Fatty Acid Metabolism in Marine Fish: Low Activity of Fatty Acyl Δ5 Desaturation in Gilthead Sea Bream (*Sparus aurata*) Cells

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Abbreviations: ANOVA, analysis of variance; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CL, cardiolipin; 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; HUFA, highly unsaturated fatty acids (≥ C_{20} with ≥ 3 double bonds); 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: Marine fish are known to have an absolute dietary requirement for C_{20} and C_{22} highly unsaturated fatty acids. Previous studies using cultured cell lines indicated that underlying this requirement in marine fish was either a deficiency in fatty acyl Δ5 desaturase or $C_{18\text{-}20}$ elongase activity. Recently, Ghioni et al. (Biochim. Biophys. Acta, 1437, 170-181, 1999) presented evidence that in turbot cells there was low activity of $C_{18\text{-}20}$ elongase whereas Δ5 desaturase had high activity. In the present study, the fatty acid desaturase/elongase pathway was investigated in a cell line (SAF-1) from another carnivorous marine fish, sea bream. The metabolic conversions of a range of radiolabelled polyunsaturated fatty acids that comprised the direct substrates for Δ6 desaturase ([1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3), $C_{18\text{-}20}$ elongase ([U-^{14}C]18:4n-3), Δ5 desaturase ([1-^{14}C]20:3n-6 and [U-^{14}C]20:4n-3) and $C_{20\text{-}22}$ elongase ([1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3) were utilized. The results showed that fatty acyl Δ6 desaturase in SAF-1 cells was highly active and there was substantial $C_{18\text{-}20}$ elongase and $C_{20\text{-}22}$ elongase activities. A deficiency in the desaturation/elongation pathway was clearly identified at the level of the fatty acyl Δ5 desaturase which was very low, particularly with 20:4n-3 as substrate. In comparison, the apparent activities of Δ6 desaturase, $C_{18\text{-}20}$ elongase and $C_{20\text{-}22}$ elongase were approximately 94-fold, 27-fold and 16-fold greater than that for Δ5 desaturase towards their respective n-3 polyunsaturated fatty acid substrates. The evidence obtained in the SAF-1 cell line is consistent with the dietary requirement for C_{20} and C_{22} highly unsaturated fatty acids in the marine fish, the sea bream, being primarily due to a deficiency in fatty acid Δ5 desaturase activity.

The essential fatty acid (EFA) requirements of freshwater and marine fish have long been known to be qualitatively different, as in freshwater fish 18:3n-3 and/or 18:2n-6 can satisfy the EFA requirement, whereas marine species required the longer chain HUFA (highly unsaturated fatty acids) 20:5n-3 and 22:6n-3 to be supplied by the diet for optimal growth (1-3). This suggested that there was a difference in the fatty acid desaturation/elongation pathway between freshwater and marine fish (4,5). Dietary conversion studies performed on turbot *in vivo* indicated that marine fish were unable to produce 20:4n-6 and 20:5n-3 from dietary 18:2n-6 and 18:3n-3, respectively (6-8). However, the *in vivo* experiments were unable to precisely distinguish the location of any potential deficiency in the fatty acid desaturation/elongation pathway.

It was shown that the difference in fatty acid metabolism between freshwater and marine fish observed *in vivo* was also present in cultured cell lines (9). Studies involving supplementation of turbot cells (TF) in culture, compared to both rainbow trout cells (RTG-2) and Atlantic salmon cells (AS), with various n-3 and n-6 polyunsaturated fatty acids (PUFA) showed that a relative deficiency in the desaturase/elongase pathway in TF cells was located either at the C_{18} to C_{20} elongase ($C_{18\text{-}20}$ elongase) multi-enzyme complex or the fatty acyl $\Delta 5$

desaturase (9,10). In order to establish more precisely a deficient step in the desaturase/elongase pathway, the direct substrates for all the component enzymes in the pathway are required so that each activity can be assayed and determined in isolation from the influence of preceding activities in the pathway. However, isotopes for two crucial fatty acid substrates, 18:4n-3 (the direct substrate for C_{18-20} elongase) and 20:4n-3 (the direct substrate for Δ5 desaturase) were not available commercially. Recently, Ghioni *et al*. (11) biosynthesized high purity [U-^{14}C]18:4n-3 and [U-^{14}C]20:4n-3 and provided good evidence that the deficiency in TF cells was located at the C_{18-20} elongase and not the Δ5 desaturase.

