Lipid and Cell Metabolic Changes Associated with Essential Fatty Acid Enrichment of Articular Chondrocytes (43149)

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Abstract. **Observations of impaired chondrocyte metabolism in essential fatty acid (EFA) deficiency as well as EFA protection against development of osteoarthrosis in inbred mice suggest the existence of a relationship between EFA, chondrocyte metabolism, and cartilage degeneration. To explore this relationship further, the fatty acid content of lipids in normal fetal bovine chondrocytes was manipulated by *in vitro* exposure to media supplemented with 100 μM arachidonic acid (20:4) or oleic acid (18:1). Chondrocytes rapidly and differentially incorporated both fatty acids into their lipid pools. The predominant acceptor was triacylglycerols. A 980% enrichment of arachidonic acid was associated with increased concentrations of fatty acids, increased $^{35}SO_4$ and [^{3}H]proline incorporation into matrix macromolecules (170% and 54–103%, respectively), and a 24-fold elevation in chondrocyte prostaglandin synthesis. No metabolic changes were observed in cells enriched by 377% with 18:1 oleic acid. The metabolic effects elicited by 20:4 arachidonic acid were abolished by pretreatment of cells with indomethacin, suggesting that the cellular responses to essential fatty acid loading may be associated with induced increases in prostaglandin synthesis. The data indicate that excessive *in vitro* accumulation of arachidonic acid is associated with an increase in synthetic activity that is causally related to increased prostaglandin synthesis and elevated levels of cellular fatty acids.** [P.S.E.B.M. 1990, Vol 195]

The accumulation of lipids in articular cartilage is an age-related phenomenon first described by Bonner *et al.* (1). The presence of intra- and extracellular lipids is considered to be a synthetic product of chondrocytes and a normal occurrence, because they are found in the absence of degenerative changes (2–6). However, excessive lipid accumulation is also a pathologic condition with a suggestive causal relationship to traumatic or degenerative arthritis. Examples include precocious arthrosis, a hereditary disorder associated with cartilage accumulation of complex lipids (7), and lipohemarthrosis, a recognized trauma-related condition associated with synovial and cartilage changes in which the lipidic component may participate (8).

Experimentally, intra-articular administration of homologous lipid in rabbits produces a state of "lipoarthrosis," indicating that lipid readily enters cartilage and produces changes mimicking osteoarthrosis (9). Increases in lipid accumulation by chondrocytes were also shown to occur, concomitant with a decrease in glycosaminoglycan synthesis. Ultrastructural studies on cartilage obtained from the Pond and Nuki canine instability model of arthritis exhibit an accumulation of amorphous lipids beneath fibrillated areas in the early stages of pathology (10). Similarly, in the Hulth rabbit model of instability, a relationship exists between mechanical perturbation and lipid accumulation in chondrocytes before histologic changes are evident (11). In similar fashion, large numbers of lipid droplets are found occupying chondrocyte cytoplasm following immobilization (12).

In contrast to these reports, the essentiality and beneficial effects of dietary lipids in articular cartilage have also been documented. The work of Silberberg and Silberberg (13) demonstrate an increase in the

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incidence of age-dependent osteoarthrosis in the C-57 inbred strain of rats with a high saturated fatty acid diet. Interestingly, addition of 3% linoleic acid (an essential fatty acid) to this diet reversed the increase in the incidence of osteoarthrosis (14). Essential fatty acid deficiency in Wistar rats results in a 50% inhibition of $^{35}SO_4$ incorporation by costal cartilage (15). This effect is abolished by injection of prostaglandin E_1 or supplementation of the diet with corn oil (rich in essential fatty acids).

A lipid source is a mandatory requirement for obtaining optimal chondrocyte proliferation in *in vitro* cultures (16). However, the influence of lipid (fatty acid) accumulation on cartilage metabolism, although often alluded to, has not been thoroughly explored. The association of this phenomenon with the initiation and/or progression of degenerative joint disease remains unknown. To establish a firmer basis for fatty acid involvement in cartilage metabolism, we have adapted an *in vitro* system whereby "fatty acid loading" of normal chondrocytes can be achieved by fatty acid supplementation of the culture medium.

