Osteoarthritis and Cartilage (2009) **17,** 896–905

doi:10.1016/j.joca.2008.12.009

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Relative efficacies of omega-3 polyunsaturated fatty acids in reducing expression of key proteins in a model system for studying osteoarthritis

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Summary

Objective: To assess the relative efficacy of three different omega-3 (n-3) polyunsaturated fatty acids (PUFAs) in suppressing the mRNA levels for important proteins involved in the etiology of osteoarthritis (OA).

Methods: A model cell culture system (bovine chondrocytes) was used. Inflammatory factors and enzymes involved in OA were induced by exposure of the chondrocyte cultures to interleukin-1α (IL-1α). The effect of pre-incubating cultures with various amounts of exogenous fatty acids on subsequent levels of mRNAs was assessed by reverse transcription-polymerase chain reactions (RT-PCR).

Results: Exposure of cultures to IL-1α induced expression of the cartilage proteinases A Disintegrin And Metalloproteinase with ThromboSpondin motifs (ADAMTS)-4 and ADAMTS-5, cyclooxygenase (COX)-2, the matrix metalloproteinase (MMP)-3 and the inflammatory cytokines IL-1α, interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α). n-3 PUFAs were able to reduce the levels of mRNA for ADAMTS-4, ADAMTS-5, MMP-3, MMP-13, COX-2 (but not COX-1), IL-1α, IL-1β and TNF-α. Eicosapentaenoic acid (EPA) was the most effective, followed by docosahexaenoic (DHA) and then α-linolenic (ALA) acid. The n-6 PUFA, arachidonic acid (AA) had no effect.

Conclusion: These results show that omega-3 (n-3) PUFAs cause a reduction in the mRNA levels for various proteins known to be important in the pathology of OA. They provide a molecular explanation, at least in part, for beneficial effects of dietary omega-3 PUFAs for the amelioration of symptoms of the disease. The relative efficacy of EPA suggests that this omega-3 PUFA may be especially useful for dietary supplementation in patients with OA.

Key words: Osteoarthritis, Chondrocyte cultures, Omega-3(n-3) polyunsaturated fatty acids, Inflammation, Proteinases.

Introduction

Osteoarthritis (OA) is a common, chronic disease, characterised by joint inflammation and, in early stages, increased catabolism of the proteoglycan aggrecan, followed by later loss of type II collagen which cumulatively leads to destruction of the joint cartilage[1]. Primary OA occurs in middle aged or elderly patients and is presumed to reflect joint 'wear and tear'. It remains a major health problem of the elderly[2]. Secondary OA occurs at any age as a result of trauma or disease[1]. One of the major causes of secondary OA is biomechanical joint instability, leading to biochemical changes within the extracellular matrices of the joint tissues[3]. A central feature is loss of articular cartilage (caused by increased matrix degradation) and a reduced capacity for repair. The cyclic disease course of OA has been proposed to be due to sequential cytokine effects elevating proteinase activity with the pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) important[1] in these metabolic processes.

Two families of metalloproteinases, matrix metalloproteinases (MMPs) and A Disintegrin And Metalloproteinase with ThromboSpondin motifs (ADAMTSs,) degrade the major components of the cartilage matrix, namely aggrecan and type II collagen[4]. The major site for cleavage of aggrecan is the Glu^{373}-Ala^{374} bond within the interglobular domain[1] of the aggrecan core protein which is hydrolysed by aggrecanases[5,6]. It is mainly cleavage at this site that is responsible for the increased aggrecan degradation seen in inflammatory joint disease[7]. The catabolites have been found in the synovial fluid of patients with OA and accumulate in model experimental systems (cartilage explants or chondrocyte cultures) when challenged with pro-inflammatory cytokines such as IL-1[8]. The two aggrecanases that act at the Glu^{373}-Ala^{374} site, and that have been isolated from articular cartilage culture systems mimicking the degradation occurring in OA, are, ADAMTS-4 and ADAMTS-5[9]. The relative involvement of these enzymes in the pathology of OA is highly controversial, particularly because ADAMTS-4-null mice did not exhibit protective effects on cartilage aggrecan loss compared with wild-type mice in two separate osteoarthritic models[10–12]. This contrasted with ADAMTS-5-null mice in which the same models showed that lack of ADAMTS-5 protected against cartilage breakdown[11,12]. However, previous publications have shown that ADAMTS-4 mRNA is more easily elevated in chondrocytes treated with IL-1 than ADAMTS-5. Contradictory evidence for the involvement of these two enzymes is also found in the relative activity of recombinant enzyme preparations on the native substrate aggrecan[13,14]. After the initial attack on aggrecan by aggrecanases, further degradation proceeds by MMP action. Two MMPs appear to be particularly important in OA, the stromelysin MMP-3 and the collagenase, MMP-13[15–17]. MMP-3, in particular,

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Received 17 June 2008; revision accepted 10 December 2008.

is readily induced by inflammatory cytokines in chondrocytes or connective tissue fibroblasts[18]. MMP-13 is chiefly involved in the late-stage catabolism of type II collagen.

Dietary polyunsaturated fatty acids (PUFAs) of both the n-3 and the n-6 series are essential for human health. However, in general these two types of PUFA have opposite effects on inflammatory responses. Thus, n-6 PUFAs give rise to inflammatory eicosanoids while n-3 PUFAs are generally anti-inflammatory[19,20] either because their eicosanoid products have this property or because they compete with n-6 PUFAs during metabolism[21]. Moreover, recently, new classes of anti-inflammatory lipid mediators have been shown to be formed from n-3 PUFAs such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids[22].

Diets containing significant levels of EPA and DHA have been shown consistently to reduce joint stiffness and tenderness in arthritic patients (see[23,24]). Fish oils are important sources of EPA and DHA and increased dietary levels are recommended for patients with arthritis to alleviate pain and inflammation in their joints[19]. Indeed, such n-3 PUFAs have been shown to also reduce the production of inflammatory cytokines such as IL-1 or TNF-α[19]. The latter cytokines have been shown to stimulate the inducible cyclooxygenase (COX), COX-2[25] which is thought responsible for the pain experienced in arthritis[26]. The non-steroidal anti-inflammatory drugs (NSAIDs), which are commonly used to relieve pain in arthritis, unfortunately inhibit both COX-2 and the constitutive COX-1[27] and, therefore, have side-effects[28,29]. However, new COX-2 selective inhibitors which were developed to obviate the known side-effects of NSAIDs have been withdrawn from the market due to safety concerns about the increased risk of cardiovascular events[30].

There have been considerable discussions about the relative efficacy of different n-3 (omega-3) PUFAs for people at risk of (or suffering from) different chronic inflammatory diseases. Although α-linolenic acid (ALA) is often the main dietary n-3 PUFA, it is rather poorly converted to EPA, the direct precursor of eicosanoids[31]. Therefore, most dietary advice specifies EPA and/or DHA (see e.g.,[32]), particularly for cardiovascular problems. In other cases such as for the alleviation of Alzheimer's disease, DHA seems more effective than EPA[33]. In most intervention trials for arthritis, both EPA and DHA have been used while in epidemiological surveys all common types of n-3 PUFAs were present in the diet. Therefore, the relative efficacy of different omega-3 PUFAs for arthritis is not known.

