Polyunsaturated fatty acid metabolism in a cell culture model of essential fatty acid deficiency in a freshwater fish, carp (*Cyprinus carpio*)

Douglas R. Tocher and James R. Dick

Unit of Aquatic Biochemistry, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK

Keywords: carp; cell culture; essential fatty acid deficiency; polyunsaturated fatty acids; desaturation; elongation; lipid.

Corresponding author: Dr Douglas R Tocher
Unit of Aquatic Biochemistry
Institute of Aquaculture
University of Stirling
Stirling FK9 4LA
Scotland, U.K.

Telephone : +44 1786 467996 Fax: +44 1786 472133 E-mail : d.r.tocher@stir.ac.uk

Abbreviations: ANOVA, analysis of variance; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CL, cardiolipin; EFA, essential fatty acid; EFAD, essential fatty acid deficient; EPC, epithelioma papillosum carp; FBS, fetal bovine serum; GC, gas chromatography; HBSS, Hank's balanced salt solution (without Ca^{2+} and Mg^{2+}); HPTLC, high-performance thin-layer chromatography; PA, phosphatidic acid; PBS, Dulbecco's modification phosphate buffered saline (without calcium and magnesium); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography; TN, total neutral lipid; TP, total polar lipid.

Abstract

Proliferation of an essential fatty acid deficient cell line from carp (EPC-EFAD; epithelioma papillosum carp-essential fatty acid deficient) is stimulated by supplementing the cells with C_{20}, but not C_{18} polyunsaturated fatty acids (PUFA). It is hypothesized that the differential ability of the PUFA to stimulate proliferation of the EPC-EFAD cells may be related to the extent of the cells' ability to desaturate and elongate C_{18} PUFA. In the present study, the metabolism of ^{14}C-labeled C_{18} and C_{20} PUFA was investigated in EPC-EFAD cells in comparison with normal EPC cells. The incorporation of all the PUFA was significantly greater in EPC-EFAD cells but the rank order, 20:5n-3 > 18:3n-3 = 18:2n-6 >20:4n-6 was the same in both cell lines. The proportion of radioactivity from all labeled PUFA recovered in phosphatidylethanolamine and total polar lipids was significantly lower in EPC-EFAD cells compared to EPC cells, whereas the proportion of radioactivity recovered in all the other phospholipid classes and total neutral lipid was greater in EPC-EFAD cells. Both cell lines desaturated[1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3 to a greater extent than the corresponding (n-6) substrates but the desaturation of all the ^{14}C-labeled PUFA was significantly greater in EPC-EFAD cells compared to EPC cells. The results showed that, although essential fatty acid deficiency had several significant effects on PUFA metabolism in EPC cells, the fatty acid desaturation/elongation pathway was not impaired in EPC-EFAD cells and so they can desaturate 18:3n-3 to 20:5n-3 and 22:6n-3, and 18:2n-6 to 20:4n-6. However, 20:4n-3 and 20:3n-6, and not 20:4n-6 and 20:5n-3, were the predominant C_{20} PUFA produced by the elongation and desaturation of [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6, respectively. Therefore, the previously reported inability of 18:3n-3 and 18:2n-6, compared to 20:5n-3 and 20:4n-6, to stimulate proliferation of the cells is apparently not due to a general deficiency in the fatty acid desaturation pathway in EPC-EFAD cells but may be related to potential differences in eicosanoid profiles in cells supplemented with C_{18} PUFA compared to C_{20} PUFA.

Introduction

Cultured cells have been an important *in vitro* tool in the study of lipid and fatty acid metabolism in mammals (Bailey and Dunbar 1973; Ferguson et al. 1975; Maeda et al. 1978; Spector et al. 1981; Masuzawa et al. 1986; Isseroff et al. 1987). Most cells in culture, including fish cells, generally require the medium to be supplemented with serum which, along with various hormones and growth factors, also provides lipid and fatty acids to the cells (Sato 1975; Bailey and Dunbar 1973). Despite this, mammalian cell lines have been developed that can grow in either serum-free medium or delipidated sera which has enabled the effects of essential fatty acid deficiency to be investigated in cell lines *in vitro* (Laposata et al. 1982; Marcelo et al. 1992; Lerner et al. 1995). The development of a fish cell line that could grow in the absence of serum, and hence exogenously added fatty acids, had added importance as the commercial mammalian sera used in cell culture, including fish cell culture, distort the fatty acid composition of the fish cells in that they display lower percentages of n-3 PUFA and are enriched in n-6 PUFA in comparison with fish tissues (Henderson and Tocher 1987; Tocher et al. 1988).

Consequently, we developed a fish cell line, EPC-EFAD, derived from the carp (*Cyprinus carpio*) epithelial papilloma line, EPC, that can survive and proliferate in essential fatty acid-deficient (EFAD) medium (Tocher et al. 1995). Proliferation of the EPC-EFAD cells was stimulated by supplementing the cells with arachidonic (20:4n-6), eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids but not by 18:2n-6 or 18:3n-3 (Tocher et al. 1996). It was hypothesized that the differential ability of the PUFA to stimulate proliferation of the EPC-EFAD cells may be related to the extent of the cells' ability to desaturate and elongate C_{18} PUFA. In the present study, the metabolism of ^{14}C-labeled C_{18} and C_{20} PUFA was investigated in EPC-EFAD cells in comparison with EPC cells that were not essential fatty acid deficient. In particular, the effects of essential fatty acid deficiency on fatty acyl desaturation and elongation activities were investigated.

Materials and methods

Cells and media

The carp (*Cyprinus carpio*) epithelioma papillosum cell (EPC) line, which retains epithelial morphology, was routinely maintained in Leibovitz L-15 medium and supplemented with 2 mM glutamine, antibiotics (50 I.U. ml^{-1} penicillin and 50 $mg.ml^{-1}$ streptomycin) and 10% fetal bovine serum (FBS). EPC-EFAD cells were derived from EPC cells by subculture in Leibovitz L-15 medium with exactly the same supplements except that the FBS was delipidated prior to use, essentially as described by Capriotti and Laposata (1986). The delipidated FBS contained approximately 10 μg of fatty acids per g of serum (0.001% by weight) with less than 2 μg/g of C_{18} PUFA, predominantly 18:2(n-6) (Tocher et al. 1995,1996). The EPC-EFAD cells used in the experiments had been maintained continuously in delipidated medium for 3 years and were at passage number 52-55.

