Essential fatty acid deficiency in freshwater fish: The effects of linoleic, α-linolenic, γ-linolenic and stearidonic acids on the metabolism of [1-^{14}C]18:3n-3 in a carp cell culture model

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Abbreviations: ANOVA, analysis of variance; BHT, butylated hydroxytoluene; BSA, bovine serum albumin;

CL, cardiolipin; EFA, essential fatty acid; EFAD, essential fatty acid deficient; 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 Ca^{2+} and Mg^{2+}); 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

The desaturation of [1-^{14}C]18:3n-3 to 20:5n-3 and 22:6n-3 is enhanced in an essential fatty acid deficient cell

line (EPC-EFAD) in comparison with the parent cell line (EPC) from carp. In the present study, the effects of competing, unlabeled C_{18} polyunsaturated fatty acids (PUFA), linoleic (18:2n-6), α-linolenic (18:3n-3), γ-linolenic (18:3n-6) and stearidonic (18:4n-3) acids, on the metabolism of [1-^{14}C]18:3n-3 were investigated in EPC-EFAD cells in comparison with EPC cells. The incorporation of [1-^{14}C]18:3n-3 in both cell lines was significantly reduced by competing C_{18} PUFA, with the rank order being 18:4n-3 > 18:3n-3 = 18:2n-6 > 18:3n-6. In the absence of competing PUFA, radioactivity from [1-^{14}C]18:3n-3 in EPC cells was predominantly recovered in phosphatidylethanolamine followed by phosphatidylcholine. This pattern was unaffected by competing n-6PUFA, but n-3PUFA reversed this pattern as did essential fatty acid deficiency in the presence of all competing PUFA. The altered lipid class distribution was most pronounced in cells supplementedwith 18:4n-3. Competing C_{18} PUFA significantly decreased the proportions of radioactivity recovered in 22:6n-3, pentaene and tetraene products, with the proportions of radioactivity recovered in 18:3n-3 and 20:3n-3 increased, in both cell lines. However, the inhibitory effect of competing C_{18} PUFA on the desaturation of [1-^{14}C]18:3n-3 was significantly greater in EPC-EFAD cells. The magnitude of the inhibitory effects of C_{18} PUFA on [1-^{14}C]18:3n-3 desaturation was dependent upon the specific fatty acid with the rank order being 18:4n-3 >18:3n-3 >18:2n-6, with 18:3n-6 having little inhibitory effect on the metabolism of [1-^{14}C]18:3n-3 in EPC cells. The differential effects of the C_{18} PUFA on [1-^{14}C]18:3n-3 metabolism were consistent with mass competition in combination with increased desaturation activity in EPC-EFAD cells and the known substrate fatty acid specificities of desaturase enzymes. However, the mechanism underpinning the greater efficacy with which the unlabeled C_{18} PUFA competed with [1-^{14}C]18:3n-3 in the desaturation pathway in EPC-EFAD cells was unclear.

Introduction

The essential fatty acid (EFA) requirement for freshwater fish can be met by the C_{18} polyunsaturated fatty acids

(PUFA), α-linolenic (18:3n-3) and/or linoleic (18:2n-6) acid at levels of approximately 1-2% of the diet (Sargent et al. 1995). Specifically, in common carp (*Cyprinus carpio*), it was determined that 1.0% each of 18:3n-3 and 18:2n-6 were required (Takeuchi and Watanabe 1977). However, there is no evidence to support either of the C_{18} EFA having an essential role themselves in freshwater fish in a similar way that 18:2n-6 has in mammals as an essential component of the lipids providing the water barrier in skin (Hansen 1986). The fact that in marine fish the EFA requirement can only be met by C_{20} and/or C_{22} highly unsaturated fatty acids (HUFA) and that the EFA requirement in rainbow trout can be supplied by n-3HUFA alone at lower levels than 18:3n-3 indicate that HUFA (20:5n-3, 22:6n-3 and 20:4n-6) have the essential roles in fish and that the role of C_{18} EFAs is solely as the metabolic precursors of HUFA (Sargent et al. 1995).