Our data suggest that chondrocytes accumulate exogenous fatty acids, which, depending on the fatty acid supplied, results in a variable alteration in the normal complement of cellular lipids. Moreover, an increased synthetic response is observed when the cells are loaded with 20:4, an essential fatty acid, an effect not seen with 18:1 oleic acid, a nonessential fatty acid. Hence, the previously demonstrated age-dependent accumulation of lipids in general and arachidonic acid in particular in human articular cartilage may be responsible for a heretofore unsuspected alteration in chondrocyte metabolism.

Materials and Methods

Chondrocytes were isolated from the metacarpal-phalangeal joints of fetal calves by a standard collagenase digestion procedure (17). Cell viability was greater than 95%, as assessed by trypan blue dye exclusion. High density primary cultures were established by seeding 60-mm petri dishes with $10–15 \times 10^6$ cells in 10 ml of Ham's F-12 medium containing 10% fetal calf serum, 100 units/ml penicillin, and 50 units/ml streptomycin. Replicate cultures were incubated at 37°C in an atmosphere of 5% CO_2. A preincubation period of 48 hr was allowed for cell attachment.

Experimental cultures were supplemented with 100 μM levels of either arachidonic acid (20:4) or oleic acid (18:1) by replacement of one half the existing medium with freshly prepared medium containing the fatty acid. Subsequent, one-half medium changes were made every 2–3 days for 14 days. Stock fatty acid-supplemented medium was prepared according to Spector *et al.* (18); with slight modification. Briefly, the fatty acid sodium salt was dissolved in Ham's F-12 and complexed with nondelipidized fetal calf serum using mild 35–40°C heating and constant stirring.

Twenty-four hours before the cultures were terminated, each group (control, 18:1, and 20:4) was divided into four subgroups. Group 1 cultures were labeled with 10 μCi/ml [^{3}H]arachidonic acid (sp act, 60–100 Ci/mmol; New England Nuclear) for 24 hr and assayed for distribution of labeled 20:4 into cellular lipids. Group 2 was treated with 5 μg/ml indomethacin (a prostaglandin synthetase inhibitor) for 24 hr before exposure of the cells to 5 μCi/ml $^{35}SO_4$ (sp act, 1200 Ci/mmol plus 5 μCi/ml [^{3}H]proline (sp act, 100 Ci/mmol). Group 3 was identical to Group 2 but without the 24-hr exposure to indomethacin. Lastly, Group 4 was utilized for fatty acid analysis of cellular lipids and radioimmunoassay of culture medium (PGE_2) and leukotriene B_4 (LTB_4) (New England Nuclear Corp. Kit NEK-020 and NET-852). The data were expressed on the basis of μg of DNA/plate, determined by the modified fluorometric method of Setaro and Morley (19).

Lipid Analysis. Confirmation of chondrocyte fatty acid loading with 20:4 and 18:1 supplement was determined by gas chromatography of fatty acid methyl esters. Lipids were extracted from washed and pooled homogenized cells by the method of Slayback *et al.* (20), after addition of β-hydroxytoluene antioxidant to a final concentration of 0.02%. Fatty acids were esterified by reaction with methanolic BF_3 solution (14%, w/v). The esters were separated and quantitated on a Hewlett Packard gas chromatograph equipped with a FID detector and interfaced with a Shimadzu CR1-A integrator. A 6-foot by 0.25-in glass column was prepacked with 10% SP-2330, 100/120-mesh Chromosorb W (Supelco Inc., Bellefonte, PA). Elution was done with a programmed 8-min temperature gradient of 175–200°C. Data were corrected for recovery based on an external standard and expressed as μg of fatty acid/μg of DNA.