In the work reported here a model cell culture system was used in which chondrocytes were pre-incubated with or without different fatty acid preparations prior to exposure to IL-1 (a cytokine known to induce catabolic changes in chondrocytes). After exposure, the direct effects of different n-3 PUFAs on relative message levels for inflammatory markers and proteinases involved in the catabolism of extracellular matrix molecules degraded during the onset and progression of OA were tested. This system has also allowed us to compare directly the relative efficiency of the three main dietary n-3 PUFAs in beginning to understand the mechanism by which n-3 PUFAs can alleviate the underlying causes of chronic cartilage destruction in arthritis. The data show clearly that all n-3 PUFAs are capable, at least at the mRNA level, of reducing the inflammatory consequences caused by IL-1 challenge to the tissue, with EPA being the most effective.

Materials and methods

MATERIALS

Clostridium histolyticum collagenase Type II, was from Worthington Biochem. Corp. (NJ 08701, USA), pronase (*Streptomyces griseus*) from Boehringer (Poole, Dorset, UK) and Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) from Gibco-BRL (now Invitrogen, Paisley

Table I
Oligonucleotide primers used for RT-PCR

Target template	PCR primer sequences (5′–3′)	Product size (bp) (bovine)	Annealing temperature (°C)
GAPDH	5′-TGGCATCGTGGAAGGGCTCAT 5′-ATGGGAGTTGCTGTTGAAGTC	370	50.0
IL-1α	5′-AAGGAGAATGTGGTGATGGTG 5′-CAGAAGAAGAGGAGGTTGGTC	470	53.2
IL-1β	5′-GCTCTCCACCTCCTCTCACAG 5′-TACATTCTTCCCTTCCCTTCT	454	54.5
TNF-α	5′-CTCAAGCCTCAAGTAACAAGC 5′-GCAATGATCCCAAAGTAGACC	454	57.6
COX-1	5′-GCCCAACACTTCACCCATCAG 5′-CCAGGAAGCAGCCCAAACACT	287	59.0
COX-2	5′-GCTCTTCCTCCTGTGCCTGAT 5′-CATGGTTCTTTCCCTTAGTGA	229	52.3
ADAMTS-4	5′-TGGATCCTGAGGAGCCCTG 5′-TGGCGGTCAGCATCATAGTC	151	55.5
ADAMTS-5	5′-GGCCTCTCCCATGAYGATTCC 5′-TGAGCGAGAACACTGGCCCCA	498	59.2
Aggrecan	5′-CGCTATGACGCCATCTGCTAC 5′-GCCTGCTTGGCCTCCTCAAA	197	57.0
Collagen type II	5′-GAATTCGGTGTGGACATAGG 5′-TACAGAGGTGTTTGACACAG	429	53.0
MMP-3	5′-CTTTTGGCGAAAATCTCTCAG 5′-AAAGAAACCCAAATGCTTCAA	404	50.0
MMP-13	5′-TTCTGGCACACGCTTTTCCTC 5′-GGTTGGGGTCTTCATCTCCTG	273	53.0
TIMP-1	5′-CCACCTTATACCAGCGTTAT 5′-CCTCACAGCCAACAGTGTAGG	282	54.0
TIMP-2	5′-GTGGACTCTGGAAACGACAT 5′-TCTTCTTCTGGGTGGTGCTCA	265	54.0
TIMP-3	5′-GGGAAGAAGCTGGTAAAGGAG	418	54.0

PA4 9RF, UK). Fatty acids were from Nu-Chek Prep. (Elysian, MN 56028, USA) and recombinant human interleukin-1α (IL-1α) from Totam Biological (Peterborough, PE1 5TX, UK). Other chemicals (best available grades) were from Sigma-Aldrich Co. (Poole, Dorset SP8 4XT, UK) or from Boehringer–Mannheim (now Roche Diagnostics Ltd., Burgess Hill RH15 9RY, UK).

ISOLATION AND CULTURE OF BOVINE CHONDROCYTES

Bovine metacarpo – and metatarsophalengeal joints (7 day-old calves) were obtained from the local abattoir. Cartilage was removed from the joint surfaces under sterile conditions and digested in 0.1% (w/v) pronase in DMEM containing 5% (v/v) FCS for 3 h at 37°C with roller agitation. It was further digested with 0.04% (w/v) collagenase in DMEM with 5% (v/v) FCS overnight at 37°C with roller agitation. After 24 h, cells were separated through a 40 μm Nitex filter (Falcon, Oxford OX4 3LY, UK) before cell numbers were estimated by microscopy. Monolayer cultures were established in 60 mm dishes at 6×10^6 cells (final density of approximately 20×10^5 cells/cm^2) in 4 ml DMEM containing 50 μg/ml gentamicin. Cells were maintained at 37°C under 5% (v/v) CO_2 for approximately 6 h to allow the chondrocytes to adhere to the tissue culture plastic prior to the addition of experimental components.

Fatty acids to be added were made up in a complex with defatted bovine serum albumin (BSA). 300 μg fatty/ml medium (20 mM HEPES, 140 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM glucose, 3.5 mg BSA, pH 7.4) was incubated overnight (16 h) at 37°C under nitrogen. After incubation, the mixture was filtered through a Sartorious (0.2 μm pore size) Minisart filter unit (Sartorius Ltd., Epsom KT19 9QQ, UK) to sterilise the solution and ensure that any unbound, excess fatty acids were removed. Gas liquid chromatography (GLC) analysis was made to quantify the fatty acid – BSA complex and was found necessary, as samples could vary significantly in their concentration of fatty acid after filtration.

The fatty acid-BSA complex was added to the adhered chondrocytes to give final concentrations of 2.5–30 μg/ml fatty acids. A number of cultures were left as controls (i.e., no addition of fatty acids). Cultures were maintained for 8 h (pre-incubation) in the absence or presence of fatty acids to allow for uptake into cells and incorporation into membrane lipids. After 8 h pre-incubation, the fatty acid media was removed and the cultures were carefully washed three times with DMEM containing 50 μg/ml gentimicin. Following this, DMEM (containing 50 μg/ml gentimicin) was added either with or without 10 ng/ml IL-1α and the cultures incubated for a further 96 h.

METABOLISM OF EXPERIMENTAL CHONDROCYTE CULTURES AS MEASURED BY LACTATE PRODUCTION

Lactate production was used as a measure of the metabolic activity of viable chondrocytes under the different experimental conditions. Lactate concentrations in the culture media were quantified with a lactate assay kit (Sigma–Aldrich) used according to the manufacturer's instructions.