Incubation conditions

The EPC and EPC-EFAD cells were cultured at 22 ºC in sealed plastic tissue culture flasks. EPC cells were cultured in standard tissue culture flasks (Corning Costar, High Wycombe, U.K.), whereas the EPC-EFAD line was cultured in surface-modified "Primaria" flasks (Falcon, Becton Dickinson UK Ltd., Oxford). Both EPC and EPC-EFAD lines were cultured in 75 cm^2 (routine culture for provision of experimental material) or 25 cm^2 (^{14}C-metabolism) flasks and were subcultured within 24 h of reaching confluence at seeding densities of 1 x 10^5 $cells.cm^{-2}$ in EPC and 2 x 10^5 $cells.cm^{-2}$ in EPC-EFAD, to account for the slower proliferation rate of the EPC-EFAD cells in delipidated FBS (Tocher et al. 1995,1996). For each experimental sample, 3 x 25 cm^2 flasks were seeded and this experiment was repeated three times to obtain the

replicates.

Incubation of cultures with ^{14}C-labeled polyunsaturated fatty acids

The medium was aspirated, cultures washed with Dulbecco's phosphate-buffered saline (PBS) and fresh Leibovitz L-15 medium, as above but without supplemental serum or delipidated serum, was added. The ^{14}C-labelled PUFAs ([1-^{14}C] 18:2n-6, [1-^{14}C] 18:3n-3, [1-^{14}C] 20:4n-6 and [1-^{14}C] 20:5n-3; 0.2 μCi per flask; concentration, 0.8 μM) were added to the cell cultures bound to fatty acid-free bovine serum albumin (BSA) in phosphate buffered saline (Ghioni et al. 1997) and incubation continued for 6 days at 22 ºC.

Lipid extraction and incorporation of radioactivity into total lipid

The medium was aspirated and cultures washed twice with 20 ml of ice-cold Hanks' balanced salt solution without calcium and magnesium (HBSS). Cells were harvested by trypsinization with 0.05 % trypsin/0.5 mM ethylenediamine tetraacetic acid (EDTA), washed with 5 ml ice-cold HBSS and a sample taken for protein determination. The cells were washed with 5 ml HBSS containing 1% fatty acid-free BSA and total lipid extracted from the cell pellets with 5 ml ice-cold chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, according to Folch et al. (1957) as described in detail previously (Tocher et al. 1988). Lipid content was determined gravimetrically after 1 h vacuum desiccation and the lipid resuspended in chloroform/methanol (2:1, v/v) containing BHT, at a lipid concentration of 10 mg.ml^{-1}, and samples stored at -20 ºC before analyses. The radioactive content of total lipid was determined in 3 aliquots of 5 μl in mini-vials containing 2.5 ml scintillation fluid (Ecoscint A; National Diagnostics, Atlanta, USA) using a TRI-CARB 2000CA liquid scintillation spectrophotometer (United Technologies Packard). Results were corrected for counting efficiency and quenching using an appropriate calibration curve. All solvents contained 0.01%

BHT as antioxidant.

Incorporation of radioactivity into glycerophospholipid classes

Samples of total lipid (100 mg) were applied as 1 cm streaks to high-performance thin-layer chromatography (HPTLC) plates, and the polar lipid classes separated using methyl acetate/propan-2-ol/ chloroform/ methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.) as developing solvent (Vitiello and Zanetta, 1978). After desiccation, the lipid classes were visualized by brief exposure to iodine vapor and areas corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid/cardiolipin (PA/CL) and total neutral lipid (TN) were scraped into scintillation vials containing 2.5 ml scintillation fluid and radioactivity determined as above.

2.6. Incorporation of radioactivity into polyunsaturated fatty acids

Total lipid extracts were transmethylated overnight at 50 ºC in methanolic sulfuric acid (Christie 1982). Fatty acid methyl esters (FAME) were extracted, after addition of 2 ml 2% $KHCO_3$, with hexane/diethyl ether (1:1, v/v) containing 0.01% BHT. Solvent was evaporated and samples were resuspended in 100 μl hexane containing BHT. Samples were applied as 1 cm streaks on HPTLC plates and then developed with hexane/diethyl ether/acetic acid (90:10:1, by vol.). FAME were detected under UV light by comparison with known standards after spraying with 2', 7'-dichlorofluorescein. FAME were eluted from the silica with hexane/diethyl ether (1:1, v/v), solvent evaporated and the purified samples resuspended in hexane/BHT. Thin layer chromatography (TLC) plates were impregnated by spraying with 2 g silver nitrate in 20 ml acetonitrile and activated at 110 ºC for 30 min. FAME were applied as 2.5 cm streaks and plates

developed with toluene/acetonitrile (95:5, v/v) to separate PUFA (Wilson and Sargent 1992). Autoradiography was performed using Kodak MR2 film for 7 days at room temperature. Silica corresponding to different FAME was scraped into scintillation vials containing 2.5 ml scintillation fluid and radioactivity determined as described above.

Lipid class and fatty acid composition

Separation and quantification of lipid classes was performed by single-dimension double-development HPTLC followed by scanning densitometry as described previously (Henderson and Tocher 1992). Fatty acid methyl esters (FAME) were prepared by acid-catalyzed transmethylation, extracted and purified by HPTLC as described previously (Tocher and Harvie 1988). Analysis of FAME was performed by GC in a Fisons GC8000 gas chromatograph (Crawley, UK) equipped with a fused-silica capillary column (30m x 0.32 mm i.d., CP Wax 52 CB, Chrompack, UK) using hydrogen as carrier gas. Temperature programming was from 50 to 150°C at 35°C/min and to 225°C at 2.5°C/min. Individual FAME were identified by comparison with known standards and published data (Ackman 1980; Bell et al. 1983).

Protein determination

The protein content of the cells was determined according to the method of Lowry et al. (1951) after incubation with 0.25 ml of 0.25% (wt/vol) sodium dodecyl sulfate/1 M NaOH for 45 min at 60 ºC.

Materials

[1-^{14}C] PUFA (all ~ 50 mCi.mmol^{-1} and 99% pure) were obtained from NEN Life Science Products (Hounslow, U.K.). Leibovitz L-15 medium, HBSS, PBS, glutamine, penicillin,

streptomycin, FBS, trypsin/EDTA , fatty acid-free BSA and BHT were obtained from Sigma Chemical Co. Ltd. (Poole, UK). TLC plates (20 cm x 20 cm x 0.25 mm) and HPTLC plates (10 cm x 10 cm x 0.15 mm), pre-coated with silica gel 60 were obtained from Merck, (Darmstadt, Germany). All solvents were of HPLC grade and were obtained from Rathburn Chemicals (Walkerburn, Peebleshire, Scotland).

Statistical analysis

All results are means $\pm$ SD of three experiments. Where indicated, data were subjected to two-way analysis of variance (ANOVA) and where appropriate the significance of differences were determined by Tukey's test. Percentage data were subjected to arc-sin transformation prior to ANOVA. When appropriate, the significance of differences between some means were determined by the Student t-test. Differences are reported as significant when $p < 0.05$ (Zar 1984).