Labeled 20:4 distribution into cellular lipids was determined by analysis of Folch-derived extracts of cell homogenates from Group 1. The extracts were subjected to Sep-Pak silica cartridge fractionation (Waters Associates, Waltham, MA) into lipid classes. The phospholipid fraction was further subjected to high-pressure liquid chromatography for isolation of individual phospholipids by the method of Chen and Kou (21), with a normal Ultrasphere 5 -μm 4.6- × 250-mm reverse phase column and an isocratic solvent system of $CHCN_3$/methanol/H_3PO_4 (130:5:1.5) delivered at a rate of 1 ml/min. Fractions monitored at 203 nm were collected at 1-min intervals and labeled 20:4 distribution into individual components was determined by addition of 8 ml of Aquasol to the eluted fractions. Samples were counted in a scintillation counter and individual compounds were identified by reference to elution times for

known standards. Data were expressed as percentage distribution of cpm in the various phospholipids.

Prostaglandin Assays. Medium from Group 4 cultures was extracted for eicosanoids using three volumes of water-saturated ethyl acetate, after acidification to pH 3.0 with 1 *M* citric acid. The assay procedures for PGE_2 and LTB_4 are highly specific, with maximum cross-reactivity of 6% for prostaglandin A_2 in the PGE_2 assay and 3% for the diasteroisomer 5,12-dihydroxy-6,8,10-*trans*-*O*-14 *cis*-eicosatetraenoic acid in the LTB_4 assay.

Metabolic Analysis. Labeled macromolecules in the media of Group 2 and 3 cultures were assayed after centrifugation and precipitation with cold 90% ethanol. Repeated washing of the precipitate was accomplished by resuspension in 90% ethanol and recentrifugation at 3000 rpm for 20 min. The final precipitate was dissolved in phosphate-buffered saline and aliquots were counted by addition of 15 ml of Aquasol II (New England Nuclear Corp., Boston, MA). The cell layer was washed repeatedly with cold saline and scraped and the resulting suspension was hand-homogenized in an all-glass Dounce homogenizer. Aliquots of the homogenate were similarly prepared and counted using Aquasol II. The data were expressed as cpm/μg of DNA.

Results

High density cultures of bovine chondrocytes were successfully loaded with the supplemental fatty acids 18:1 and 20:4 (Table I). Oleic acid addition resulted in a 377% increase in cellular oleic acid, whereas arachidonic acid enriched cell levels by 980%. Interestingly, an examination of the profile of the more common fatty acids in supplemented cells revealed some alterations in distribution and content, compared with control cultures. Enrichment with 18;1 had no effect on fatty acid levels, whereas 20:4 enrichment increased 16:0 palmitic (+218%, $P < 0.025$), 16:1 palmitoleic (+225%, $P < 0.01$), 18:0 stearic (+133%, $P < 0.025$), and 18:1 oleic (+185%, $P < 0.01$) acids. No significant changes in the other fatty acids were detected. Total fatty acid content/μg of DNA was increased by 32% in oleic acid supplements (primarily from increases in oleic acid) and by 172% in 20:4-supplemented cells.

Examination of several indices of metabolism, as indicated by incorporation of labeled precursors, is presented in Table II. Only 20:4 supplementation significantly increased incorporation of both labels (+170% for $^{35}SO_4$ and 54–103% for [^{3}H]proline). Similar effects were seen in the media and cell layer compartments, with perhaps increased stimulation of [^{3}H] proline-labeled material located in the medium. No change was noted in the DNA content in control versus fatty acid-supplemented cells. Preconditioning of 20:4-supplemented cells with indomethacin for 20 hr abolished the fatty acid-stimulatory response. At a dose of 5 μg/ml or less, indomethacin alone has no effect on proteoglycan synthesis (22). Elevated levels of PGE_2, the major eicosanoid synthesized by chondrocytes (23), were found in cultures supplemented with 20:4 (+2413%) and, to a lesser extent, in cultures with added 18:1 (+77%) (Table III). The elevation in PGE_2 seen with 20:4 exposure was expected but the magnitude of the increase was surprising (3.9 pg/μg of DNA). Cultures exposed to 18:1 also synthesized greater amounts of PGE_2 (0.275 pg/μg of DNA), doubling the control value of 0.156 pg/μg of DNA, but still much less than that seen in 20:4-enriched cells. LTB_4 assays were done because leukotrienes are an alternate metabolic path-