LIPID ANALYSIS

Chondrocyte monolayers were extracted by a method[34] which ensures quantitative recovery even of very polar membrane lipids. The extracted lipids were transmethylated using 2.5% (v/v) H_2SO_4 in dry methanol/toluene (2:1, v/v) for 2 h at 70°C. After extraction into petroleum ether, the fatty acid methyl esters were separated with 10% SP-2330 on 100/120 Supelcoport

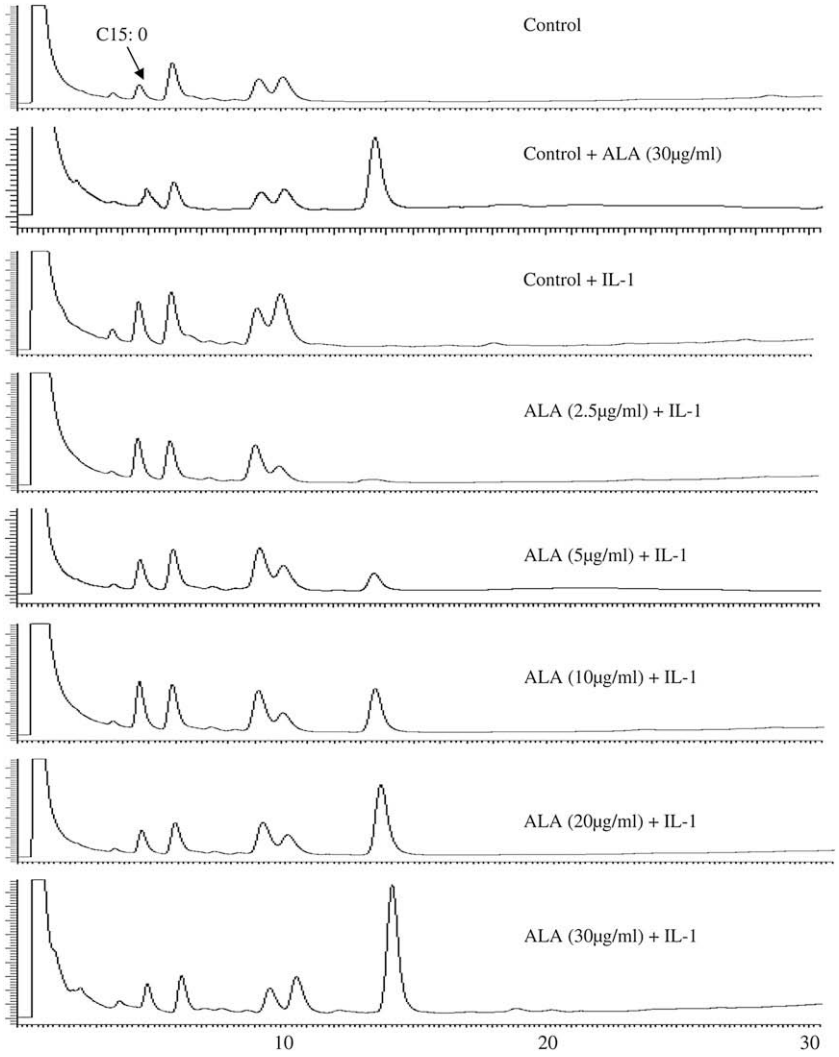

Fig. 1. Uptake of α-linolenate into chondrocyte cultures. GLC traces are shown with control and IL-1α treated cultures and increasing amounts of α-linolenate added. See Materials and methods for procedures. Incubations with added linolenate were for 8 h.

(Supelco) using a 1.5 m × 0.4 mm column at 185°C with a Perkin–Elmer autosystem XL gas chromatograph. Fatty acid methyl ester(s) (FAMEs) were identified using standard mixtures (Nu-Chek) and quantification performed using Perkin–Elmer internal software and a pentadecanoate internal standard.

RNA ISOLATION, RT-PCR AND SEMI-QUANTIFICATION OF MESSAGE LEVELS BY DENSITOMETRIC ANALYSIS OF ETHIDIUM BROMIDE-STAINED GELS

For the amplification of cDNA, total RNA was isolated from chondrocyte cultures using Tri-reagent (1 ml/60 mm dish). 200 μl of $CHCl_3$ was added, inverted and left to stand for 15 min at room temperature, prior to centrifugation at 13,200 rpm for 15 min. The upper aqueous phase was removed and mixed with an equal volume of 70% (v/v) ethanol and mRNA was isolated using the RNeasy miniprep kit and reagents (Qiagen Ltd., Crawley, UK) according to the manufacturer's protocol. Endogenous DNA was removed using an on-column digest with DNase I (80 μl, Qiagen). Purified RNA was eluted in RNase-free water. Reverse transcription-polymerase chain reactions (RT-PCR) were performed using an RNA PCR core kit (Applied Biosystems). Typically, a concentration of 500 ng of RNA was used for cDNA amplification. Primers used, along with annealing temperatures are listed in Table I. The RT reaction mix was subjected to a cycle of 42°C for 30 min, 99°C for 5 min and soaked at 4°C. PCR cycling parameters were as follows: initial denaturation at 95°C for 60 s, amplification at 95°C for 30 s, 45 s at the specified annealing temperature of the primers, 72°C for 45 s (X40) and a final extension step of 5 min at 72°C.

Following PCR, the reaction products and a DNA ladder of standards were size fractionated on 2% (w/v) agarose gels containing ethidium bromide. Bands were visualised under UV light, photographed and stored as image files