Results

The lipid content of the essential fatty acid deficient cells (EPC-EFAD) was significantly lower than the normal cells (EPC) (Table 1). Due to this, the absolute amounts of all lipid classes were lower in EPC-EFAD cells (data not shown). In relative terms, both cell lines showed lipid class compositions that were predominantly polar lipids with cholesterol as the only major neutral lipid with the EPC-EFAD cells having a significantly higher percentage of polar lipids and lower cholesterol compared to EPC cells (Table 1). The proportions of the main phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), in both cell lines were unchanged although the EPC-EFAD cells had significantly increased percentages of many of the more minor polar lipids. Due to the decreased amount of lipid, the absolute amounts of all the fatty acid groups were significantly reduced in EPC-EFAD cells (Table 2). In particular, the lipid of

EPC-EFAD cells contained very little (n-3) or (n-6) PUFA, with the totals and all individual PUFA being very significantly lower than in EPC cells (Table 2). The relative proportions of total saturated fatty acids, in particular the predominant 16:0 and 18:0 acids, were also significantly lower in EPC-EFAD cells although shorter chain 12:0 and 14:0 were significantly higher. In contrast, the relative amount of total monounsaturated fatty acids was significantly increased in EPC-EFAD cells although the proportion of 18:1n-9 was reduced while the proportions of (n-9) PUFA were increased (Table 2).

Two-way analysis of variance (ANOVA) showed that the incorporation of ^{14}C-labeled PUFA was significantly greater in EPC-EFAD cells irrespective of the PUFA added or the way the results were expressed (Table 3). Irrespective of cell line, i.e. EPC or EPC-EFAD, the incorporation [1-^{14}C]20:5n-3 was significantly greater than any other fatty acid and the incorporation of [1-^{14}C]20:4n-6 was lower than the other PUFA with the rank order for the incorporation being

20:5n-3 > 18:3n-3 = 18:2n-6 > 20:4n-6.

The distribution of radioactivity from the added ^{14}C-labeled PUFA in individual lipid classes in EPC and EPC-EFAD cells is shown in Table 4. Two-way ANOVA showed that this distribution was significantly affected by the cell line and was dependent upon the specific ^{14}C-fatty acid (Table 5). Qualitatively, the distribution of the PUFA was similar in both cell lines, with all PUFAs preferentially incorporated into polar lipids. The incorporation of the ^{14}C-labeled C_{18} PUFAs was greater into PC, phosphatidic acid/cardiolipin (PA/CL) and total neutral lipids, compared to the ^{14}C-labeled C_{20} PUFAs (Tables 4 & 5). [1-^{14}C]20:5n-3 was preferentially incorporated into PE and [1-^{14}C]20:4n-6 was preferentially incorporated into phosphatidylinositol (PI). Quantitatively though, there were significant differences between the cell lines. The proportion of radioactivity from all labeled PUFA recovered in PE and total polar lipids was significantly lower in EPC-EFAD cells compared to EPC cells, whereas the proportion of radioactivity recovered in all the other phospholipid classes and total neutral lipid was increased in EPC-EFAD cells (Tables 4 & 5).

EPC cells metabolized the n-3 PUFA, [1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3, to a greater extent than the corresponding (n-6) substrates (Table 6). Very little tetraene and pentaene products were formed from [1-^{14}C]18:2n-6 and [1-^{14}C]20:4n-6, respectively, in EPC cells. In contrast, [1-^{14}C]18:3n-3 was metabolized to 20:5n-3 and 22:6n-3, and [1-^{14}C]20:5n-3 was metabolized to 22:6n-3 (Table 6). Desaturation of all the ^{14}C-labeled PUFA was significantly greater in EPC-EFAD cells compared to EPC cells (Table 6). With [1-^{14}C]18:2n-6, the proportion of radioactivity recovered in the main product of it's desaturation, 20:3n-6, was significantly increased in EPC-EFAD cells, as was 20:4n-6 and 22:4n-6, whereas the proportion of radioactivity recovered in 18:2n-6 was significantly reduced. With [1-^{14}C]18:3n-3, the proportions of radioactivity recovered in 20:5n-3, 22:5n-3 and 22:6n-3 were all significantly greater and the proportions recovered in almost all the trienes and tetraenes were reduced in EPC-EFAD cells (Table 6). The recovery of radioactivity in 22:6n-3 was significantly greater in EPC-EFAD cells incubated with [1-^{14}C]20:5n-3 compared to EPC cells, whereas the proportions of radioactivity recovered in 20:5n-3 and it's elongation products, 22:5n-3 and 22:4n-3, were significantly lower. There was significantly more radioactivity recovered in 22:5n-6 in EPC-EFAD cells incubated with [1-^{14}C]20:4n-6 compared to EPC cells.

The apparent activity at each step in the desaturation/elongation pathway was estimated by adding the percentages of radioactivity recovered in all fatty acid metabolites for each step, irrespective of subsequent metabolism by another activity (Table 7). This showed that the metabolism of [1-^{14}C]18:2n-6 in EPC-EFAD cells was increased at the level of Δ6 desaturase whereas the metabolism [1-^{14}C]18:3n-3 did not appear to be greatly increased at this level. However, the desaturation of [1-^{14}C]18:3n-3 appeared to be increased in EPC-EFAD cells at Δ5 desaturase and subsequent steps although preceding activities may still be influencial. The metabolism of [1-^{14}C]20:5n-3 appeared to be primarily increased in EPC-EFAD cells at a step after the $C_{20\text{-}22}$ elongase.

Discussion

The main effects on lipid composition of three years continuous culture in delipidated serum were decreased lipid content and decreased levels of n-3 and n-6 PUFA in EPC-EFAD cells. The very low levels of n-3 and n-6 PUFA in EPC-EFAD cells were accompanied by decreased proportions of cholesterol, saturates (other than short chain 12:0 and 14:0) and increased proportions of n-9 PUFA and monoenes which would compensate for the effects on membrane unsaturation and fluidity. These compositional effects in EPC-EFAD cells were accompanied by increased incorporation of the labeled PUFA. Net "incorporation" is actually based on the recovery of radioactivity and represents uptake minus the amount oxidized or lost via other metabolic pathways. However, all fatty acids were used at the same concentration and so the amount taken up and oxidized will only be dependent on the fatty acid and the cell type. The differences in incorporation between the fatty acids were similar in the two cell lines showing that essential fatty acid deficiency had affected these processes similarly for all fatty acids.

However, the primary aims of the present study were to precisely determine the ability of EPC cells to desaturate and elongate PUFA, particularly C_{18} PUFA, and to investigate the effects of essential fatty acid deficiency on that pathway. The results of the present study demonstrated that the EPC cell line is capable of desaturating 18:3n-3 to 20:5n-3 and, to a lesser extent, 22:6n-3. Similarly, 18:2n-6 was converted to 20:4n-6 in EPC cells although 20:3n-6 was the main product. EPC-EFAD cells also exhibited the full desaturation/elongation pathway and indeed essential fatty acid deficiency significantly increased the desaturation of 18:3n-3 to 20:5n-3 and 22:6n-3, and 18:2n-6 to 20:4n-6.