Table I. Degree of Chondrocyte Enrichment Following Supplementation with 100 μM 18:1 or 20:4 Fatty Acids[a]

Fatty acid	Supplemented fatty acid					
	Control		Oleic acid		Arachidonic acid	
	μg	%	μg	%	μg	%
16:0	1.1	19	1.6	16	3.5[b]	17
16:1	1.2	20	1.3	14	3.9[b]	19
18:0	2.4	41	2.7	28	5.6[b]	27
18:1	0.7	11	3.1[c]	32	1.9[b]	9
18:2	1.2	2	0.2	2	0.4	2
20:4	0.4	3	0.4	5	4.0[c]	23
Total fatty acids	7.09		9.34		19.25	

[a] Values represent μg of fatty acid/μg of DNA and are expressed as the mean ± SD of triplicate determinations. Significance was determined by Student's *t* test.
[b] Significantly different from controls at $P < 0.01$.
[c] $P < 0.001$.

Table II. Effect of 18:1 and 20:4 Fatty Acids on Incorporation of $^{35}SO_4$ and [^{3}H]Proline by Fetal Bovine Chondrocytes[a]

Label	100 μM 18:1 Oleic acid	100 μM 20:4 Arachidonic acid	100 μM 20:4 + 5 μg/ml indomethacin
$^{35}SO_4$			
Cell layer	−10 ± 21% NS[b]	+170 ± 24% $P < 0.001$	+11 ± 19% NS
Media	+10 ± 19% NS	+169 ± 55% $P < 0.001$	+8 ± 10% NS
[^{3}H]Proline			
Cell layer	−1 ± 16% NS	+54 ± 18% $P < 0.001$	+10 ± 10% NS
Media	+12 ± 12% NS	+103 ± 38% $P < 0.001$	+10 ± 10% NS

[a] Metabolic data calculated as cpm/μg of DNA are expressed as the mean percentage change from control values for nine cultures. Indomethacin data were calculated from three cultures. Significance was determined by Student's two-tailed *t* test. Media counts represent ethanol-precipitable material.
[b] NS, not significant.

Table III. Effect of 18:1 and 20:4 Fatty Acids on PGE_2 and LTB_4 Synthesis by Fetal Bovine Chondrocytes[a]

	PGE_2 (pg/μg DNA)	Multiple of control value	LTB_4 (pg/μg DNA)	Multiple of control value
Control	156 ± 64	—	1.98 ± 0.21	—
100 μM 20:4	3918 ± 780 $P < 0.001$	24.1	0.62 ± 0.11 $P < 0.005$	0.32
100 μM 18:1	275 ± 89 $P < 0.025$	1.77	1.08 ± 0.04 $P < 0.005$	0.54

[a] Values represent mean ± SD for nine cultures (PGE_2) or three cultures (LTB_4). Significance was determined by Student's two-tailed *t* test.

way for 20:4 metabolism and recent evidence confirms their presence in normal chondrocytes (24). The levels we detected were in the less sensitive and nonaccurate range of the assay and, although not altogether reliable, suggest a lower level than control values (Table III).

The substantial increase in 20:4 levels obtained with exogenous supplementation prompted an analysis of its distribution into various lipid classes. Group 1 cultures labeled with tritiated 20:4 were analyzed by gas chromatography and high-pressure liquid chromatography, as described. Distribution of tritium label in control cultures was predominantly in phospholipids (90%) but 20:4 enrichment increased the distribution into neutral lipids and free fatty acids (+950%) and prostaglandins (+165%), while decreasing label in the phospholipid fraction (−32%) (Table IV).