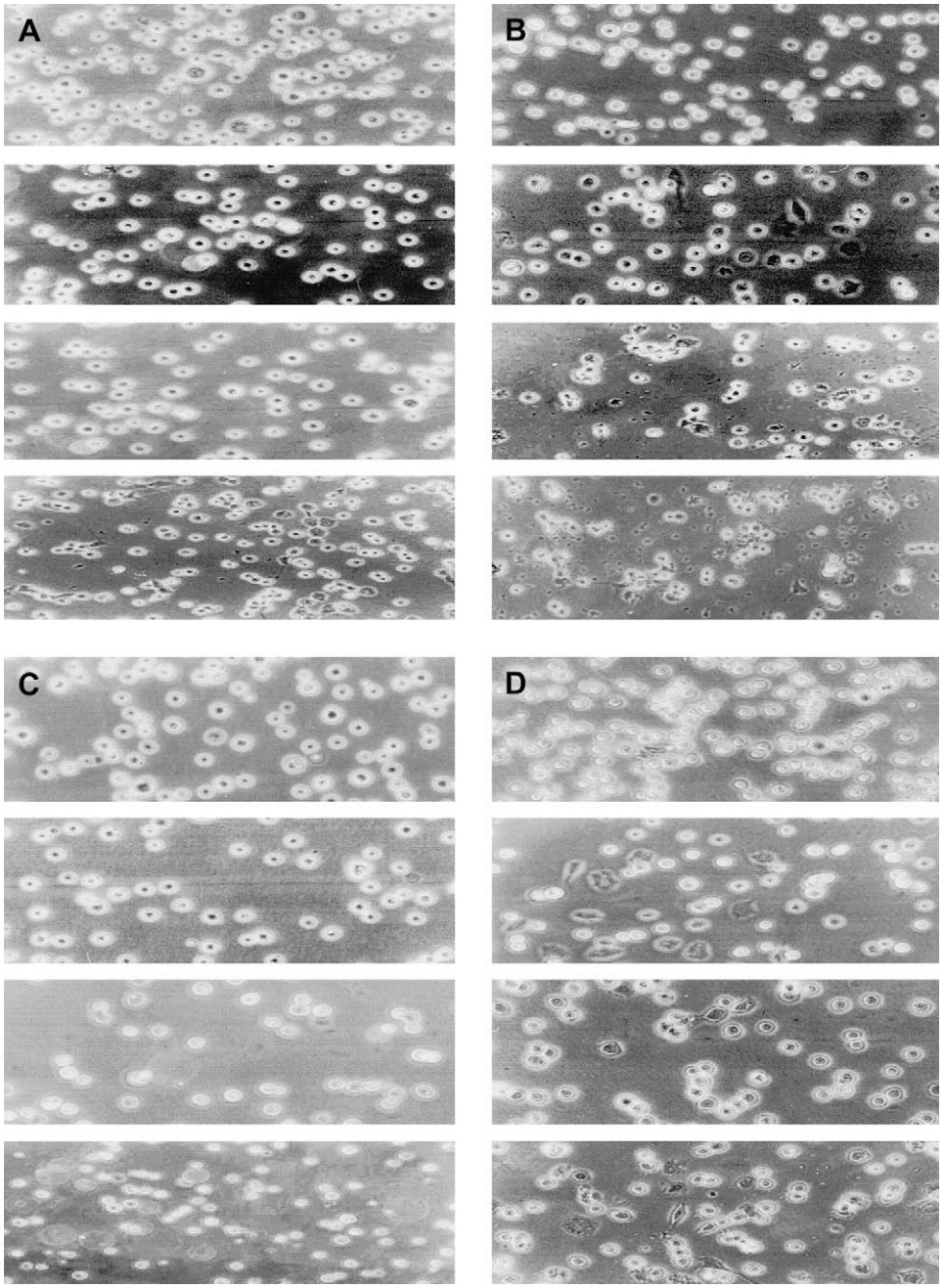

Fig. 2. Morphology of chondrocytes in culture. Pictures were taken by light microscopy of control cells (A), cells treated with IL-1α (B), cells pre-incubated with 10 μg/ml EPA followed by IL-1α treatment (C) and cells pre-incubated with 30 μg/ml EPA followed by IL-1α treatment (D). Time points represent 0, 24, 48 and 72 h treatment, respectively.

for further analysis. Individual gels ($n = 3$) were scanned using Kodak Digital IB Image Analysis apparatus to provide densitometric values for each of the PCR products of interest. These densitometric values were normalised to the densitometric values obtained for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (used as a constitutive gene) from the same cDNA samples.

Results

UPTAKE OF EXOGENOUS FATTY ACIDS

In experiments with various tissues where fatty acid supplementation is required, the fatty acids are usually added where complexed to serum albumin, since this is the way in which they are carried within the body. Such *in vitro* experiments have generally not reported tissue damage provided that the levels of fatty acids added are not excessive. Although joint connective tissue is avascular, it receives nutrients indirectly from the blood. Recently the transport of fatty acids from albumin complexes has been demonstrated and the mechanism studied. These experiments showed clearly that fatty acids moved through the cartilage matrix at measurable rates[35]. Therefore, fatty acid-BSA mixtures were used in our experiments.

Preliminary experiments indicated that 8 h was sufficient time for uptake of fatty acids into chondrocytes to reach equilibrium and would, therefore, be appropriate for further experiments. Uptake into chondrocytes was examined with different fatty acids (ALA, EPA, DHA and arachidonic acid [AA]) and, at different concentrations, uptake was proportional to the exogenous concentration added and there was no detectable difference in the total uptake between the four fatty acids[36]. A typical analysis (for ALA) is shown in Fig. 1. The data showed that fatty acids were efficiently taken up by the chondrocyte cultures in a dose-dependent manner and it was, therefore, possible to test for the effects of different concentrations of added fatty acids on the expression levels of inflammatory mediators and proteinases up-regulated in chondrocytes by IL-1.

CHONDROCYTE MORPHOLOGY AND METABOLIC ACTIVITY

The morphology of the chondrocytes was examined using light microscopy to determine if the cells maintained the typical rounded chondrocytic morphology and did not become fibroblastic-like during the experimental culture period. Following the pre-incubation period (8 h) of chondrocytes in the presence or absence of fatty acid, a typical spherical morphology was observed [Fig. 2(A–D), Plate 1]. Following the experimental culture period, this morphological appearance remained the same for control cultures maintained in serum-free media [Fig. 2(A), Plate 2–4]. However, chondrocytes challenged with 10 ng/ml IL-1α [Fig. 2(B), Plate 2–4] changed shape considerably over the experimental culture period of 96 h. IL-1 treated cells were heterogeneous and by 72 h their cell shapes became more spindle-like, sharp or stillate, possibly as a result of the heterogeneity reported amongst articular chondrocytes[37]. In addition, cell debris and cell death could be seen at 48 h [Fig. 2(B), Plate 3] with accumulation occurring through the remaining culture period. In contrast, chondrocyte cultures pre-treated with either 10 or 30 μg/ml EPA prior to the addition of IL-1 [Fig. 2(C and D)] remained as morphologically rounded cells following 48 h of IL-1 treatment. At 72 h there was an appearance of fibroblastic-like cells, cell debris and cell death. However, these features were markedly reduced compared to those seen in the IL-1 only cultures suggesting that the pre-incubation of chondrocytes with the n-3 PUFA, EPA has a protective effect from some of the adverse effects of the inflammatory cytokine, IL-1α.