The aims of the present study arose from previous studies that had shown that C_{18} PUFA (18:3n-3 and 18:2n-6) did not stimulate proliferation of EPC-EFAD cells, whereas C_{20} and C_{22} PUFA (20:4n-6, 20:5n-3 and 22:6n-3) were effective in increasing the proliferation rate of the essential fatty acid deficient cells (Tocher et al. 1996). The fatty acid compositional data in the previous study had suggested that the EPC-EFAD cells had only limited $\Delta 5$ desaturase activity based on the fact that the level of 20:4n-6 in total lipid was not significantly increased after

supplementation with 18:2n-6 and that the levels of 20:5n-3 (and 22:6n-3) were not increased after supplementing with 18:3n-3 (Tocher et al. 1996). This was unexpected as, firstly, the EFA requirements of common carp are known to be met by C_{18} PUFA alone, specifically about 1% each of 18:3n-3 and 18:2n-6 (Takeuchi and Watanabe 1977). Secondly, a deficiency in the desaturase pathway had been widely perceived to be a trait of marine fish, especially carnivorous marine fish, and so it was unexpected to have an apparent deficiency in a cell line from a largely herbivorous freshwater species (Sargent et al. 1995; Tocher et al. 1998). However, it was known that many cultured cell lines appear to lack, or display low levels of activity, of certain enzymes of the fatty acid desaturation/elongation pathway (Tocher et al. 1998). Therefore, it was possible that EPC (and EPC-EFAD) cells no longer reflected the situation in carp and had lost an enzyme activity in the fatty acid desaturation/elongation pathway. The present study has demonstrated that this is not the case and that both the EPC and EPC-EFAD cell lines have the fatty acid desaturation/elongation profile expected of cells from a freshwater fish.

In the previous study, levels of 20:4n-6 and 20:5n-3 were increased by supplementing EPC-EFAD cells with 20:4n-6 and 20:5n-3, and 20:5n-3 was also very significantly increased by supplementation with 22:6n-3 (Tocher et al. 1996). In contrast, 20:5n-3 and 20:4n-6 were not increased by supplementing EPC-EFAD cells with 50 μM unlabeled 18:3n-3 and 18:2n-6, respectively. This had suggested that eicosanoids may be implicated in the mechanism of stimulation of proliferation (Tocher et al. 1996). The data from the present study is not inconsistent with the data from the previous study and can support the view that eicosanoids are involved. In the previous study, 20:3n-6 was significantly increased by supplementing with 18:2n-6 and this is supported by the data in the present study which shows that 20:3n-6 was the main product of [1-^{14}C]18:2n-6 desaturation and that the residual 18:2n-6 (~1.5 μg/g) in delipidated serum is converted to 20:3n-6. A relative accumulation of radioactivity in EFA deficient human Hep G2 cells incubated with tracer amounts of ^{14}C-labelled 18:2n-6 was reported previously (Melin and Nilsson 1997). Similarly, supplementing EPC-EFAD cells with 18:3n-3 in the previous study significantly increased the levels of both 20:3n-3 and 20:4n-3, and

20:4n-3 was a major product of [1-^{14}C]18:3n-3 desaturation in the present study. It is well known that 20:3n-6 can modulate the production and efficacy of 20:4n-6-derived eicosanoids via competitive inhibition and as the precursor of eicosanoids with attenuated biological properties (Horrobin 1992; Bell et al. 1994). Similarly, 20:4n-3 can be converted to eicosanoids with attenuated properties in mammalian tissues and inhibit 20:4n-6 metabolism in human leukocytes (Oliw et al. 1986a,b; Samel et al. 1987; Careaga and Sprecher 1987; Guichardant et al. 1993). Eicosanoids, particularly prostaglandins, are known to be highly involved in the regulation of cellular proliferation (Wickremasinghe 1988; Skouteris et al. 1988) including fish cells (Secombes et al. 1994; Rowley et al. 1995).

Therefore, the data in the present study are consistent with 18:2n-6 failing to stimulate proliferation of EPC-EFAD cells due to the accumulation of 20:3n-6 rather than 20:4n-6. However, although the present data can explain the accumulation of both 20:3n-3 and 20:4n-3 in EPC-EFAD cells supplemented with 18:3n-3 it is unclear why 18:3n-3 did not appear to be significantly converted to 20:5n-3 and 22:6n-3 in the earlier study. This discrepancy between the two studies may be related to fatty acid concentration as a relatively high concentration of 50 μM was used previously whereas the labeled fatty acids in the present study were used at tracer concentrations of less than 1 μM. Differences in metabolism due to fatty acid concentration have been noted previously (Melin and Nilsson 1997).

Essential fatty acid deficiency had other effects on PUFA metabolism in EPC cells. In EPC-EFAD cells, the distribution of the PUFA in phospholipid classes was altered with the main effect, observed with all the PUFA, being decreased amounts incorporated in PE and increased in PC. This may reflect lowered specificity between phospholipid classes in EPC-EFAD cells, in which all the phospholipids are essentially lacking their normal complement of PUFA. Therefore, PC, the predominant phospholipid class, accumulates relatively more PUFA in EPC-EFAD cells than in EPC cells. However, alterations in the relative incorporation of PUFA into PC and PE have been observed before in fish cell lines in response to other factors such as culture temperature (Tocher and Sargent 1990) and xenobiotics (Ghioni et al. 1998). Alterations

in the relative proportions of PC and PE are also common in adaptation of fish to environmental factors such as temperature, salinity and hydrostatic pressure (Tocher 1995) but no such alteration was observed in response to EFA deficiency in the present study.

In comparison with salmonid cell lines, EPC cells were similar to Atlantic salmon (AS) cells in which 20:4n-3 was the predominant product of 18:3n-3 desaturation rather than 20:5n-3, as in rainbow trout (RTG-2) cells (Tocher and Sargent 1990; Ghioni et al. 1998). However, EPC cells and, especially, EPC-EFAD cells produced far more 22:6n-3, both from [1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3, than the other freshwater cell lines investigated previously (Tocher and Sargent 1990; Ghioni et al. 1998). It was noteworthy that the effect of essential fatty acid deficiency on the desaturation and elongation of [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 was different. The production of Δ5 products of [1-^{14}C]18:3n-3 was doubled in EPC-EFAD cells and the radioactivity recovered in 22:6n-3 was over 4-fold higher whereas there was apparently less effect on Δ6 desaturase and C_{18-20} elongase. With [1-^{14}C]18:2n-6, Δ5 products were also increased by about 10-fold in EPC-EFAD cells but Δ6 desaturase products were also doubled. The precise reason for the apparent difference in the pathway using the different C_{18} PUFA substrates is not known but it is important to appreciate that the data for C_{18-20} elongase and all subsequent enzymes are, of course, not independent of preceding activities and labeled versions of the direct substrates of each activity are required to further investigate this apparent effect. Previously, it was shown that both Δ6 and Δ5 desaturases in human Hep G2 cells incubated with ^{14}C-labeled 18:2n-6 were apparently both rate-limiting and upregulated in EFA deficiency (Melin and Nilsson 1997).