Cultured cell lines have often been utilized in the study of lipid and fatty acid metabolism in mammals and lines have been developed that can grow in either serum-free medium or delipidated serum which has enabled the effects of EFA deficiency to be investigated *in vitro* (Laposata et al. 1982; Marcelo et al. 1992; Lerner et al. 1995). We developed a fish cell line (EPC-EFAD) derived from the carp epithelial papilloma line, EPC, that can survive and proliferate in an essential fatty acid-deficient (EFAD) medium based on delipidated serum (Tocher et al. 1995). The EPC-EFAD cells contain only very low levels of n-3 and n-6PUFA and HUFA and displayed a slower growth rate than EPC cells which was not stimulated by supplementing the culture medium with C_{18} PUFA, although it was stimulated by supplementation with C_{20} and C_{22} HUFA (Tocher et al. 1996). Recently, we studied the metabolism of ^{14}C-labelled C_{18} and C_{20} PUFA in EPC-EFAD cells in comparison with EPC cells (Tocher and Dick 1999). Both cell lines desaturated[1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3 to a greater extent than [1-^{14}C]18:2n-6 and [1-^{14}C]20:4n-6 but the desaturation of all the ^{14}C-labeled PUFA was significantly greater in EPC-EFAD cells compared to EPC cells (Tocher and Dick 1999). The results suggested that all the steps in the desaturation/elongation pathway were enhanced in EPC-EFAD cells although the metabolism of [1-^{14}C]18:3n-3 was primarily increased in EPC-EFAD cells at the level of Δ5 desaturase and subsequent steps in the pathway. However, the results could not fully explain the inability of C_{18} PUFA, compared to $C_{20/22}$ HUFA, to stimulate growth in EPC-EFAD cells.

In the present study, the effects of competing C_{18} PUFA on the metabolism of [1-^{14}C]18:3n-3 were investigated in EPC and EPC-EFAD cells. Specifically both cell lines were incubated with [1-^{14}C]18:3n-3 in the absence or presence of unlabeled linoleic (18:2n-6), α-linolenic (18:3n-3), γ-linolenic (18:3n-6) and

stearidonic (18:4n-3) acids and the effects on incorporation, lipid class distribution, desaturation and elongation determined.

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 over years and were at passage number 54-58.

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, and 2 x 10^5 $cells.cm^{-2}$, respectively, 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 [1- ^{14}C]18:3n-3 with or without supplementary unlabeled 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 serum or delipidated serum, was added. The [1-^{14}C]18:3n-3 (0.2 μCi per flask; concentration, 0.8 μM) was added to the cell cultures bound to fatty acid-free bovine serum albumin (BSA) in PBS (Ghioni et al. 1997) and incubation continued for 6 days at 22 ºC. For experiments performed in the presence of unlabeled PUFA, the supplementary PUFA were also added as fatty acid-free BSA complexes. Flasks received 20 μM unlabeled PUFA, 24 h before the addition of [1-^{14}C]18:3n-3 and incubation continued at 22 ºC for a further 6 days. Flasks incubated without supplementary unlabelled PUFA received an equal volume of fatty acid-free BSA-PBS.

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 first with 5 ml ice-cold HBSS and then with 5 ml HBSS containing 1% fatty acid-free BSA. Total lipid was extracted from the cell pellets with 5 ml ice-cold chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, essentially 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, U.S.A.) 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 μg) 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 vapour 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.

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, samples resuspended in 100 μl hexane containing BHT, loaded as 1 cm streaks on HPTLC plates and 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 6 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.

Materials

[1-^{14}C]18:3n-3 (50 mCi.mmol^{-1} and 99% pure) was obtained from NEN Life Science Products (Hounslow, U.K.). Unlabeled PUFA (all approx. 99% pure except for 18:4n-3, 90%), 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) to determine the significance of effects due to cell line and fatty acid supplement, and where appropriate the significance of differences were determined by Tukey's post-test. Percentage data were subjected to arc-sin transformation prior to further statistical analysis. 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

Net "incorporation" is based on the recovery of radioactivity and will be dependent on the initial uptake of fatty acid less the amount of fatty acid oxidized or lost through other metabolic pathways. As all concentrations used were the same, the amount taken up and oxidized will only be dependent on acid and cell line, not on concentration. Two-way analysis of variance (ANOVA) showed that the incorporation of [1-^{14}C]18:3n-3 was significantly greater in EPC-EFAD cells irrespective of the presence of unlabeled C_{18} PUFA (Table 1). Irrespective of cell line, i.e. EPC or EPC-EFAD, the incorporation of [1-^{14}C]18:3n-3 was significantly greater in the absence of unlabeled PUFA. The rank order for inhibition of the incorporation of [1-^{14}C]18:3n-3 being 18:4n-3 > 18:3n-3 = 18:2n-6 > 18:3n-6 (Table 1).