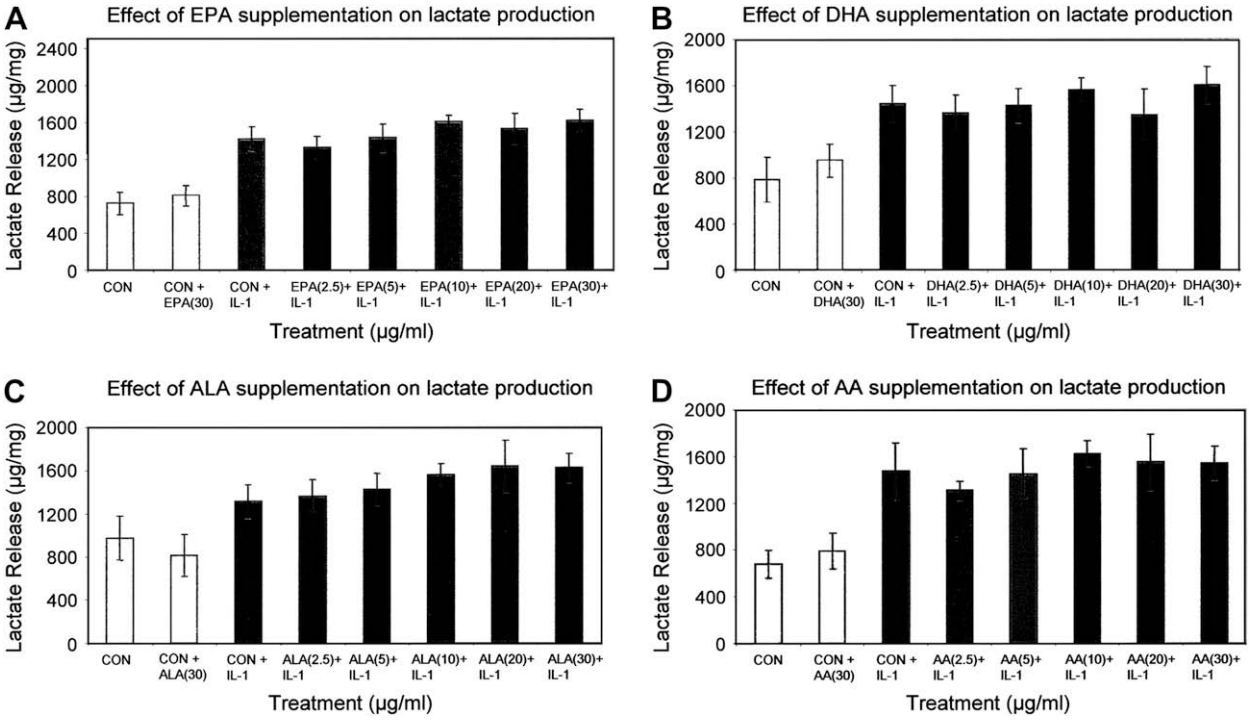

Fig. 3. Chondrocyte metabolism as measured by lactate production. Lactate release into serum-free culture medium with or without IL-1 was measured after the experimental period of 96 h. Cultures were either pre-treated with or without fatty acids or BSA solution alone (as labelled in A–D). Means $\pm$ S.D. are shown ($n = 3$).

Lactate production by chondrocytes under control conditions (serum-free media containing no IL-1) for 96 h was unaffected by an 8 h pre-incubation with fatty acids in a concentration range of 2.5–30 μg/ml. Figure 3, (open bars) shows the lactate concentrations obtained for control cultures and cultures pre-treated with 30 μg/ml fatty acid. However, during the experimental period (96 h) challenge with IL-1α increased lactate production and this increased amount was not altered by pre-incubation with any of the fatty acids [Fig. 3(A–D), solid bars]. These results show that general metabolism, at least as measured by lactate production, was not affected by addition of any of the fatty acids used.

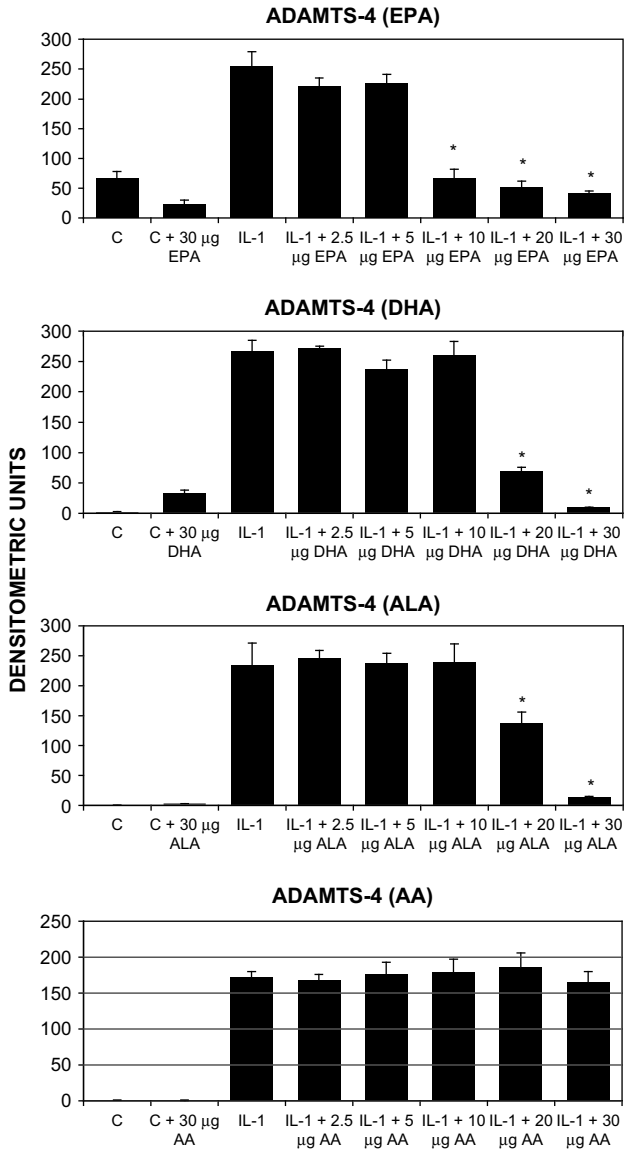

Fig. 4. Variable effects of different PUFAs on mRNA levels for the proteinase ADAMTS-4. For procedures see Materials and methods. (A) shows RT-PCRs for ADAMTS-4 whose expression is induced by IL-1α and reduced by n-3 PUFAs. Pre-exposure to fatty acids or BSA alone (controls) was for 8 h and incubation with or without 10 ng/ml IL-1α was for 4 days. The histogram shows data from scans of gels from three separate experiments with means ± S.D. given. Individual lanes show (L to R): controls (C), controls +30 μg/ml fatty acid (FA), +IL-1α, +IL-1α and 2.5 μg/ml FA, +IL-α and 5 μg/ml FA, +IL-1α and 10 μg/ml FA, +20 μg/ml FA, +IL-1α and 30 μg/ml FA. * indicate significantly different from +IL-1α alone ($P < 0.05$) by Student's t test.

