubated for 30 min at 20 °C. Following the addition of 1.25 ml of chloroform and 1.25 ml of Garbus solution (2 M KCl, 0.5 M KPO_4, pH 7.4), the chloroform phase of all mixtures was dried down in a stream of nitrogen. Generation of fatty acid methyl esters was achieved by addition of H_2SO_4 (2.5%) in anhydrous methanol (1%). As an internal standard an appropriate quantity of pentadecaenoic acid (15:0) was added. After sealing, the tubes were heated for 2 h at 70 °C. The tubes were then cooled and 2.5 ml of 5% NaCl was added. The methyl esters were extracted three times with 3 ml of petroleum ether, dried down in a stream of nitrogen, and then redissolved in chromatographically pure petroleum ether. The methyl esters were analyzed using gas chromatography, with the yields of fatty acid being calculated from the known amount of internal standard.

Analyses of Chondrocyte Metabolism and Phenotype—Cellular DNA content of all chondrocyte cultures was measured using the Hoechst 33258 dye DNA assay (25). Proteoglycan synthesis was measured by radiolabeling cultures for 96 h with [^{35}S]sulfuric acid (20 μCi/ml). Unincorporated radiolabel was removed from the culture media and cell layer extracts using a Sephadex G-50 column (Amersham Pharmacia Biotech) and total counts/min in the void volume measured. The concentration of lactate in culture media was measured using a commercial lactate assay kit (Sigma-Aldrich Co.). The expression of mRNAs for two genes which are characteristic of the chondrocyte phenotype (*i.e.* aggrecan and collagen type II) was assessed by RT-PCR as described below.

Western Blot Analyses of Aggrecanase- or Matrix Metalloproteinase-generated Aggrecan Catabolites—To detect the occurrence of aggrecanase or matrix metalloproteinase activity in the monolayer cultures, portions of conditioned media containing an equivalent quantity of proteoglycan metabolites (measured by dimethylmethylene blue assay (26)) were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting as described previously (9). Briefly, after deglycosylation with chondroitinase ABC (Sigma-Aldrich Co.), keratanase and keratanase II (Seikagaku/AMS Biotechnology, Abingdon, UK), aggrecan fragments were separated on 4–12% gradient gels (Novex, Frankfurt, Germany) and electrophoretically transferred to nitrocellulose. Membranes were then probed using monoclonal antibody BC-3 (which specifically recognizes the aggrecanase-generated neoepitope N-terminal sequence ^{374}ARGSV . . . on aggrecan metabolites) or monoclonal antibody BC-14 (which specifically recognizes the matrix metalloproteinase-generated neoepitope N-terminal sequence ^{342}FFGVG . . . on aggrecan metabolites) (9).

RNA Extraction and RT-PCR Analyses—Bovine chondrocyte monolayer cultures were extracted by direct addition of Tri-Reagent (Sigma-Aldrich Co.). Following the addition of chloroform (0.2 ml/1 ml of Tri-Reagent) and centrifugation for 20 min at ~16,000 × *g*, total RNA from the aqueous phase of all extracts was isolated using Rneasy mini-columns and reagents (Qiagen, Crawley, Ltd., Crawley, UK). Because spectrophotometric analyses revealed a reasonable, but relatively low, yield of total RNA (approximately 20 μg/60-mm culture dish), we utilized RT-PCR methods to examine chondrocyte gene transcription. First strand cDNA was synthesized by reverse transcription and PCR amplification was performed as described (27) using oligonucleotide primers corresponding to cDNA sequences for aggrecan (CGCTACGACGCCATCTGCTAC and GCCTGCTGTGCCTCCTCAAA; GenBank™ accession number M55172), collagen II-αI (TGCCTGGTGCTCCTGGTCTGA and CTTCTCCCTTCTCGCCGTTAG; GenBank™ accession number X16711), aggrecanase-1 (ACCACTTTGACACAGCCATTC and ACCCCCACAGGTCCGAGAGCA; GenBank™ accession number AF148213), aggrecanase-2 (TGTGCTGTGATTGAAGACGAT and GACTGCAGGAGCGGTAGATGG; GenBank™ accession number AF142099), COX-1 (GCCCAACACTTCACCCATCAG and CCAGGAAGCAGCCCAAACACT; GenBank™ accession number AF004943), COX-2 (GCTCTTCCTCCTGTGCCTGAT and CATGGTTCTTTCCCTTAGTGA; GenBank™ accession number AF004944), IL-1α (AAGGAGAATGTGGTGATGGTG and CAGAAGAAGAGGAGGTTGGTC; GenBank™ accession number M37210), TNF-α (CTCAAGCCTCAAGTAACAAGC and GCAATGATCCCAAAGTAGACC; GenBank™ accession number Z48808), and GAPDH (TGGTATCGTGGAAGGACTCAT and GTGGGTGTCGCTGTTGAAGTC; GenBank™ accession number X01677). PCR products were separated on 3% agarose gels, stained with ethidium bromide, and their nucleotide sequences verified using an Applied Biosystems 310 Genetic Analyzer. The cDNA sequences obtained for bovine aggrecanase-1 and aggrecanase-2 have been deposited to GenBank™ under accession numbers AF192770 and AF192771.