The distribution of radioactivity from [1-^{14}C]18:3n-3 recovered in EPC and EPC-EFAD cell lipids is shown in Table 2. Two-way ANOVA showed that there were significant differences in this distribution between the two cell lines, and that inclusion of unlabeled C_{18} PUFA also significantly affected the distribution of radioactivity recovered in the cell lipids (Table 3). In the absence of unlabeled PUFA, radioactivity from [1-^{14}C]18:3n-3 in EPC cells was predominantly recovered in PE followed by PC (Table 2). This pattern was unaffected by the inclusion of unlabeled n-6 PUFA but inclusion of unlabeled n-3 PUFA reversed this pattern (recovery in PC > PE) as did EFA deficiency in the presence of all unlabeled PUFA. Overall, the proportion of radioactivity recovered in PE, PS, PA/CL and total polar lipids was significantly lower in EPC-EFAD cells compared to EPC cells, whereas the proportion of radioactivity recovered in PC and total neutral lipid was greater in EPC-EFAD cells (Tables 2 & 3). The altered distribution was most pronounced in cells supplementedwith 18:4n-3 as evidenced by the fact that it was generally at the opposite end of the rank order to unsupplemented cells (none) in Table 3.

The metabolism of [1-^{14}C]18:3n-3 by desaturation and elongation in EPC and EPC-EFAD cells is shown in Table 4. The statistically correct analysis for the whole data set in Table 4 is two-way ANOVA where the effects of both factors, EFA deficiency and the presence of unlabeled PUFA, and their interaction can be determined. This showed that the recovery of radioactivity in all elongated and desaturated products of 18:3n-3 was significantly decreased, and the proportion of radioactivity recovered as 18:3n-3 significantly increased, in EPC-EFAD cells compared to EPC cells (Table 5). Similarly, the presence of unlabeled PUFA significantly decreased the proportions of radioactivity recovered in 22:6n-3 and almost all tetraene, pentaene products in both cell lines with the proportions of radioactivity recovered in 18:3n-3 and its immediate elongation product, 20:3n-3, increased (Tables 4 & 5). The magnitude of the above effects were dependent upon the specific unlabeled fatty acid used with the rank order for the decreased recovery of radioactivity in all desaturated metabolites of [1-^{14}C]18:3n-3 generally being 18:4n-3 >18:3n-3 >18:2n-6 > 18:3n-6.

However, it obvious from the data in Table 4 that the effect of EFA deficiency is different in the presence or absence of added unlabeled PUFA and, similarly, the effect of added unlabeled 18:3n-6 is different in EPC cells compared to EPC-EFAD cells. Individual differences such as these can be masked by the nature of two-way ANOVA where data are combined before comparison. Therefore, the fact that EFA deficiency clearly increased the desaturation and elongation of [1-^{14}C]18:3n-3 in EPC cells in the absence of competing PUFA,

with the proportions of radioactivity recovered in 20:5n-3, 22:5n-3 and 22:6n-3 all significantly greater and the proportions recovered in trienes and tetraenes significantly reduced, in EPC-EFAD cells compared to EPC cells, was not obvious from the two-way ANOVA analysis (Table 4). The presence of unlabeled C_{18} PUFA completely reversed this effect of EFA deficiency, with the desaturation and elongation of [1-^{14}C]18:3n-3 being greatly reduced in EPC-EFAD cells in the presence of unlabeled C_{18} PUFA, an effect that dominated the ANOVA analysis (Tables 4 and 5). This effect of EFA deficiency also dominated the ANOVA analysis of the effects of added unlabeled PUFA. Therefore, it is noteworthy that in the absence of EFA deficiency, the effects of unlabeled C_{18} PUFA were dependent on the specific PUFA with 18:3n-3, 18:4n-3 and 18:2n-6 all greatly inhibiting desaturation and elongation of [1-^{14}C]18:3n-3 whereas 18:3n-6 significantly increased the production of 22:5n-3 and 22:6n-3 in EPC cells (Table 4).