EFFECTS OF FATTY ACIDS ON EXPRESSION OF CARTILAGE MATRIX PROTEINASES

As discussed earlier, the initial cleavage of aggrecan during cartilage damage in arthritis is thought to be due to the activity of ADAMTS-4 and/or ADAMTS-5. Arbitrary densitometric values were obtained from gels using three separate cultures of each experimental condition and these values were normalised to densitometric values obtained for the reference gene GAPDH by PCR of cDNA from the same experimental cultures. For ADAMTS-4 this analysis is shown in Fig. 4 (Panel I, EPA, II, DHA, III, ALA, IV, AA) where it can be seen in all control cultures following 96 h in serum free medium there was negligible mRNA levels and furthermore, the addition of fatty acids for an 8 h period prior to the 96 h experimental time in the form of EPA, DHA, ALA or AA did not alter the expression level of ADAMTS-4. However, after a 96 h exposure of control cultures to IL-1 expression of ADAMTS-4 was increased. In contrast, the IL-1 induced mRNA levels following pre-exposure to n-3 PUFAs were reduced in the range 10–30 μg/ml EPA, 20–30 μg/ml DHA and 30 μg/ml ALA. The n-6 PUFA AA had no detectable effect in

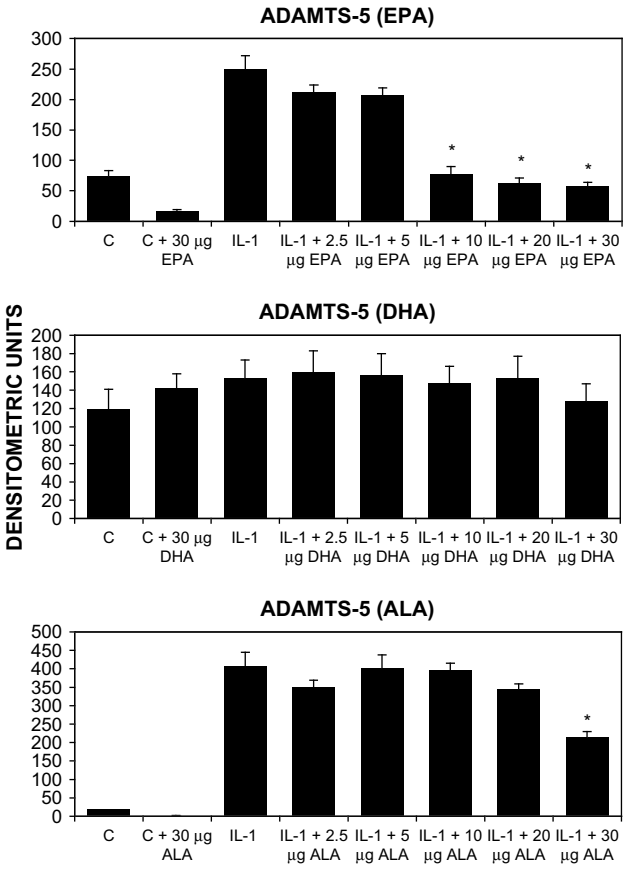

Fig. 5. Effects of exogenous PUFAs on mRNA levels for the proteinase ADAMTS-5. For procedures see Materials and methods. The results for three experiments (means ± S.D.) are shown with EPA, DHA or ALA being compared. See legend to Fig. 4 for details.

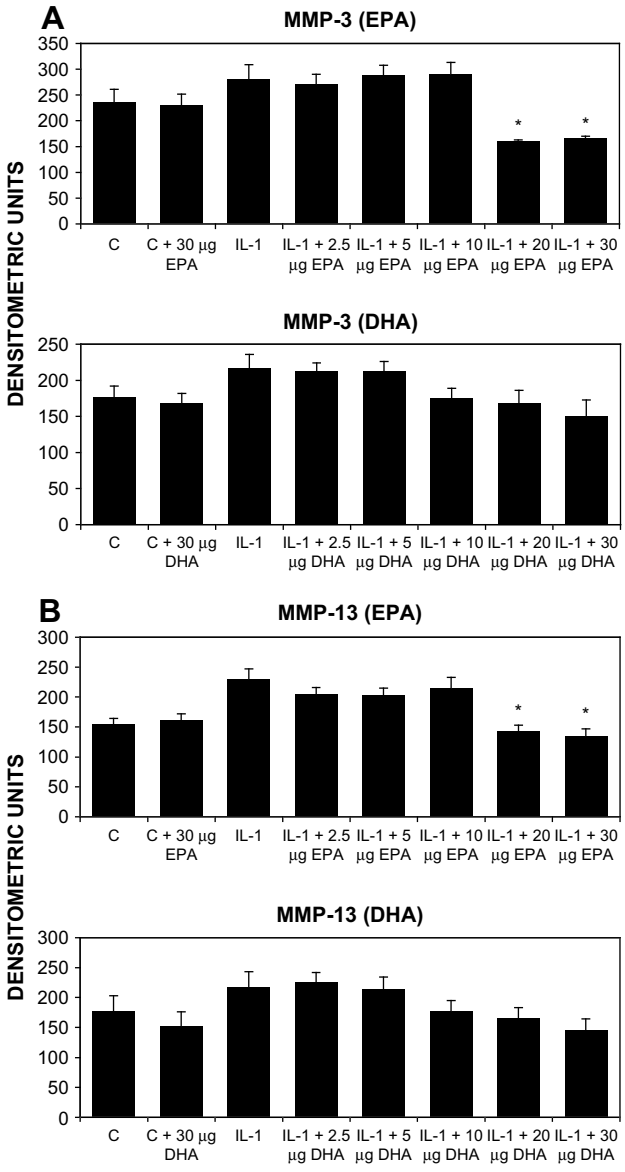

Fig. 6. Effects of exogenous fatty acids on mRNA levels for the MMP-3 and MMP-13. (A) shows results for MMP-3 and (B) data for MMP-13. Exposure to EPA or DHA was for 8 h and incubations with or without 10 ng/ml IL-1α were for 4 days. Abbreviations and further details as for Fig. 4.

the experiments [Fig. 4, Panel IV]. Indeed, this acid did not alter IL-1 induced mRNA levels for any gene tested. Therefore, these data are omitted from Figs. 5–8.

In separate experiments a similar study was carried out to examine the effects of pre-culture of chondrocytes with n-3 PUFAs on the expression levels of ADAMTS-5 following exposure to IL-1. ADAMTS-5, expression in control cultures (having no exposure to fatty acids) was very variable. In some cultures it was constitutively expressed [Fig. 5, Panel II] whilst in others there appeared to be little or no expression [Fig. 5, Panel I & III]. However, the pre-treatment of these chondrocyte cultures expressing high levels of ADAMTS-5 [Fig. 5, Panel II] with n-3 PUFAs maintained this level of expression of ADAMTS-5. In those control cultures showing little expression of ADAMTS-5, the addition of IL-1 caused a significant increase in the expression