To date, a deficiency in marine fish fatty acid metabolism has only been characterized in one species, turbot. Results from various *in vivo* studies involving injection of radiolabelled fatty acid precursors such as [1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3 with other species such as gilthead sea bream (*Sparus aurata*) and golden grey mullet (*Liza aurata*) confirmed that an apparent deficiency in the desaturation/elongation pathway was present in those species and supported the view that it was common to all marine fish (12-14). However, as with the *in vivo* studies in turbot, they were unable to identify the specific enzymic location responsible for a deficiency in the pathway. Recently, the availability of a cell line from sea bream, SAF-1, combined with the availability of [U-^{14}C]18:4n-3 and [U-^{14}C]20:4n-3 isotopes, has enabled the deficiency in the pathway to be characterized in another marine fish. We report here that, in contrast to turbot cells, the primary deficiency in the desaturation/elongation pathway in SAF-1 cells from gilthead sea bream was clearly identified at the level of the fatty acyl Δ5 desaturase.

MATERIALS AND METHODS

Cell line and culture conditions. The gilthead seabream (*Sparus aurata* L.) cell line, SAF-1, developed from fin tissue without immortalization was kindly provided by Dr. M.C. Alvarez (Department of Cell Biology and Genetics, University of Malaga, Spain) (15). The cell line

displays fibroblast-like morphology and had been subcultured approximately 120 times over a two year period. Karyotype and DNA content indicate a predominantly euploid and stable cell population. The cells were routinely maintained in Leibovitz L-15 medium supplemented with 2 mM glutamine, antibiotics (50 I.U. ml^{-1} penicillin and 50 $mg.ml^{-1}$ streptomycin) and 10% fetal bovine serum (FBS). Cells were cultured at 22°C in 75 cm^2 sealed plastic tissue culture flasks (Corning Costar, High Wycombe, U.K.) and were routinely subcultured within 24 h of reaching confluence at a seeding density of 1 x 10^5 $cells.cm^{-2}$. Unless otherwise stated, for each experimental sample, 3 x 75 cm^2 flasks were seeded and this experiment was repeated three times to obtain the replicates. Therefore, all results are means $\pm$ SD of three such experiments.

Cell harvesting and lipid extraction. Medium was aspirated and the 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 samples taken for cell counting (16) and protein determination according to the method of Lowry *et al*. (17) after incubation with 0.25 ml of 0.25% (wt/vol) sodium dodecyl sulphate/1 M NaOH for 45 min at 60°C. The cells were washed with 5 ml HBSS containing 1% fatty acid-free bovine serum albumin (FAF-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*. (18) as described in detail previously (19). 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 concentration of 10 $mg.ml^{-1}$, and samples stored at -20°C prior to analyses.

Lipid class analysis. Lipid classes were separated by one-dimensional, double-development high-performance thin-layer chromatography (HPTLC) using methyl acetate/propan-2-ol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.) and hexane/diethyl ether/acetic acid (85:15;1.5, by vol.) as developing solvents as described

previously (20). Lipid classes were quantified by charring (21) followed by scanning densitometry using a Shimadzu CS-9000 dual wavelength flying spot scanner and DR-2 recorder (22).

Fatty acid analyses. Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transmethylation at 50°C for 16 h (23) and extracted and purified as described previously (20). FAME were analyzed in a Carlo Erba Vega GC6000 gas chromatograph (Carlo Erba Strumentazione, Milan, Italy) equipped with a CP Wax 51 fused silica capillary column (50 m x 0.32 mm i.d., Chrompack U.K. Ltd., London), with on-column injection using hydrogen as carrier gas and a biphasic thermal gradient from 50°C to 225°C. FAME were identified by comparison with known standards and a well-characterized fish oil and by reference to published data as described previously and were quantified using a Carlo Erba DP800 data processor (20). All solvents contained 0.01% BHT as an antioxidant.

Incubation of cultures with ^{14}C-labelled polyunsaturated fatty acids and incorporation of radioactivity into total lipid. Cell cultures were routinely grown in 75 cm^2 flasks in medium containing 10% FBS and subcultured into medium containing 2% FBS 24 h prior to experimentation. All experiments were performed in triplicate with three flasks (for [1-^{14}C]PUFAs) and one flask (for [U-^{14}C]PUFAs) per sample per experiment. The medium was aspirated, cultures washed with Dulbecco's phosphate-buffered saline (PBS) and fresh Leibovitz L-15 medium, as above, was added. The [1-^{14}C]PUFAs (18:2n-6, 18:3n-3, 20:3n-6, 20:4n-6 and 20:5n-3) (0.25 μCi per flask; concentration, 0.35 μM) were added to the cell cultures bound to FAF-BSA in medium (24). The [U-^{14}C]PUFAs (18:4n-3 and 20:4n-3) were both obtained as methyl esters and because of the low yield that characterized their production, with little mass available, these substrates were added directly as methyl esters in 50 μl of ethanol) (0.25 μCi per flask; concentration approximately 0.1 μM), to avoid the losses associated with saponification, purification of unesterified fatty acid and sterile filtration (11). Preliminary