In conclusion, the present study has demonstrated that the carp cell line, EPC, has a complete fatty acid desaturation and elongation pathway, and can biosynthesize 22:6n-3 from 18:3n-3 and 20:4n-6 from 18:2n-6. The overall desaturation of PUFA was increased by essential fatty acid deficiency with apparently increased desaturase activities, rather than increased elongase activities, in EPC-EFAD cells. Therefore, the previously reported inability of 18:3n-3 and 18:2n-6, in comparison to C_{20} and C_{22} PUFA, to stimulate proliferation of EPC-EFAD cells

was not due to a general deficiency in the desaturase pathway. However, it may be related to accumulation of 20:4n-3 and 20:3n-6, with their associated potential to alter eicosanoid profiles, in cells supplemented with C_{18} PUFA although this requires further investigation.

References

Ackman, R.G. 1980. Fish lipids, part 1. *In* Advances in Fish Science and Technology. pp. 86-103. Edited by J.J. Connell. Fishing New Books, Farnham, Surrey, UK.

Bailey, J.M. and Dunbar, L.M. 1973. Essential fatty acid requirements of cells in tissue culture: A review. Exp. Mol. Pathol. 18: 142-161.

Bell, J.G., Tocher, D.R. and Sargent, J.R. 1994. Effects of supplementation with 20:3(n-6), 20:4(n-6) and 20:5(n-3) on the production of prostaglandins E and F of the 1-, 2- and 3-series in turbot (*Scophthalmus maximus*) brain astroglial cells in primary culture. Biochim. Biophys. Acta 1211: 335-342.

Bell, M.V., Simpson, C.M.F. and Sargent, J.R. 1983. (n-3) and (n-6) polyunsaturated fatty acids in the phosphoglycerides of salt-secreting epithelia from two marine fish species. Lipids 18: 720-726.

Capriotti, A.M. and Laposta, M. 1986. Identification of variables critical to reproducible delipidation of serum. J. Tissue Culture Methods 10: 219-221.

Careaga, M.M. and Sprecher, H. 1987. Metabolism of 8,11,14,17-eicosatetraenoic acid by human platelet lipoxygenase and cyclooxygenase. Biochim. Biophys. Acta 920: 94-101.

Christie, W.W. 1982. Lipid Analysis, 2nd Edn. Pergamon Press, Oxford.

Ferguson, K.A., Glaser, M., Bayer, W.H. and Vagelos, P.R. 1975. Alteration of fatty acid composition of LM cells by lipid supplementation and temperature. Biochemistry 14: 146-151.

Folch, J., M. Lees, M., Sloane Stanley, G.H. 1957. A simple method for the isolation and

purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509.

Ghioni, C., Tocher, D.R. and Sargent, J.R. 1997. The effect of culture on morphology, lipid and fatty acid composition, and polyunsaturated fatty acid metabolism of rainbow trout (*Oncorhynchus mykiss*) skin cells. Fish Physiol. Biochem. 16: 499-513.

Ghioni, C., Tocher, D.R. and Sargent, J.R. 1998. Effects of dichlorvos and formalin on fatty acid metabolism of rainbow trout (*Oncorhynchus mykiss*) skin cells in primary culture. Fish Physiol. Biochem. 18: 241-252.

Guichardant, M., Traitler, H., Spielmann, D., Sprecher, H. and Finot, P.A. 1993. Stearidonic acid, an inhibitor of the 5-lipoxygenase pathway - A comparison with timnodonic and dihomogammalinolenic acid. Lipids 28: 321-324.

Henderson, R.J and Tocher, D.R. 1987. The lipid composition and biochemistry of freshwater fish. Prog. Lipid Res. 26: 281-347.

Henderson, R.J. and Tocher, D.R. 1992. Thin-layer chromatography. *In* Lipid analysis: A Practical Approach. pp.65-111. Edited by R.J. Hamilton and S. Hamilton. IRL Press, Oxford

Horrobin, D.F. 1992. Nutritional and medical importance of gamma-linolenic acid. Prog. Lipid Res. 31: 163-194.

Isseroff, R.R., Ziboh, V.A., Chapkin, R.S. and Martinez, D.T. 1987. Conversion of linoleic acid into arachidonic acid by cultured murine and human keratinocytes. J. Lipid Res. 28: 1342-1349.

Laposata, M., Prescott, S.M., Bross, T.E. andMajerus, P.W. 1982. Development and characterization of a tissue culture cell line with essential fatty acid deficiency. Proc. Natl. Acad. Sci. USA 79: 7654-7658.

Lerner, R., Lindstrom, P., Berg, A., Johansson, E., Rosendahl, K. and Palmblad, J. 1995. Development and characterization of essential fatty acid deficiency in human endothelial cells in culture. Proc. Natl. Acad. Sci. USA 92: 1147-1151.

Lowry, O.H., Rosebrough, N.J. Farr, N.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Maeda, M., Doi, O. and Akamatsu, Y. 1978. Metabolic conversion of polyunsaturated fatty acids in mammalian cultured cells. Biochim. Biophys. Acta 530: 153-164.

Marcelo, C.L., Duell, E.A., Rhodes, L.M. and Dunham, W.R. 1992. *In vitro* model of essential fatty acid deficiency. J. Invest. Dermatol. 99: 703-708.

Masuzawa, Y, Okano, S. Waku, K., Sprecher, H. and Lands, W.E.M. 1986. Selective incorporation of various C-22 polyunsaturated fatty acids in Ehrlich ascites tumor cells. J. Lipid Res. 27: 1145-1153.

Melin, T. and Nilsson, A. (1997) Δ6 desaturase and Δ5 desaturase in human Hep G2 cells are both fatty acid interconversion rate limiting and are upregulated under essential fatty acid deficient conditions. Prostaglandins Leukotrienes Essent. Fatty Acids 56: 437-442.

Oliw, E.H., Sprecher, H. and Hamberg M. 1986a. Biosynthesis of a novel prostaglandin, Δ^{17}-PGE_1 in the ram. Acta Physiol. Scand. 127: 45-49.

Oliw, E.H., Sprecher, H. and Hamberg, M. 1986b. Isolation of two novel prostaglandins in human seminal fluid.J. Biol. Chem. 261: 2675-2683.