TABLE I

Fatty acid analysis of chondrocyte membranes from cultures treated with (+) or without (−) 100 μg/ml n-3 polyunsaturated or saturated fatty acids

Data are the mean values for cells harvested from two different animals.

Fatty acid measured	Percentage of total fatty acids in membranes			
	n-3 fatty acid-treated (α18:3 linolenate)		Saturated fatty acid-treated (16:0 palmitate)	
	−	+	−	+
α18:3 linolenate[a]	ND[b]	14.0	ND	ND
γ18:3 linolenate[a]	6.1	3.1	9.3	5.0
18:2 linoleate[a]	13.2	10.4	10.1	3.2
20:4 arachidonate[a]	2.8	1.2	4.1	1.6
16:1 palmitoleate[c]	7.0	5.4	9.7	9.3
18:1 oleate[c]	32.8	31.2	32.3	35.8
16:0 palmitate[d]	21.5	19.6	18.6	31.7
18:0 stearate[d]	16.5	15.1	16.1	13.4

[a] Polyunsaturated fatty acid.
[b] ND, none detected.
[c] Monounsaturated fatty acid.
[d] Saturated fatty acid.

RESULTS AND DISCUSSION

In this study, articular cartilage chondrocytes were exposed in culture to fatty acids at concentrations which cover the typical range (50–70 μg/ml) for free fatty acid levels in human plasma (28). The results for measurements of the lipid content of chondrocytes supplemented without or with a polyunsaturated *n*-3 (α18:3 linolenate) or a saturated (16:0 palmitate) fatty acid are shown in Table I. Exposure to the *n*-3 fatty acid markedly changed the overall lipid composition profile of the chondrocyte with major changes occurring in the supplemented fatty acid (*e.g.* α18:3 linolenate), with a concomitant reduction in other polyunsaturated fatty acids. Similarly, supplementation with 16:0 palmitate markedly altered the level of this fatty acid in the chondrocyte lipid composition profile, again at the expense of polyunsaturated fatty acid components. Replicate cultures, with or without prior fatty acid supplementation, were then cultured for a further 96 h in the absence or presence of IL-1α. Proteoglycan synthesis, as measured by [^{35}S]sulfate incorporation, was decreased in chondrocyte cultures treated with IL-1α compared with controls as has been reported previously (29). However, prior addition of fatty acids to the cultures had no effect on these changes in proteoglycan synthesis or in cell numbers, DNA content or morphology, nor in levels of lactate secreted into the medium (results not shown), thus demonstrating that fatty acid supplementation was not toxic to the chondrocytes. Furthermore, unaltered expression of mRNAs for aggrecan and collagen type II confirmed maintenance of the chondrocyte phenotype in the experimental cultures (results not shown).

The effect of fatty acid supplementation on chondrocyte aggrecanase activity was then investigated (Fig. 1). There was no evidence for aggrecanase activity in conditioned medium from control cultures supplemented with either *n*-3 (Fig. 1*A*, *lanes 1*

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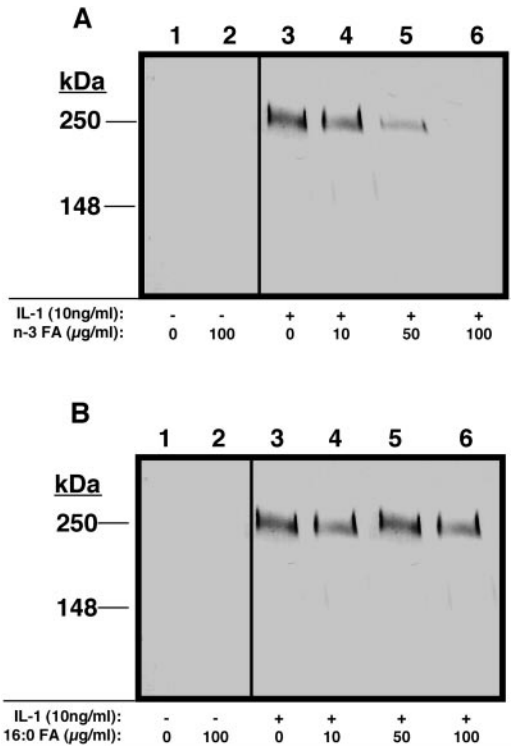