Further characterization of radiolabeled 20:4 distribution into phospholipids revealed that 20:4 enrichment slightly increased 20:4 uptake into phosphotidylserine and slightly decreased 20:4 uptake into phosphotidylethanolamine (Table V).

Discussion

High density cultures of fetal bovine articular chondrocytes readily and somewhat selectively take up supplemental fatty acids. As a consequence of 20:4 enrichment, PGE_2 is elevated by several orders of magnitude and matrix synthesis is accelerated, effects not seen with 18:1 supplementation.

Arachidonic acid is not a major constituent of mammalian cell lipids like palmitic (16:0), stearic (18:0), or oleic (18:1) acids. However, it was specifically chosen for this study because it is an essential fatty acid, a major component of cell membranes, the precursor to the series of prostaglandins, and an important component of the second messenger diacylglycerol (25). Arachidonic acid has also been shown to accumulate in aged human articular cartilage (1). Oleic acid is a monounsaturated fatty acid that is endogenously synthesized, is present in cells at high levels, and is a nonprostaglandin precursor. The magnitude of accumulation of 18:1 and 20:4 and their effects on levels of endogenously synthesized fatty acids differed, even though it is generally recognized that regulatory controls in cells are capable of maintaining a relatively normal fatty acid lipid profile. Such control has been shown in other studies not to be effective when the supplemented fatty acids are odd-numbered chain saturated or polyunsaturated fatty acids (26). Alterations of fatty acid synthesis in cultured cells by exposure to medium containing lipids has been a useful technique in studies of lipid nutrition. Such alterations arise from an effect on either reducing activities of acetylcoenzyme A carboxylase or fatty acid synthetase (26).

Distribution of 20:4 into cellular lipid fractions is a function of the fatty acid concentration. At low levels (5 n*M* to 1 μ*M*) available 20:4 binds to phospholipids, but at levels greater than 20 μ*M* esterification shifts to favor triacylglycerols (27). The neutral lipid fraction of cells (triacylglycerols) is a storage form of fatty acids

Table IV. Effect of 20:4 Fatty Acid on Tritiated Arachidonic Acid Distribution into Lipid Classes[a]

Lipid class	Control		100 μM 20:4	
	cpm	%	cpm	%
Neutral/triacylglycerols	3025 ± 450	2.2	13,495 ± 1845 $P < 0.001$	23.2
Prostaglandins	6640 ± 910	4.9	7480 ± 235 $P < 0.001$	12.9
Glycolipid	2675 ± 300	2.0	1015 ± 720 NS[b]	1.8
Phospholipids	124,560 ± 14,150	91.0	36,135 ± 3325 $P < 0.05$	62.2

[a] Values represent mean ± SD of cpm for determinations in three cultures. Significance was determined by Student's two-tailed *t* test.
[b] NS, not significant.

Table V. Effect of 20:4 Arachidonic Acid on Tritiated Arachidonic Acid Distribution into Phospholipids[a]

Lipid	Control		100 μM 20:4	
	cpm	%	cpm	%
PI	2213 ± 210	24.8	562 ± 28 NS[b]	24.1
PS	144 ± 22	1.6	69 ± 6 $P < 0.005$	3.0
PE	2000 ± 96	22.4	411 ± 25 $P < 0.05$	17.7
PC	4367 ± 277	48.9	1200 ± 140 NS	51.6

[a] PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine. Values represent mean ± SD of cpm in three cultures. Significance was determined by comparison of percentage of distribution using the Student's two-tailed t test.
[b] NS, not significant.

that can be used either as an energy source or for membrane lipid synthesis. Free fatty acids are the preferred energy substrate for myocardium and, similarly, fatty acids are proposed to be the preferred substrate for chondrocytes (28). Hence, accumulation of fatty acids would be expected to "vitalize" an energy-poor cell. In this study, 20:4 but not 18:1 altered cell metabolism, suggesting that a simple explanation of increased energy substrate may not be the mechanism by which metabolism has been altered.