Discussion

There were two very interesting effects observed in this study. The first is that the different C_{18} PUFA had differential effects on the metabolism of [1-^{14}C]18:3n-3, with 18:2n-6, 18:3n-3 and 18:4n-3 all inhibiting desaturation whereas 18:3n-6 increased desaturation of [1-^{14}C]18:3n-3 in EPC cells. The second is that EFA deficiency had opposite effects in cells in the absence of C_{18} PUFA compared to cells in the presence of C_{18} PUFA. That is, in the absence of supplemental C_{18} PUFA, EFA deficiency increased the desaturation of [1-^{14}C]18:3n-3 to 20:5n-3, 22:5n-3 and 22:6n-3 as evidenced by the increased proportions of radioactivity recovered in those fatty acid fractions in EPC-EFAD cells. In contrast, in the presence of supplemental C_{18} PUFA, EFA deficiency considerably reduced desaturation of [1-^{14}C]18:3n-3.

In order to interpret the data correctly, it is important to note that the results are not simply reflecting dilution of the isotope with cold fatty acids, lowering the specific activity so that the counts recovered in each fraction are decreased. For instance, EPC and EPC-EFAD both have very little 18:3n-3 in their lipids (Tocher et al. 1995), and so the increased flux in the fatty acid desaturation/elongation pathway in EPC-EFAD cells is not due to less dilution of the isotope in EPC-EFAD compared to EPC cells. When cold 18:3n-3, 18:4n-3 and 18:2n-6 are added there is decreased recovery of radioactivity in the products of [1-^{14}C]18:3n-3 metabolism in both EPC and EPC-EFAD cells. Dilution of the isotope and competitive effects will contribute to this.

However, the recovery of radioactivity in the products of [1-^{14}C]18:3n-3 metabolism in EPC cells was increased in the presence of 18:3n-6, and this cannot be explained by dilution or competitive effects. Isotope dilution would be identical in both cell lines as the same concentration of isotope was used and the same concentration of C_{18} PUFA added to both EPC and EPC-EFAD cells and so simple dilution would have identical effects in both EPC and EPC-EFAD cells. The results expected in that case would be that EPC-EFAD cells would show a reduced flux in the pathway but that it would still be greater than in EPC cells. These are not the results obtained in the present study, in which the increased flux through the pathway observed in EPC-EFAD cells compared to EPC cells in the absence of added PUFA was completely reversed such that the metabolism of [1-^{14}C]18:3n-3 in EPC-EFAD cells was very significantly lower than in EPC cells. Therefore, other factors are involved in the reduced metabolism of ^{14}C-18:3n-3 in EPC-EFAD cells in the presence of unlabeled C_{18} PUFA.

The precise mechanism for the decreased desaturation of [1-^{14}C]18:3n-3 in the presence of unlabeled C_{18} PUFA being greater in EPC-EFAD cells compared to EPC cells is unclear. The incorporation data suggest that in EPC-EFAD cells there may be greater uptake of the C_{18} PUFA and, therefore, more available in the cell to compete. However, there would similarly be more [1-^{14}C]18:3n-3 present in EPC-EFAD cells negating this effect, i.e. both EPC and EPC-EFAD would have a similar relative proportion of [1-^{14}C]18:3n-3 and unlabeled competing PUFA. Therefore, although differences in the amount of competing unlabeled PUFA may play a role it is unlikely to be responsible for such a large difference between EPC and EPC-EFAD cells. In addition, the flux in the pathway for the desaturation of [1-^{14}C]18:3n-3 is greater in EPC-EFAD cells and this may also apply to other substrates resulting in the unlabeled C_{18} PUFA competing more effectively. However, an earlier study showed that [1-^{14}C]18:3n-3 was a much better substrate for the pathway than [1-^{14}C]18:2n-6 in both EPC or EPC-EFAD cells and so there is little evidence for higher activity of the pathway in EPC-EFAD playing a role in the greater efficacy of inhibition (Tocher and Dick 1999). The completely opposite effect of 18:3n-6 in EPC and EPC-EFAD cells also suggests that the effects of EFA deficiency go beyond simple differences in metabolic substrate concentrations.