experiments incubating cells with 25 μM unlabelled 18:4n-3 as fatty acid salt/BSA complex and as methyl ester in ethanol, showed no difference in the metabolism to 20:4n-3, after 6 days of culture and no accumulation of methyl ester in cellular lipids (11). Therefore these two ways of presenting and delivering radiolabelled PUFA to the cells in culture can be considered equivalent under the conditions used in this study.

After addition of isotope, incubation continued for 6 days at 22°C. Cells were harvested, lipid extracted as above and the radioactive content of total lipid 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 HPTLC plates, and lipid classes separated by the one-dimensional, double-development method as described above. 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; the combined free fatty acid, triacylglycerol and steryl ester areas) 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°C in methanolic sulfuric acid (23) and FAME extracted and purified as described above. TLC plates were impregnated by spraying with 2 g silver nitrate in 20 ml acetonitrile and activated at 110°C for 30 min. FAME were applied as 1 cm streaks and plates developed with toluene/acetonitrile (95:5, v/v) to separate PUFA (25). Autoradiography

was performed using Kodak MR2 film for 7 days at room temperature. Silica corresponding to individual FAME was scraped into scintillation vials containing 2.5 ml scintillation fluid and radioactivity determined as described above.

Preparation of [U-^{14}C]18:4n-3 and [U-^{14}C]20:4n-3. [U-^{14}C]18:4n-3 was prepared as described in detail by Ghioni *et al*. (1999). Briefly, the unicellular marine alga *Isochrysis galbana* (Parke) (S.M.B.A. strain No. 58 C.C.A.P. strain 927/1) was incubated with sodium [^{14}C]bicarbonate, total lipid extracted, the 18:4-rich glycerolipids (mono- and di-galactosyldiacylglycerols) purified by TLC, transmethylated and FAME extracted as described previously (11, 20). FAME were separated by argentation TLC, individual FAME identified by autoradiography and the [U-^{14}C]18:4n-3 methyl ester eluted and quantified (11). [U-^{14}C]20:4n-3 was biosynthesized from [U-^{14}C]18:4n-3 as described by Ghioni *et al*. (1999). Briefly, Atlantic salmon (AS) cells were incubated with [U-^{14}C]18:4n-3 methyl ester, total lipid extracted, transmethylated, and FAME separated by argentation TLC as above. Both [U-^{14}C] isotopes had specific activities of approximately 12 mCi/mmol and were > 99% pure, determined by radio-gas chromatography as described by Buzzi *et al*. (26).

Materials. Sodium [^{14}C]bicarbonate (~ 50 mCi.mmol^{-1}) was purchased from ICN Biomedicals Ltd. (Thame, U.K.). [1-^{14}C] PUFA (all 50-55 mCi.mmol^{-1} and >98% pure) were obtained from NEN Life Science Products (Hounslow, U.K.). Leibovitz L-15 medium, HBSS, PBS, glutamine, penicillin, streptomycin, FBS, trypsin/EDTA , FAF-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 one-way analysis of variance (ANOVA) and where appropriate the

significance of differences were determined by Tukey's test. When necessary, data were subjected to arc-sin transformation prior to statistical analysis. Differences are reported as significant when $p < 0.05$ (27).

RESULTS

The lipid content and class composition of SAF-1 cells cultured in 10% FBS are shown in Table 1. Almost 75% of the total lipid was polar lipid with almost 30% PC, 19% PE, 7.3% PI and 6.3% PS whereas the neutral lipid was predominantly cholesterol (20.3%). The fatty acid composition of total lipid of SAF-1 cells contained over 41% monounsaturated fatty acids, with 18:1n-9 comprising 25.2%, and approximately 25% saturated fatty acids, predominantly 16:0 (15%) and 18:0 (8.5%) (Table 2). The PUFA were characterized by 10% n-9PUFA, 90% of which was 18:2n-9 the single most abundant PUFA in the cells, with 11.1% n-3PUFA, mainly 22:6n-3 and 20:5n-3, and 9.5% n-6PUFA, primarily 20:4n-6.