Accumulation of fatty acids and/or lipids by chondrocytes is a well-documented phenomenon. Information on the consequences of 20:4 accumulation in chondrocytes is lacking but the data presented here strongly suggest that cell metabolism may be significantly altered. It is generally recognized that myocardial metabolism of 20:4 is of major importance in pathology of cardiac muscle (28). In a similar study, Kirkpatrick *et al.* (29, 30) suggest that excessive lipid accumulation (arachidonic acid) is not toxic to chondrocytes but that nonlethal accumulation of 20:4 inhibits proteoglycan synthesis. These studies used from 1 to 100 nM levels of supplemented 20:4, and levels of prostaglandins resulting from this exposure or the degree of chondrocyte enrichment were not measured. Because both stimulatory and inhibitory effects have been observed in systems where exogenous prostaglandins have been added, the differences observed in our study may be related to a dose-dependency function that prostaglandins have on cells (24, 31) or variations in biologic effect between exogenously supplied prostaglandins and those produced endogenously. Most prostaglandin-mediated metabolic effects, including those in chondrocytes, have been reported as being a function of induced changes in cellular cAMP levels (32).

There is little doubt that lipids appear in old or injured cells (fatty degeneration/infiltration), but there is little indication of their origin or why they appear. Studies of this process in cultured L cells suggest a relationship between aging and a decrease in cell division, with accumulation of lipids and retardation of protein synthesis (33). Metabolic inhibitors also effect an increase in cell lipid content (34). Hence, there is little doubt that it is possible to produce an accumulation of lipid in cells by interfering with growth and protein synthesis. In contrast to these data, it has also been demonstrated that the appearance of lipid is not a reliable indicator, per se, of aging, retarded growth, or degeneration (27). In fact, lipid accumulation has been associated with rabbit ear cartilage regeneration (35), and increases in lipid content have been found in active chondrocytes in tissues showing no signs of degeneration in the early stages of the Pond and Nuki model of osteoarthrosis (10). It has been suggested that fatty acids exert both growth-promotion and growth-inhibiting activity in other cells depending on their concentration (36).

We interpret these data to indicate that chondrocytes, *in vitro*, can readily and somewhat selectively accumulate fatty acids. In the case of 20:4, such accumulation can have substantial metabolic consequences on prostaglandin synthesis and sulfate incorporation into ethanol-precipitable material. Previous observations indicate that lipids, as well as 20:4, accumulate in aged articular cartilage and may have profound consequences on the health and viability of the tissue. Extending these observations further, the increased metabolic activity specifically associated with 20:4 accumulation may have a protective effect in articular cartilage. In fact, studies by Silberberg *et al.* (14) support this view. Addition of 3% linoleic acid or corn oil (a rich source of essential fatty acids) prevented the injurious action that a high saturated fatty acid diet had on articular aging changes. If the results of accumulation of 20:4 *in vivo* mimic the increased synthetic activity observed *in vitro*, such activity may have a protective effect on viability of aging chondrocytes.

Additional support for the requirement of arachidonic acid in articular cartilage homeostasis can be derived from studies where 20:4 is displaced from cell lipids by exposure to ω-3 fatty acids. In a recently reported study, such exposure resulted in a significant inhibition of metabolic activity and alteration in cell ultrastructure (37).

In contrast to the beneficial effect of essential fatty acids, the relationship between lipid deposition and articular cartilage degeneration has also been alluded to in the introductory paragraphs. There is ample opportunity for cartilage to be exposed to increased lipid concentrations in synovial fluid. In instances of joint trauma and rheumatoid arthritis, increased levels of lipid material have been documented (38, 39). Intracellular lipid accumulation resulting from tissue exposure to a traumatic environment is a characteristic

phenomenon of other cells as well and cell protection against such accumulation does not appear to exist. Because articular cartilage appears to have an avidity for lipids, the resultant effects on metabolism (protective or deleterious) would appear to be dependent on the type of fatty acid accumulated.

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