A relative reduction in the proportion of [1-^{14}C]18:3n-3 desaturated must concomitantly result in either increased proportions of radioactivity recovered unmetabolized (as [1-^{14}C]18:3n-3) or elongated to the so-called "dead-end" product 20:3n-3. Therefore, it was noteworthy that the reduction in desaturation of [1-^{14}C]18:3n-3 by 18:2n-6, 18:3n-3 and 18:4n-3 in EPC cells was accompanied specifically by increased elongation to 20:3n-3.

In contrast, the reduced desaturation of [1-^{14}C]18:3n-3 in EPC-EFAD cells supplemented with C_{18} PUFA, compared to EPC cells supplemented with C_{18} PUFA, was accompanied by very increased proportions of [1-^{14}C]18:3n-3 recovered unmetabolized. Again this illustrates the wide ranging effects of EFA deficiency in the cells.

A consistent effect of EFA deficiency was that [1-^{14}C]18:3n-3 was acylated into PC rather than PE and this effect was primarily observed in cells supplemented with C_{18} PUFA. However, this cannot play a major role in the greater inhibition of [1-^{14}C]18:3n-3 metabolism by C_{18} PUFA in EPC-EFAD cells as supplementation with unlabeled 18:3n-3 and 18:4n-3 also altered the distribution of [1-^{14}C]18:3n-3 from PE to PC in EPC cells and it was with 18:3n-3 and 18:4n-3 supplementation that the greatest difference between EPC and EPC-EFAD cells was observed with respect to [1-^{14}C]18:3n-3 desaturation.

A very interesting result from the present study is that, in complete contrast to the other C_{18} PUFA, γ-linolenic acid (18:3n-6) did not inhibit desaturation of [1-^{14}C]18:3n-3 in EPC cells and rather increased the recovery of radioactivity in 22:6n-3 and 22:5n-3. Previously, we had shown that the desaturation of both [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 in Atlantic salmon hepatocytes was stimulated by feeding borage oil, containing 18:3n-6, compared to diets containing fish oil (Tocher et al. 1997). This effect was not due to reduced dietary C_{20} and C_{22} PUFA in the borage oil diet as other vegetable oil diets containing sunflower or olive oils failed to stimulate desaturation. The effects of dietary 18:3n-6 in rats has given conflicting results with no effect (Hoy et al. 1983), depressed (Blond et al. 1986; Choi and Sugano 1988; Ullman et al. 1991) and increased (Ullman et al. 1991) desaturase activities all reported. The different fatty acid compositions of the oils used (evening primrose and borage oils) and the dffering ages of the animals used in these mammalian trials have contributed to the conflicting data obtained. However, rat liver Δ6-desaturase activity decreases with age (Brenner 1981) and dietary supplementation with 18:3n-6 in the form of evening primrose oil could reverse this effect (Biagi et al. 1991).

The present study also demonstrated the potential value of a cell line containing little in the way of endogenous PUFA/HUFA. In particular, the way in which specific C_{18} PUFA competed with [1-^{14}C]18:3n-3 in the desaturation/elongation pathway were clearer in the EPC-EFAD cells. Thus, 18:4n-3 was very effective in inhibiting the conversion of 18:3n-3 to 20:3n-3, carried out by the C_{18-20} elongase for which 18:4n-3 is a direct substrate. A qualitatively similar effect was observed with 18:3n-6. On the other hand, supplemental 18:2n-6

and 18:3n-3 inhibited the first destauration step (Δ6 desaturase) rather than the elongation to 20:3n-3. The effects of 18:2n-6 and 18:3n-3 on inhibiting desaturation rather than elongation were also observed in EPC cells but the effects of 18:3n-6 and 18:4n-3 were not so clear in EPC cells where the endogenous complement of PUFA and HUFA combined with the exogenously added C_{18} PUFA in exerting effects.