The recovery of radioactivity in total lipid from SAF-1 cells incubated with [1-^{14}C]20:5n-3 was significantly greater than any of the other PUFA investigated (Table 3). There were numerical differences between the other PUFA but, irrespective of the way that the recovery data were presented, few were statistically significant although there was a trend for the recovery of radioactivity from cells incubated with [1-^{14}C]18:2n-6, [1-^{14}C]18:3n-3 and [1-^{14}C]20:4n-6 to be higher than the recovery of radioactivity from incubations with [1-^{14}C]18:4n-3, [1-^{14}C]20:3n-6 and [1-^{14}C]20:4n-3. All the PUFA were predominantly esterified into the main glycerophospholipid classes with between 88% and 98% of radioactivity being recovered in these classes (Table 4). Higher incorporation into the glycerophospholipids was generally observed with the C_{20} PUFA compared to the C_{18} PUFA. Both [1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3 were predominantly incorporated into PE whereas all the other PUFA were incorporated predominantly into PC, these differences being statistically significant (Table 4). Significantly more [U-^{14}C]18:4n-3 and [U-^{14}C]20:4n-3 were incorporated into PC compared to the other

PUFA and significantly greater proportions of [1-^{14}C]20:4n-6 and, to a lesser extent, [1-^{14}C]20:3n-6, were incorporated into PI compared to the other PUFA.

The main metabolic product of [1-^{14}C]18:2n-6 recovered from SAF-1 cells was 18:3n-6 (over 35% of radioactivity recovered in this fraction) whereas the main metabolic products of [1-^{14}C]18:3n-3 were 18:4n-3 and 20:4n-3 (almost 41% and 21% of radioactivity recovered in these products, respectively) (Table 5). With all the other PUFA, over 80% of the radioactivity was recovered in the form of the fatty acid originally added to the cells. With [U-^{14}C]18:4n-3, the only major products were the elongation products 20:4n-3 (13.2%) and 22:4n-3 (5.1%), with only 0.7% being desaturated to 20:5n-3. Similarly, with [1-^{14}C]20:3n-6, elongation products comprised almost 85% of the total metabolized. The main products of the metabolism of [U-^{14}C]20:4n-3 in SAF-1 cells were 22:4n-3 and the chain shortened product, 18:4n-3 (Table 5). Both [1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3 were mainly elongated with only a small fraction of radioactivity being recovered as 22:6n-3 in [1-^{14}C]20:5n-3-supplemented cells. Table 6 is a summary of the results obtained in the isotope experiments and is derived from the data contained in Table 5 but presented as sums of products of each individual step in the deaturation/elongation pathway taking into account all the fatty acids that are derived from that step, irrespective of subsequent conversions. The results obtained for each enzyme activity when incubating the cells with the fatty acid which is its direct substrate (i.e. 18:4n-3 for C_{18-20} elongase and 20:4n-3 for Δ5 desaturase) are of particular interest as they show the potential of each activity independent of the previous enzymes. Table 6 shows that considerable amounts of radioactivity are recovered as Δ6 desaturase products in SAF-1 cells incubated with the main fatty acid substates for Δ6 desaturase, [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6. Although the majority of the [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 metabolized to Δ6 desaturase products were not further metabolized, about 27% and 9% of the total radioactivity recovered in the case of [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6, respectively, was elongated by C_{18-20} elongase. Very little radioactivity

from [1-^{14}C]18:2n-6 and, especially, [1-^{14}C]18:3n-3, was recovered in Δ5 desaturated products. When SAF-1 cells were incubated with the direct n-3 substrate of C_{18-20} elongase, [U-^{14}C]18:4n-3, 19% was elongated but only 0.7% was recovered in Δ5 desaturated products. The very low activity of fatty acyl Δ5 desaturase in SAF-1 cells was confirmed using direct substrates for Δ5 desaturase, [1-^{14}C]20:3n-6 and [U-^{14}C]20:4n-3, where little radioactivity was recovered in Δ5 desaturated products (Table 6). C_{20-22} elongase products were found in the cells with all the fatty acids used and in higher percentages compared to Δ5 desaturation products.

DISCUSSION

The results of the present study indicate that the sea bream cell line has a deficiency in the desaturation/elongation pathway similar in effect to that observed in turbot and sea bream *in vivo* and turbot cells *in vitro*. Specifically, the results show; firstly, that the lipid and fatty acid composition of cell lines derived from marine fish have several features which distinguish them from cell lines from freshwater fish; secondly, that one of the main factors underpinning this is a relative deficiency in the pathway for the desaturation and elongation of EFA in marine fish cells; and thirdly, that although the effect of the deficiency is similar in different marine fish, its location in the pathway is different.