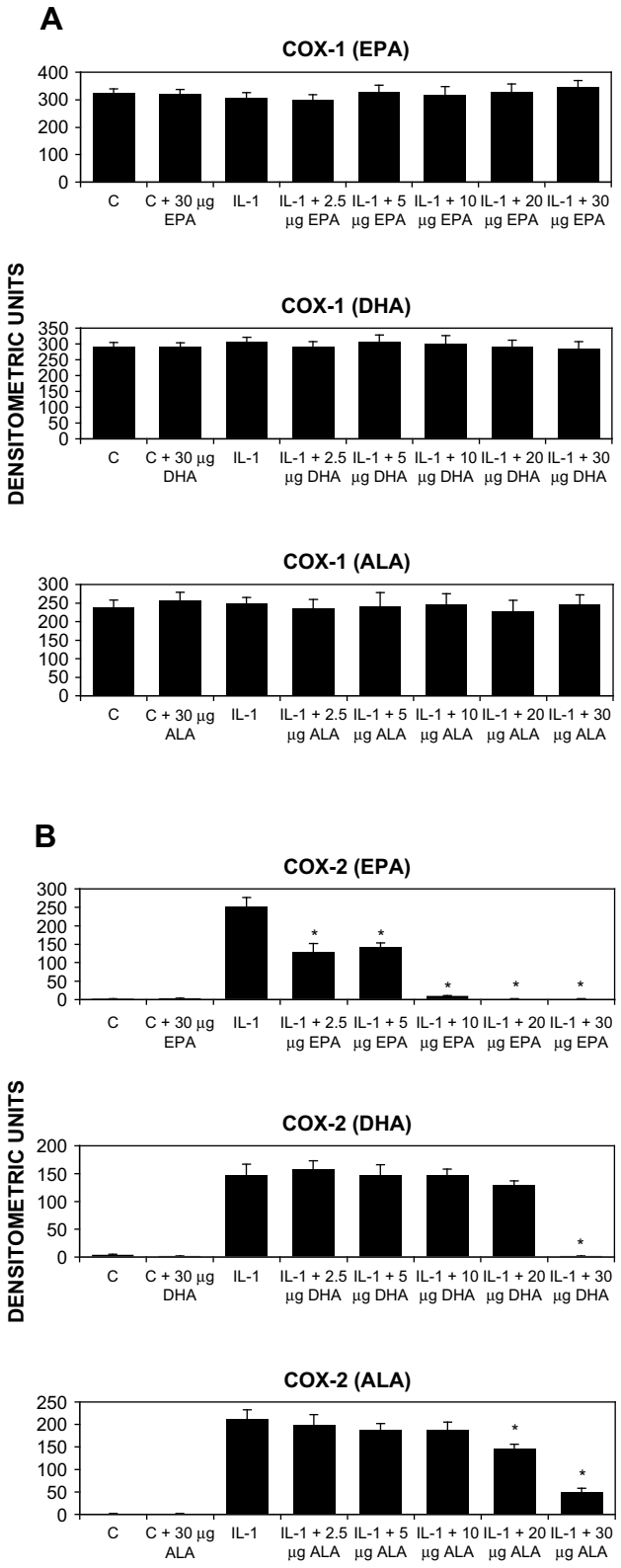

Fig. 7. n-3 PUFAs reduce mRNA levels for the inducible COX-2 but not for COX-1 in chondrocyte cultures. (A) shows results for COX-1 and (B) data for COX-2. Exposure to fatty acids was for 8 h and incubations with or without 10 ng/ml IL-1α were for 4 days. Abbreviations and further details as for Fig. 4.

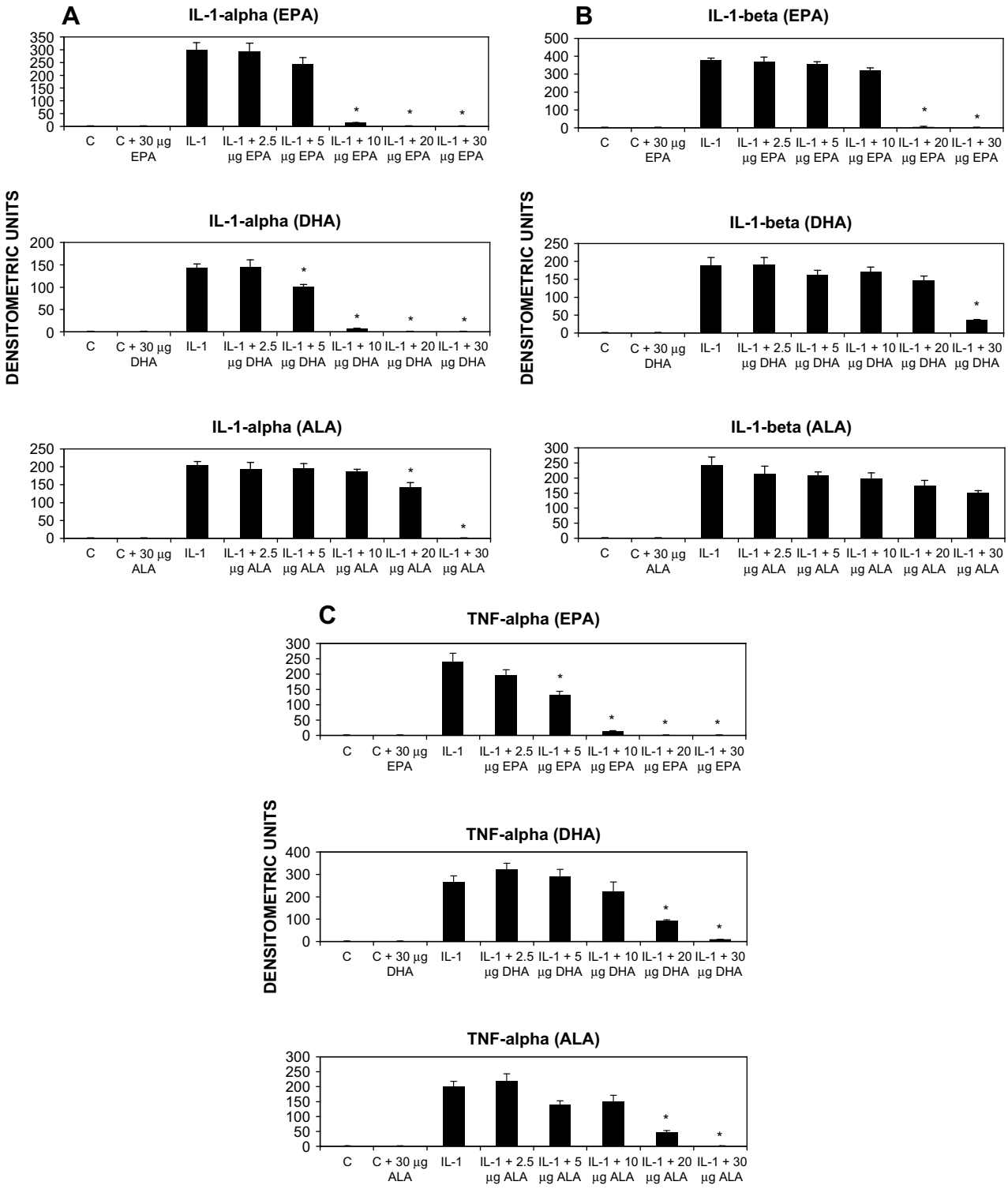

Fig. 8. n-3 PUFAs reduce mRNA levels for inflammatory cytokines in chondrocyte cultures. RT-PCRs are shown for IL-1α (A), IL-1β (B) and TNF-α (C). Exposure to fatty acids was for 8 h and incubations with 10 ng/ml IL-1α for 4 days. The histograms show means ± S.D. ($n = 3$). Abbreviations as for Fig. 4 and IL-α, IL-1α; IL-1β; TNF-α. Individual conditions are as for Fig. 4 with results for EPA, DHA and ALA shown.

levels of its mRNA [Fig. 5 Panel I & III] and this was reduced by 10–30 μg/ml EPA, and by 30 μg/ml ALA. In contrast, the addition of IL-1 to chondrocyte cultures showing high expression of ADAMTS-5 in the control cultures caused no significant increase or decrease in expression after a 96 h exposure with IL-1 either in the presence or absence of an 8 h pre-incubation with fatty acid. Because of the high constitutive levels in the controls and little increase with the addition of IL-1, no definitive conclusions can be made on the effects of DHA in this culture system.