In conclusion, the differences in fatty acid metabolism observed in EPC-EFAD cells cannot be explained simply by differences in PUFA composition affecting their roles as substrates and intermediates in metabolic pathways. Indeed, perhaps as expected, the results suggested that the metabolic effects of EFA deficiency are more wide ranging and probably highly dependent upon physicochemical parameters and effects on membrane fatty acid composition which can directly affect the activities of membrane-bound enzymes, receptors and other proteins.

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Table 1. Effect of essential fatty acid deficiency on the incorporation of [1-^{14}C]18:3n-3 into EPC cells and the effects of supplementation with unlabeled C_{18} PUFA.

	Incorporation (pmol / million cells)		
Unlabeled PUFA	EPC	EPC-EFAD	Sign.
none	0.54 ± 0.05	0.64 ± 0.04	a
18:2n-6	0.17 ± 0.03	0.33 ± 0.08	bc
18:3n-6	0.20 ± 0.03	0.43 ± 0.05	b
18:3n-3	0.15 ± 0.01	0.31 ± 0.04	bc
18:4n-3	0.11 ± 0.02	0.17 ± 0.02	c

Results are means ± SD (n = 3). The significance of differences between cell types (EPC and EPC-EFAD) and treatments (unlabelled fatty acids) were analysed by two-way analysis of variance (ANOVA) and Tukey's multiple comparison test. Incorporation of radioactive PUFA was significantly greater in EPC-EFAD cells ($p < 0.05$). Differences between treatments are indicated by letters.

Table 2. Effect of C_{18} polyunsaturated fatty acids (PUFA) on the incorporation of [1-^{14}C] 18:3n-3 into phospholipid classes in EPC and EPC-EFAD cells.

	none		18:2n-6		18:3n-6	
Lipid class	EPC	EPC-EFAD	EPC	EPC-EFAD	EPC	EPC-EFAD
PC	33.2 ± 1.0	34.1 ± 0.4	37.2 ± 1.0	37.6 ± 0.9	36.3 ± 0.5	51.0 ± 5.0
PE	45.1 ± 0.9	39.1 ± 0.6	44.2 ± 1.4	23.4 ± 0.8	44.1 ± 0.6	25.0 ± 1.1
PS	7.5 ± 0.2	9.8 ± 0.2	8.2 ± 0.7	5.9 ± 0.4	6.6 ± 0.2	4.0 ± 0.2
PI	2.5 ± 0.0	3.0 ± 0.2	2.7 ± 0.3	2.0 ± 0.2	2.2 ± 0.1	2.3 ± 0.5
PA/CL	9.1 ± 0.2	11.6 ± 0.2	4.1 ± 0.3	5.1 ± 0.5	7.6 ± 0.3	9.5 ± 1.1
TP	97.4 ± 0.1	97.6 ± 0.2	96.4 ± 0.3	74.0 ± 2.5	96.8 ± 0.1	91.8 ± 2.4
TN	2.6 ± 0.1	2.4 ± 0.2	3.6 ± 0.3	26.0 ± 2.5	3.2 ± 0.1	8.2 ± 2.4

	18:3n-3		18:4n-3	
Lipid class	EPC	EPC-EFAD	EPC	EPC-EFAD
PC	46.4 ± 0.9	46.8 ± 0.5	43.1 ± 1.1	57.0 ± 1.5
PE	36.2 ± 0.6	27.9 ± 0.5	29.3 ± 1.2	17.3 ± 1.0
PS	5.9 ± 0.4	5.8 ± 0.2	4.3 ± 0.2	2.7 ± 0.3
PI	2.6 ± 0.2	1.9 ± 0.2	2.6 ± 0.2	3.3 ± 0.2
PA/CL	6.1 ± 0.1	5.0 ± 0.3	15.7 ± 0.3	3.6 ± 0.3
TP	96.2 ± 0.1	87.4 ± 0.9	95.0 ± 0.2	83.9 ± 1.0
TN	3.8 ± 0.1	12.6 ± 0.9	5.0 ± 0.2	16.1 ± 1.0

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 2. Effect of C_{18} polyunsaturated fatty acids (PUFA) on the incorporation of [1-^{14}C] 18:3n-3 into phospholipid classes in EPC and EPC-EFAD cells.