Rowley, A.F., Knight, J., Lloyd-Evans, P., Holland, J.W. and Vickers, P.J. 1995. Eicosanoids and their role in immune modulation in fish - a brief overview. Fish Shellfish Immunol. 5: 549-567.

Samel, N. Jarving, I., Lohmus, M., Lopp, A., Kobzar, G., Sadovskaya, V., Valimal, T. and Lille, U. 1987. Identification and biological activity of a novel natural prostaglandin, 5,6-dihydroprostaglandin E_3. Prostaglandins 33: 137-146.

Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R. 1995. Requirement criteria for essential fatty acids. J.Appl. Icthyol. 11: 183-198.

Sato, G. 1975. The role of serum in culture. *In* Biochemical Action of Hormones. Vol.3, pp. 391-396. Edited by G. Litwak. Academic Press, New York.

Secombes, C.J., K. Clements, K., Ashton, I., and Rowley, A.F. 1994. The effect of eicosanoids on rainbow trout, Oncorhynchus mykiss, leucocyte proliferation. Vet. Immunol. Immunopathol. 42: 367-378.

Skouteris, G.G., Ord, M.G. and Stocken, L.A. 1988. Regulation of the proliferation of primary rat hepatocytes by eicosanoids. J. Cellular Physiol. 135: 516-520.

Spector, A.A., Mathur, S.N., Kaduce, T.L. and Hyman, B.T. 1981. Lipid nutrition and metabolism of cultured mammalian cells. Prog. Lipid Res. 19: 155-186.

Takeuchi, T. and Watanabe, T. 1977. Requirement of carp for essential fatty acids. Bull. Jap. Soc. Sci. Fish. 43: 541-551.

Tocher, D.R. 1995. Glycerophospholipid metabolism. *In* Biochemistry and Molecular Biology of Fishes. Vol. 4, pp. 119-157. Edited by P.W. Hochachka and T.P. Mommsen. Elsevier Press, Amsterdam.

Tocher, D.R. Dick, J.R. and Sargent, J.R. 1995. Development of an in vitro model of essential fatty acid deficiency in fish cells. Prostaglandins Leukotrienes Essent. Fatty Acids 53: 365-375.

Tocher, D.R., Dick, J.R. and Sargent, J.R. 1996. Stimulation of proliferation of an essential fatty acid deficienct fish cell line by C_{20} and C_{22} polyunsaturated fatty acids and effects on fatty acid composition. Prostaglandins Leukotrienes Essent. Fatty Acids 55: 345-356.

Tocher, D.R. and Harvie, D.G. 1988. Fatty acid compositions of the major phosphoglycerides from fish neural tissues; (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri*) and cod (*Gadus morhua*) brains and retinas. Fish Physiol. Biochem. 5: 229-239.

Tocher, D.R., Leaver, M.J. and Hodgson, P.A. 1998. Recent developments in the molecular biology and biochemistry of fatty acyl desaturases. Prog. Lipid Res. 37: 73-117.

Tocher, D.R. and Sargent, J.R. 1990. Effect of temperature on the incorporation into phospholipid classes and metabolism via desaturation and elongation of n-3 and n-6 polyunsaturated fatty acids in fish cells in culture. Lipids 25: 435-442.

Tocher, D.R., Sargent, J.R. and Frerichs, G.N. 1988. The fatty acid compositions of established

fish cell lines after long-term culture in mammalian sera. Fish Physiol. Biochem. 5: 219-227.

Vitiello, F., and Zanetta, J.-P. 1978. Thin-layer chromatography of phospholipids. J. Chromatogr. 166: 637-640.

Wickremasinghe, R.G. 1988. The role of prostaglandins in the regulation of cell proliferation. Prostaglandins Leukotrienes and Essent. Fatty Acids 31: 171-180.

Wilson, R. and Sargent, J.R. 1992. High resolution separation of polyunsaturated fatty acids by argentation thin-layer chromatography. J. Chromatogr. 623: 403-407.

Zar, J.H. 1984. Biostatistical Analysis, 2nd Edition. Prentice-Hall, Englewood Cliffs.

Table 1. Effect of essential fatty acid deficiency on lipid content and lipid class composition (percentage of total lipid) of EPC cells grown at 22 °C in 10% FBS (EPC) or for three years in delipidated FBS (EPC-EFAD).

Lipid	EPC	EPC-EFAD
Total lipid content		
μg lipid /10^6 cells	17.9 ± 1.3	11.0 ± 0.8*
Lipid class composition		
Phosphatidylcholine	27.2 ± 1.1	26.4 ± 0.1
Phosphatidylethanolamine	23.6 ± 0.1	24.6 ± 0.4
Phosphatidylserine	6.8 ± 0.1	8.7 ± 0.2*
Phosphatidylinositol	6.1 ± 0.0	5.0 ± 0.1*
Phosphatidic acid/cardiolipin	3.1 ± 0.3	3.2 ± 0.2
Sphingomyelin	7.9 ± 0.3	9.5 ± 0.3*
Sulfatides/cerebrosides	1.4 ± 0.2	2.8 ± 0.1*
Total polar lipids	76.1 ± 1.7	80.2 ± 0.8*
Total neutral lipids	23.9 ± 1.7	19.8 ± 0.8*
Cholesterol	22.1 ± 1.0	19.0 ± 0.6*
Triacylglycerol	1.2 ± 0.1	0.8 ± 0.2
Steryl ester	0.6 ± 0.5	n.d.
Free fatty acids	tr	n.d.

Results are means ± S.D. (n=3); n.d., not detected; tr, trace (< 0.05%).
* EPC-EFAD cells significantly different to EPC cells ($p < 0.05$) as determined by the Student's t-test.

Table 2. Effect of essential fatty acid deficiency on fatty acid compositions of total lipid from EPC cells grown at 22°C in 10% FBS (EPC) or for three years in delipidated FBS (EPC-EFAD).