FIG. 1. **Effect of fatty acid supplementation on chondrocyte aggrecanase activity.** Cultures were supplemented with or without increasing amounts of *n*-3 α18:3 linolenate (*A*) or 16:0 palmitate (*B*) and subsequently treated with (+) or without (−) IL-1. Aggrecan fragments released into the culture media were immunodetected on Western blots using the neoepitope monoclonal antibody BC-3, which specifically detects products generated by aggrecanase cleavage at the Glu^{373}-Ala^{374} peptide bond.

and *2*) or saturated fatty acids (Fig. 1*B*, *lanes 1* and *2*). Exposure to IL-1, as expected (8), did induce aggrecanase activity in these conditioned media (Fig. 1, *A* and *B*, *lane 3*, respectively). However, addition of the *n*-3 fatty acid α18:3 linolenate abolished this IL-1-induced aggrecanase activity in a dose-dependent manner (Fig. 1*A*, *lanes 4–6*). In contrast, supplementation with 16:0 palmitate had no effect on aggrecanase activity (Fig. 1*B*, *lanes 4–6*). In replicate studies, no evidence for matrix metalloproteinase-mediated aggrecan cleavage was observed for cultures maintained in the absence or presence of either *n*-3 or saturated fatty acids (results not shown). In keeping with the observed loss of aggrecanase-mediated proteolytic activity in IL-1-treated chondrocyte conditioned medium, there was also a decrease in the levels of mRNA transcripts for aggrecanase-1 and aggrecanase-2 in response to *n*-3 fatty acid supplementation (Fig. 2). Aggrecanase-1 mRNA was detected in IL-1-treated, but not control, cultures, whereas mRNA for aggrecanase-2 was detected both in the presence and absence of IL-1 (Fig. 2, *A* and *B*, *lanes 1* and *2*). However, addition of the *n*-3 fatty acid α18:3 linolenate caused a decrease in both aggrecanase-1 and aggrecanase-2 transcript levels (Fig. 2*A*, *lanes 3–5*). In contrast, cultures supplemented with 16:0 palmitate expressed aggrecanase-1 and aggrecanase-2 mRNAs at all concentrations of fatty acid tested (Fig. 2*B*, *lanes 3–5*). The addition of the vehicle for fatty acid supplementation (fatty acid-free albumin) had no effect on either aggrecanase-1 or -2 expression in the control or IL-1-treated cultures (results not shown). Expression of GAPDH was used to normalize the amount of mRNA present in all samples.

We next examined the effect of supplementation with *n*-3 fatty acids *versus* other fatty acids on COX-1 and COX-2 mRNA expression and on the expression of IL-1α and TNF-α mRNAs

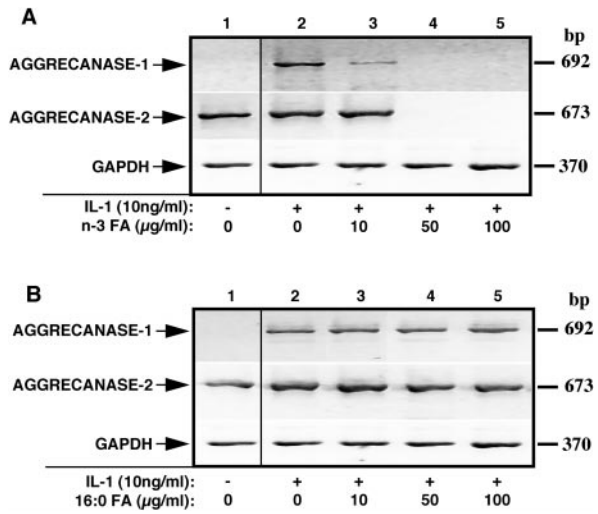

FIG. 2. **Effect of fatty acid supplementation on expression of chondrocyte aggrecanase-1 and aggrecanase-2 mRNAs.** Cultures were supplemented with or without increasing amounts of *n*-3 α18:3 linolenate (*A*) or 16:0 palmitate (*B*) and subsequently treated with (+) or without (−) IL-1. Total RNA was extracted and amplified by RT-PCR. The size of PCR products (in base pairs) relative to the migration of DNA size standards is indicated to the *right* of each panel.