MMPs carry out further catabolism of cartilage components after the initial attack by aggrecanases. Two isoforms, MMP-3 and MMP-13, are thought to be particularly active in OA. Expression levels for their mRNAs were examined by RT-PCR and data are shown in Fig. 6 for EPA and DHA. MMP-3 was significantly expressed in control tissues but the levels of its mRNA were increased during inflammatory stimulation by IL-1α. EPA at 20 and 30 μg/ml reduced mRNA levels but no other fatty acid (including ALA, AA: data not shown) appeared to affect these [Fig. 6(A)]. For MMP-13, expression was again significant in control tissues but exposure to IL-1α had little effect in this case. While EPA at 20 or 30 μg/ml was able to reduce mRNA levels for MMP-13 [Fig. 6(B)], exposure to other PUFAs (DHA, ALA, AA) had no significant effect.

EFFECTS OF PUFAs ON COX mRNA LEVELS

COXs are important for the conversion of the n-3 or n-6 PUFAs to eicosanoids. While COX-1 has a constitutive (house-keeping) role, COX-2 is thought to be responsible for increased eicosanoid production during inflammation[38]. In accordance with the above, mRNA levels for COX-1 were significant in control tissues and were unaffected by inflammatory stimulation [Fig. 7(A)]. No change in COX-1 mRNA levels was found on treatment of the chondrocyte cultures with any PUFA. In contrast, there was little expression of COX-2 mRNA in control tissues and this was strongly induced by exposure to the inflammatory cytokine, IL-1α [Fig. 7(B)]. Levels of COX-2 mRNA were abrogated effectively by 10–30 μg/ml EPA, by 30 μg/ml DHA and partly by 30 μg/ml ALA. AA had no effect. These data showed that, in bovine chondrocyte cultures, only COX-2 was affected by inflammatory stimulation and only this COX was reduced by exposure to n-3 PUFAs.

MODULATION OF INFLAMMATORY CYTOKINES BY n-3 PUFAs

A number of cytokines are thought to be involved in the pathology of OA. Of these, IL-1 and TNF-α are preeminent[39]. Accordingly, RT-PCR was used to examine expression levels for IL-1α, IL-1β and TNF-α. Induction of inflammation, with exogenous IL-1α, caused significant expression of IL-1α mRNA [Fig. 8(A)]. This was reduced to negligible amounts by 10–30 μg/ml EPA or DHA and by 30 μg/ml ALA. Arachidonate treatment did not affect the mRNA levels induced by inflammation (data not shown).

Like IL-1α, there was little if any IL-1β mRNA in control chondrocyte cultures [Fig. 8(B)]. This was strongly induced by exposure of cells to exogenous IL-1α. The induced mRNA levels were reduced to negligible levels by preexposure of cells to 20 and 30 μg/ml EPA, partly by 30 μg/ml DHA and were unchanged by exposure to 2.5–30 μg/ml ALA or AA (data not shown).

mRNA for TNF-α was also absent from control cultures and was induced by inflammatory stimulation of the chondrocyte cultures [Fig. 8(C)]. This mRNA was reduced strongly by preexposure to 10–30 μg/ml EPA, and by 20–30 μg DHA or ALA. Exposure to the n-6 PUFA, arachidonate, did not affect mRNA levels for TNF-α (data not shown).

Discussion

In this study, we have shown that chondrocytes are able to take up exogenous PUFAs speedily, in a dose-dependent manner. We added the fatty acids in a complex to BSA and at concentrations appropriate to physiological levels[35]. Moreover, additionally, after challenge of chondrocyte monolayer cultures with the cytokine IL-1, fatty acid pre-treated chondrocytes appeared to have altered expression of mRNAs for proteins involved in the pathology of OA.

Of particular note was the ability of different n-3 PUFAs to reduce mRNA expression for cartilage-degrading proteinases (aggrecanases, MMP) and inflammatory cytokines. Moreover, n-3 PUFAs reduce expression of the 'inflammatory' COX-2 but not the constitutive COX-1 [Fig. 7] and, therefore, in theory produce a much better outcome than, say, the use of NSAIDS where both are reduced[28]. This result gives an initial reason why dietary n-3 PUFAs are efficacious for arthritis sufferers. That is, not only do n-3 PUFAs compete with the inflammatory n-6 PUFAs for enzymes that produce eicosanoids (and also themselves produce less inflammatory eicosanoids)[21,40,41] but they also reduce COX-2 mRNA levels. This is also consistent with the effects of n-3 PUFAs in other tissues where they are selective for COX-2 but not COX-1[42,43].

There has been considerable discussion about the relative effectiveness of different n-3 PUFAs for good health (cf. 31). It is, for example, well known that dietary ALA is less effective than EPA and DHA (on a g/g basis) both because of the former's rapid β-oxidation and also its rather poor conversion to EPA and, especially, DHA[31]. Here we have tested the relative efficacy directly and it is very clear that, in our experimental system, EPA was more effective than DHA which in turn was better than ALA. This was true for all of the message levels that we examined.

The relatively poor efficiency of ALA compared to EPA can be explained if the mechanism by which mRNA expression is regulated is *via* eicosanoid production since EPA can produce eicosanoids directly[40]. If that is the case then DHA is likely to have its effects *via* the newly discovered mediators such as resolvins D[22] because DHA is not metabolised directly to eicosanoids[41] but only after retroconversion to EPA.

However, the n-3 PUFAs are also likely to have more direct effects on gene expression and differences between individual PUFAs on such expression have been extensively documented[44,45]. This has included, for example, a direct effect of DHA on IL-1α-induced expression of COX-2 (but not COX-1) in endothelial cells[43]. Work in our laboratory has suggested that signalling pathways using MAP kinases such as ERK may be involved[46] and this agrees with recent data in other inflammatory tissues[43].

Thus, it seems most likely that n-3 PUFAs can have a beneficial effect on the inflammation and cartilage degradation associated with OA in several ways. Moreover, our results in showing the relative efficacy of EPA in such situations provides a molecular mechanism to justify conclusions drawn from dietary studies (see[24]). This should also provide impetus to efforts being made to ensure supplies of EPA for the nutrition industry[47,48] at a time when some traditional fish stock sources are under threat.

Conflict of interest

All authors have no conflict of interest relating to this manuscript in any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work.

Acknowledgments

This work was supported by grants from the BBSRC (D16961) and the Malaysian Palm Oil Board for which we are grateful.

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