	Percentage of weight		μg/million cells	
Fatty acid	EPC	EPC-EFAD	EPC	EPC-EFAD
14:0	0.7 ± 0.1	2.9 ± 0.1*	0.08 ± 0.01	0.21 ± 0.01*
15:0	0.1 ± 0.0	0.4 ± 0.0*	0.01 ± 0.00	0.03 ± 0.00*
16:0	13.1 ± 0.1	9.4 ± 0.1*	1.47 ± 0.01	0.68 ± 0.01*
17:0	0.3 ± 0.1	n.d.	0.03 ± 0.01	n.d.
18:0	7.1 ± 0.1	2.1 ± 0.1*	0.80 ± 0.01	0.15 ± 0.01*
20:0	0.2 ± 0.0	0.2 ± 0.0	0.02 ± 0.01	0.01 ± 0.00
22:0	n.d.	1.0 ± 0.1*	n.d.	0.07 ± 0.01*
24:0	n.d.	0.5 ± 0.0*	n.d.	0.04 ± 0.00*
Total saturated	21.5 ± 0.5	16.5 ± 0.1*	2.41 ± 0.06	1.19 ± 0.01*
16:1(n-9)	2.7 ± 0.1	1.8 ± 0.0*	0.30 ± 0.01	0.12 ± 0.00*
16:1(n-7)	2.2 ± 0.1	5.7 ± 0.1*	0.25 ± 0.01	0.41 ± 0.01*
18:1(n-9)	34.9 ± 0.1	31.9 ± 0.1*	3.91 ± 0.01	2.30 ± 0.01*
18:1(n-7)	4.7 ± 0.2	10.8 ± 0.1*	0.53 ± 0.02	0.78 ± 0.01*
18:1(n-5)	n.d.	2.3 ± 0.1*	n.d.	0.14 ± 0.01*
20:1(n-11)	n.d.	0.8 ± 0.0*	n.d.	0.06 ± 0.00*
20:1(n-9)	3.5 ± 0.1	2.8 ± 0.0*	0.34 ± 0.01	0.20 ± 0.00*
20:1(n-7)	0.3 ± 0.1	0.9 ± 0.1*	0.03 ± 0.01	0.06 ± 0.01
22:1	0.5 ± 0.0	3.2 ± 0.1*	0.06 ± 0.00	0.23 ± 0.01*
24:1	1.2 ± 0.1	2.0 ± 0.0*	0.13 ± 0.01	0.14 ± 0.00
Total monounsaturated	50.1 ± 0.3	62.2 ± 0.0*	5.61 ± 0.03	4.48 ± 0.00*
18:2(n-9)	0.8 ± 0.1	0.7 ± 0.0	0.09 ± 0.01	0.05 ± 0.00*
20:2(n-9)	4.2 ± 0.0	5.4 ± 0.1*	0.47 ± 0.00	0.39 ± 0.01*
20:3(n-9)	2.7 ± 0.1	3.4 ± 0.0*	0.30 ± 0.01	0.25 ± 0.00*
C22(n-9)	0.2 ± 0.0	1.0 ± 0.0*	0.02 ± 0.00	0.07 ± 0.00*
Total (n-9) PUFA	7.9 ± 0.1	10.5 ± 0.1*	0.88 ± 0.01	0.76 ± 0.01*
18:2(n-6)	0.9 ± 0.1	0.7 ± 0.1	0.10 ± 0.01	0.05 ± 0.01*
18:3(n-6)	n.d.	n.d.	n.d.	n.d.
20:2(n-6)	0.3 ± 0.1	n.d.	0.03 ± 0.01	n.d.
20:3(n-6)	1.4 ± 0.0	0.5 ± 0.1*	0.16 ± 0.00	0.04 ± 0.0*
20:4(n-6)	3.8 ± 0.0	n.d.*	0.43 ± 0.00	n.d.*
C22(n-6)	0.4 ± 0.1	n.d.*	0.05 ± 0.01	n.d.*
Total (n-6) PUFA	6.8 ± 0.2	1.2 ± 0.2*	0.67 ± 0.02	0.09 ± 0.01*
18:3(n-3)	n.d.	n.d.	n.d.	n.d.
18:4(n-3)	n.d.	n.d.	n.d.	n.d.
20:4(n-3)	n.d.	n.d.	n.d.	n.d.
20:5(n-3)	0.3 ± 0.0	n.d.*	0.03 ± 0.00	n.d.*
22:5(n-3)	1.5 ± 0.0	n.d.*	0.17 ± 0.00	n.d.*
22:6(n-3)	2.6 ± 0.1	0.2 ± 0.0*	0.29 ± 0.01	0.01 ± 0.00*
Total (n-3) PUFA	4.4 ± 0.1	0.2 ± 0.0*	0.49 ± 0.01	0.01 ± 0.00*
Total dimethylacetals	4.9 ± 0.1	5.8 ± 0.4*	0.55 ± 0.01	0.42 ± 0.03*
Total PUFA	19.1 ± 0.2	11.9 ± 0.3*	2.04 ± 0.02	0.86 ± 0.02*
Total unidentified	4.4 ± 0.6	3.6 ± 0.4	0.49 ± 0.07	0.26 ± 0.03*

Results are means ± S.D. (n=3); n.d., not detected; PUFA, polyunsaturated fatty acids. * EPC-EFAD cells significantly different to EPC cells ($p < 0.05$) as determined by the Student's t-test.

Table 3. Effect of essential fatty acid deficiency on the incorporation of [1-^{14}C] polyunsaturated fatty acids (PUFA) into EPC cells.

	Incorporation (pmol / mg total lipid)			Incorporation (pmol / million cells)		
[1-^{14}C]PUFA	EPC	EPC-EFAD	Sign.	EPC	EPC-EFAD	Sign.
[1-^{14}C]18:3n-3	4.54 ± 0.40	7.81 ± 0.83	b	0.54 ± 0.05	0.64 ± 0.04	b
[1-^{14}C]18:2n-6	4.61 ± 0.59	6.19 ± 1.59	bc	0.47 ± 0.05	0.71 ± 0.13	b
[1-^{14}C]20:4n-6	3.28 ± 0.14	4.92 ± 0.78	c	0.32 ± 0.05	0.48 ± 0.04	c
[1-^{14}C]20:5n-3	6.77 ± 0.18	12.90 ± 0.69	a	0.72 ± 0.18	1.05 ± 0.02	a

Results are means ± SD (n = 3). For each way of expressing the data (pmol incorporated per mg total lipid or million cells), the significance of differences between cell types (EPC and EPC-EFAD) and treatments (labelled fatty acids) were analysed by two-way analysis of variance (ANOVA) and, where appropriate, Tukey's multiple comparison test. Incorporation of radioactive PUFA was significantly greater in EPC-EFAD cells ($p < 0.05$) irrespective of the way the incorporation was expressed. Differences between fatty acids for each way of expressing incorporation are indicated by letters, e.g. incorporation of [1-^{14}C]20:5n-3 was significantly greater than the incorporation of any other fatty acid whichever way that incorporation was expressed.

Table 4. Effect of essential fatty acid deficiency on the incorporation of [1-^{14}C] polyunsaturated fatty acids (PUFA) into phospholipid classes in EPC cells.