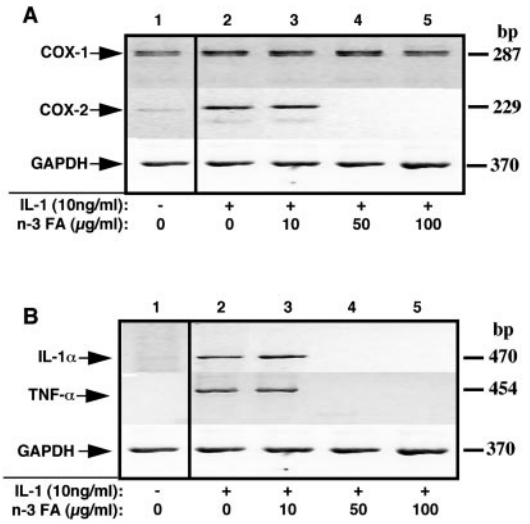

FIG. 3. **Effect of fatty acid supplementation on expression of chondrocyte COX-1 and COX-2 mRNAs (*A*) and IL-1α and TNFα mRNAs (*B*).** Cultures were supplemented with or without increasing amounts of *n*-3 α18:3 linolenate and subsequently treated with (+) or without (−) IL-1. Total RNA was extracted and amplified by RT-PCR. The size of PCR products (in base pairs) relative to the migration of DNA size standards is indicated to the *right* of each panel.

in chondrocyte cultures exposed to exogenous IL-1 (Fig. 3). Chondrocyte COX-1 mRNA was present in all culture systems, with or without fatty acid supplementation (Fig. 3*A*, *lanes 1–5*). However, chondrocyte COX-2 expression was detected in IL-1-treated, but not control, cultures (Fig. 3*A*, *lanes 1* and *2*). Significantly, COX-2 mRNA expression in IL-1-treated cultures was decreased by *n*-3 fatty acid (α18:3 linolenate) supplementation (Fig. 3*A*, *lanes 3–5*). In contrast, addition of a saturated fatty acid (16:0 palmitate) had no such effect on IL-1-induced COX-2 expression (data not shown). Examination of chondrocyte-derived cytokine expression revealed that the mRNAs for IL-1α and TNF-α were absent in control cultures (Fig. 3*B*, *lane 1*), but these were detected following exposure to exogenous IL-1α (Fig. 3*B*, *lane 2*). However, expression of chondrocyte IL-1α and TNF-α mRNAs was decreased in *n*-3 fatty

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acid-treated cultures (Fig. 3*B*, *lanes 3–5*), but not in cultures supplemented with saturated fatty acids (data not shown).

Identical results to those shown in Figs. 1–3 were obtained when two other *n*-3 fatty acids found in fish oils (eicosapentaenoic acid and docosahexaenoic acid) were added to the culture systems, and no effects were observed when either an *n*-6 fatty acid (linoleic acid) or a monounsaturated fatty acid (oleic acid) were supplemented (data not shown). These results conclusively indicate that the observed effects on the expression of chondrocyte aggrecanases, COX-2, and autocrine cytokines are specific to supplementation with *n*-3 (omega-3) fatty acids.

Collectively, these data provide informative novel information on the biological and molecular mechanisms whereby dietary fish oil supplementation can reduce inflammatory and degradative aspects of articular joint disease and thus modulate disease progression. The balance of dietary *n*-3/*n*-6 polyunsaturated fatty acids is well known to affect inflammatory responses (30). Polyunsaturated fatty acids of the *n*-6 series, such as linoleic acid, are intimately involved in the production of inflammatory eicosanoids (31), and *n*-3 polyunsaturated fatty acids can interfere with this process at, for example, the desaturation steps or by producing alternative eicosanoids with different activities (32). On the other hand, accumulating evidence suggests that *n*-3 fatty acids can regulate gene expression via activation of transcription factors such as peroxisome proliferator-activated receptor-α or by affecting expression levels of sterol regulatory element-binding proteins (33). Whereas such mechanisms remain to be investigated in chondrocytes, our current findings demonstrate that *n*-3 fatty acid supplementation can specifically affect molecular mechanisms that regulate the expression of catabolic factors involved in articular cartilage degradation and thus further advocate a beneficial role for dietary fish oils in alleviation of several of the physiological parameters that cause and propogate arthritic disease.

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