	none		18:2n-6		18:3n-6	
Lipid class	EPC	EPC-EFAD	EPC	EPC-EFAD	EPC	EPC-EFAD
PC	33.2 ± 1.0	34.1 ± 0.4	37.2 ± 1.0	37.6 ± 0.9	36.3 ± 0.5	51.0 ± 5.0
PE	45.1 ± 0.9	39.1 ± 0.6	44.2 ± 1.4	23.4 ± 0.8	44.1 ± 0.6	25.0 ± 1.1
PS	7.5 ± 0.2	9.8 ± 0.2	8.2 ± 0.7	5.9 ± 0.4	6.6 ± 0.2	4.0 ± 0.2
PI	2.5 ± 0.0	3.0 ± 0.2	2.7 ± 0.3	2.0 ± 0.2	2.2 ± 0.1	2.3 ± 0.5
PA/CL	9.1 ± 0.2	11.6 ± 0.2	4.1 ± 0.3	5.1 ± 0.5	7.6 ± 0.3	9.5 ± 1.1
TP	97.4 ± 0.1	97.6 ± 0.2	96.4 ± 0.3	74.0 ± 2.5	96.8 ± 0.1	91.8 ± 2.4
TN	2.6 ± 0.1	2.4 ± 0.2	3.6 ± 0.3	26.0 ± 2.5	3.2 ± 0.1	8.2 ± 2.4

	18:3n-3		18:4n-3	
Lipid class	EPC	EPC-EFAD	EPC	EPC-EFAD
PC	46.4 ± 0.9	46.8 ± 0.5	43.1 ± 1.1	57.0 ± 1.5
PE	36.2 ± 0.6	27.9 ± 0.5	29.3 ± 1.2	17.3 ± 1.0
PS	5.9 ± 0.4	5.8 ± 0.2	4.3 ± 0.2	2.7 ± 0.3
PI	2.6 ± 0.2	1.9 ± 0.2	2.6 ± 0.2	3.3 ± 0.2
PA/CL	6.1 ± 0.1	5.0 ± 0.3	15.7 ± 0.3	3.6 ± 0.3
TP	96.2 ± 0.1	87.4 ± 0.9	95.0 ± 0.2	83.9 ± 1.0
TN	3.8 ± 0.1	12.6 ± 0.9	5.0 ± 0.2	16.1 ± 1.0

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 3. Results of two-way analysis of variance (ANOVA) for data in Table 2 for the effect of unlabeled C_{18} PUFA on the incorporation of radioactivity from [1-^{14}C]18:3n-3 into phospholipid classes.

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	18:4 >18:3n-6 = 18:3n-3 > 18:2 > none
PE	*	*	*	EPC > EPC-EFAD	none >18:2 = 18:3n-6 > 18:3n-3 > 18:4
PS	*	*	*	EPC> EPC-EFAD	none >18:2 > 18:3n-6 = 18:3n-3 > 18:4
PI	ns	*	*	-	none =18:4 > 18:2 = 18:3n-6 = 18:3n-3
PA/CL	*	*	*	EPC > EPC-EFAD	none =18:4 > 18:3n-6 > 18:3n-3 > 18:2
TP	*	*	*	EPC > EPC-EFAD	none =18:3n-6 > 18:3n-3 = 18:4 > 18:2
TN	*	*	*	EPC < EPC-EFAD	18:2 > 18:4 > 18:3n-3 > 18:3n-6 >none

CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TN, total neutral lipids; TP, total polar lipids.

*, significant at $p < 0.05$; ns, not significant.