	[1-^{14}C]18:2n-6		[1-^{14}C]20:4n-6	
Lipid class	EPC	EPC-EFAD	EPC	EPC-EFAD
PC	35.7 ± 0.6	38.5 ± 0.2	11.0 ± 0.5	13.9 ± 0.2
PE	34.7 ± 0.6	24.3 ± 0.3	48.6 ± 2.2	34.2 ± 0.3
PS	10.1 ± 0.3	8.3 ± 0.2	3.3 ± 0.1	3.8 ± 0.1
PI	3.7 ± 0.1	8.5 ± 0.2	29.7 ± 2.2	40.6 ± 1.2
PA/CL	12.6 ± 0.5	15.9 ± 0.2	5.3 ± 0.6	6.1 ± 0.6
TP	96.8 ± 0.2	95.5 ± 0.2	97.9 ± 0.1	98.6 ± 0.2
TN	3.2 ± 0.2	4.5 ± 0.2	2.1 ± 0.1	1.4 ± 0.2

	[1-^{14}C]18:3n-3		[1-^{14}C]20:5n-3	
Lipid class	EPC	EPC-EFAD	EPC	EPC-EFAD
PC	33.2 ± 1.0	34.1 ± 0.4	19.7 ± 0.1	28.1 ± 1.4
PE	45.1 ± 0.9	39.1 ± 0.6	62.9 ± 0.4	50.7 ± 0.9
PS	7.5 ± 0.2	9.8 ± 0.2	9.2 ± 0.4	10.9 ± 0.2
PI	2.5 ± 0.0	3.0 ± 0.2	3.7 ± 0.4	4.3 ± 0.2
PA/CL	9.1 ± 0.2	11.6 ± 0.2	3.2 ± 0.5	4.3 ± 0.1
TP	97.4 ± 0.1	97.6 ± 0.2	98.7 ± 0.1	98.3 ± 0.2
TN	2.6 ± 0.1	2.4 ± 0.2	1.3 ± 0.1	1.7 ± 0.2

Results are expressed as percentages of total radioactivity recovered and are means ± SD (n = 3).
CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TN, total neutral lipids; TP, total polar lipids.

Table 5. Results of two-way analysis of variance (ANOVA) for the data in Table 4.

Lipid class	Effect of cell line	Effects of fatty acids	Interaction	Multiple range tests: cell line	Multiple range tests: fatty acids
PC	*	*	*	EPC < EPC-EFAD	18:2 > 18:3 > 20:5 > 20:4
PE	*	*	*	EPC > EPC-EFAD	20:5 > 18:3 = 20:4 > 18:2
PS	*	*	*	EPC < EPC-EFAD	20:5 > 18:2 > 18:3 > 20:4
PI	*	*	*	EPC < EPC-EFAD	20:4 > 18:2 > 20:5 = 18:3
PA/CL	*	*	*	EPC < EPC-EFAD	18:2 > 18:3 > 20:4 > 20:5
TP	*	*	*	EPC > EPC-EFAD	20:5 > 20:4 > 18:3 > 18:2
TN	*	*	*	EPC < EPC-EFAD	18:2 > 18:3 > 20:4 > 20:5

CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TN, total neutral lipids; TP, total polar lipids.
*, significant at $p < 0.05$.

Table 6. Effect of essential fatty acid deficiency on the metabolism of [1-^{14}C] polyunsaturated fatty acids (PUFA) by desaturation and elongation in EPC cells.

	[1-^{14}C]18:2n-6		[1-^{14}C]20:4n-6	
Fatty acid	EPC	EPC-EFAD	EPC	EPC-EFAD
18:2n-6	74.4 ± 0.8	55.0 ± 0.5*	n.d.	n.d.
20:2n-6	8.5 ± 0.6	10.5 ± 0.2*	n.d.	n.d.
18:3n-6	2.3 ± 0.1	3.9 ± 0.2*	n.d.	n.d.
20:3n-6	14.3 ± 0.2	24.8 ± 0.5*	n.d.	n.d.
22:3n-6	t	0.5 ± 0.1*	n.d.	n.d.
20:4n-6	0.5 ± 0.1	2.6 ± 0.1*	96.3 ± 0.9	94.9 ± 0.8
22:4n-6	n.d.	2.5 ± 0.2*	1.5 ± 0.4	1.9 ± 0.3
24:4n-6	n.d.	n.d.	1.9 ± 0.5	2.0 ± 0.2
22:5n-6	n.d.	0.2 ± 0.1	0.3 ± 0.1	1.2 ± 0.4*
	[1-^{14}C]18:3n-3		[1-^{14}C]20:5n-3	
Fatty acid	EPC	EPC-EFAD	EPC	EPC-EFAD
18:3n-3	21.1 ± 0.3	17.6 ± 0.5*	n.d.	n.d.
20:3n-3	17.6 ± 0.8	13.5 ± 0.6*	n.d.	n.d.
18:4n-3	7.0 ± 0.1	8.2 ± 0.4*	n.d.	n.d.
20:4n-3	28.4 ± 1.0	18.2 ± 0.7*	n.d.	n.d.
22:4n-3	4.1 ± 0.5	1.9 ± 0.2*	n.d.	n.d.
20:5n-3	8.1 ± 0.3	13.7 ± 0.4*	29.8 ± 3.5	39.3 ± 1.3*
22:5n-3	11.0 ± 0.6	15.0 ± 0.1*	40.7 ± 0.1	32.0 ± 1.2*
24:5n-3	n.d.	n.d.	17.8 ± 0.4	6.7 ± 0.2*
22:6n-3	2.7 ± 0.2	11.9 ± 0.2*	11.7 ± 4.0	22.1 ± 0.9*

Results are expressed as percentages of total radioactivity recovered in total lipid and are means ± SD (n = 3). Differences between values for EPC and EPC-EFAD cells for each [1-^{14}C] PUFA were significantly different where indicated (*) as determined by the Student t-test ($p < 0.05$).
n.d., not detected.

Table 7. Products of desaturase and elongase activities in EPC and EPC-EFAD cells incubated with various ^{14}C-labelled polyunsaturated fatty acids

	Δ6 desaturase products		C_{18-20} elongase products		Δ5 desaturase products		C_{20-22} elongase products		n-3hexaene/n-6pentaene products	
Fatty acid	EPC	EPC-EFAD	EPC	EPC-EFAD	EPC	EPC-EFAD	EPC	EPC-EFAD	EPC	EPC-EFAD
[1-^{14}C]18:2n-6	17.1	34.5	23.3	41.1	0.5	5.3	n.d.	2.7	n.d.	0.2
[1-^{14}C]18:3n-3	61.3	68.9	71.9	74.2	21.8	40.6	13.7	26.9	2.7	11.9
[1-^{14}C]20:4n-6	-	-	-	-	-	-	3.7	5.1	0.3	1.2
[1-^{14}C]20:5n-3	-	-	-	-	-	-	70.2	60.8	11.7	22.1

Results are presented as percentages of total radioactivity recovered. All fatty acid metabolites for a given step in the pathway were summed, irrespective of subsequent metabolism by another activity (e.g. for [1-^{14}C]18:2n-6, "Δ6 products" represents the sum of radioactivity recovered as 18:3n-6, 20:3n-6, 22:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6). n.d., not detected.