Table 4. Effect of C_{18} polyunsaturated fatty acids (PUFA) on the metabolism of [1-^{14}C] 18:3n-3 by desaturation and elongation in EPC and EPC-EFAD cells.

	none		18:2n-6		18:3n-6	
Fatty acid	EPC	EPC-EFAD	EPC	EPC-EFAD	EPC	EPC-EFAD
18:3n-3	21.1 ± 0.3	17.6 ± 0.5*	15.7 ± 1.3	48.5 ± 1.0	16.7 ± 0.4*	62.0 ± 3.2
20:3n-3	17.6 ± 0.8	13.5 ± 0.6*	43.1 ± 1.1	42.8 ± 0.7	15.6 ± 0.5	21.2 ± 4.7
18:4n-3	7.0 ± 0.1	8.2 ± 0.4*	5.8 ± 0.6	0.4 ± 0.1	10.6 ± 0.3*	2.4 ± 0.2
20:4n-3	28.4 ± 1.0	18.2 ± 0.7*	18.1 ± 0.3	5.7 ± 0.7	23.8 ± 0.5*	9.5 ± 2.0
22:4n-3	4.1 ± 0.5	1.9 ± 0.2*	7.8 ± 0.1	1.8 ± 0.1	5.9 ± 0.1*	2.2 ± 0.3
20:5n-3	8.1 ± 0.3	13.7 ± 0.4*	1.9 ± 0.2	0.3 ± 0.0	8.4 ± 0.5	1.0 ± 0.1
22:5n-3	11.0 ± 0.6	15.0 ± 0.1*	6.7 ± 0.8	0.3 ± 0.1	14.7 ± 0.8*	1.0 ± 0.2
22:6n-3	2.7 ± 0.2	11.9 ± 0.2*	0.9 ± 0.2	0.2 ± 0.0	4.3 ± 0.2*	0.7 ± 0.4

	18:3n-3		18:4n-3	
Fatty acid	EPC	EPC-EFAD	EPC	EPC-EFAD
18:3n-3	24.6 ± 1.9	46.1 ± 2.2	30.7 ± 1.9	77.8 ± 3.2
20:3n-3	54.7 ± 1.5	51.5 ± 2.4	57.2 ± 0.9	18.1 ± 3.1
18:4n-3	1.9 ± 0.3	0.1 ± 0.1	1.1 ± 0.3	0.3 ± 0.1
20:4n-3	11.7 ± 0.7	1.8 ± 0.2	6.7 ± 0.7	2.8 ± 0.3
22:4n-3	4.1 ± 0.4	0.4 ± 0.1	2.0 ± 0.2	0.7 ± 0.1
20:5n-3	1.0 ± 0.1		0.8 ± 0.2	
22:5n-3	1.4 ± 0.3	0.1 ± 0.1	0.9 ± 0.2	0.3 ± 0.2
22:6n-3	0.6 ± 0.3		0.6 ± 0.2	

Results are expressed as percentages of total radioactivity recovered and are means ± SD (n = 3). Two sets of students t-tests were performed, EPC (none) versus EPC-EFAD (none) and EPC (none) versus EPC (+18:3n-6). Significant differences ($p < 0.05$) between the values for EPC-EFAD (none) and EPC (+18:3n-6) and the values for EPC (none) were marked (*).

Table 5. Results of two-way analysis of variance (ANOVA) for data in Table 4 for the effect of unlabeled C_{18} PUFA on the desaturation and elongation of [1-^{14}C]18:3n-3.

Fatty acid	Effect of cell line	Effects of fatty acids	Interaction	Multiple range tests: cell line	Multiple range tests: fatty acids
18:3 >none	*	*	*	EPC < EPC-EFAD	18:4 > 18:3n-6 > 18:3n-3 = 18:2
20:3 >none	*	*	*	EPC > EPC-EFAD	18:3n-3 > 18:2 > 18:4 > 18:3n-6
18:4 18:4	*	*	*	EPC > EPC-EFAD	none >18:3n-6 > 18:2 > 18:3n-3 =
20:4 18:4	*	*	*	EPC > EPC-EFAD	none >18:3n-6 > 18:2 > 18:3n-3 >
22:4 18:4	*	*	*	EPC > EPC-EFAD	18:2 >18:3n-6 = none >18:3n-3 >
20:5 18:4	*	*	*	EPC > EPC-EFAD	none >18:3n-6 > 18:2 >18:3n-3 =
22:5 18:4	*	*	*	EPC > EPC-EFAD	none >18:3n-6 > 18:2 >18:3n-3 >
22:6 =18:4	*	*	*	EPC > EPC-EFAD	none >18:3n-6 > 18:2 = 18:3n-3

*, significant at $p < 0.05$.