The first feature distinguishing cells from a marine fish was the lipid class composition. The SAF-1 cells cultured in 2% serum displayed a lipid content (59.8 ± 4.7 μg/10^{6}cells) comparable to levels previously reported in other fish cell lines cultured in 2% serum, being similar to that observed in Atlantic salmon (AS) cells (50.4 ± 10.0 μg/10^6 cells) but slightly higher than that found in another marine fish cell line, TF, (41.4 ± 3.3) and lower than that found in rainbow trout cells (RTG-2) (87.2 ± 6.0) (9,28). However, the proportion of neutral lipids in SAF-1 cells (25.8%) was very similar to that found in TF cells (27.3%), both of which were much lower than the levels found in the freshwater fish cell lines, 37.2% and 39.2% for AS (cell line prepared

from freshwater fry stage) and RTG-2 cells, respectively, which were characterized by higher percentages of triacylglycerols (9,28).

Importantly however, the fatty acid composition of SAF-1 cells was characterized by the presence of substantial amounts of n-9PUFA, 90% of which was 18:2n-9, the most abundant PUFA in SAF-1 cells. This is an identical feature to that observed in TF cells, in which 18:2n-9 was also the most abundant PUFA and its level was increased to over 22% by reducing the serum supplement to 2% (9). In contrast, 18:2n-9 is a relatively minor PUFA in AS and RTG-2 cells amounting to only 1.2% and 1.8%, respectively, in cells cultured in 2% serum (9,28). The presence of large amounts of this unusual PUFA (18:2n-9) in marine fish cells, compared to freshwater fish cells and mammalian cell lines, on its own strongly suggests a deficiency in fatty acid desaturation/elongation as a principal factor responsible for the differences in EFA requirements between marine and freshwater fish.

The higher level of total n-9PUFA in TF cells compared to AS and RTG-2 cells was hypothesized to be due to the marine fish cells having a higher requirement for n-3HUFA (4, 29) and thus experiencing a greater EFA deficiency when grown in relatively n-3HUFA-deficient, n-6PUFA-rich mammalian serum (9). This, combined with the specific location of the apparent deficiency in the EFA desaturation/elongation pathway in marine fish, resulted in the specific accumulation of 18:2n-9 in TF cells. Although the level of 18:2n-9 is lower than in TF cells, the present data in SAF-1 cells support this hypothesis and indicate that high levels of 18:2n-9 may be a characteristic fatty acid marker for all marine fish cells in culture. Consistent with the hypothesis that marine fish cells require higher levels of n-3HUFA, the two marine fish cell lines retained the highest proportions of n-3PUFA in their total lipid, with n-3PUFA accounting for 54% and 45% of total PUFA (not counting n-9PUFA) in SAF-1 and TF cells, respectively, but only 38% and 34% in AS and RTG-2 cells, respectively (9,28). These observations in cell lines derived from marine fish are supported by the dietary traits of the

marine species with both sea bream and turbot being carnivorous, specifically piscivorous, species with diets consisting almost entirely of smaller fish and as such their natural diets are rich in the long-chain n-3HUFA, 20:5n-3 and 22:6n-3, (5,29,30). In contrast, freshwater salmonids, such as rainbow trout, have diets richer in C_{18} PUFA and much lower long-chain HUFA (4, 29,31).

In contrast to the lipid and fatty acid composition data, the fatty acid "incorporation" data show surprisingly little difference between marine and freshwater cell lines. The data in Table 3 suggest that the SAF-1 cells retained [1-^{14}C]20:5n-3 in their lipids to a greater extent than any other PUFA studied and that the PUFA which are present normally in only very small amounts in fish lipids, and are generally regarded as more transient intermediates in the PUFA pathway, specifically 18:4n-3, 20:3n-6 and 20:4n-3, were retained the least. This is consistent with the observed fatty acid composition of fish in general, with 20:5n-3 and 22:6n-3 being the major PUFAs. However, these data represent the net recovery of radioactivity in the cells after incubation with the fatty acid isotopes and that the radioactivity recovered will be dependent upon the amount of fatty acid initially taken up by the cells less the amount of fatty acid lost due to other metabolic processes such as oxidation or conversion to eicosanoids. Due to the limited amount of pure U-^{14}C-labelled isotopes available, they were not able to be used at exactly the same concentration as the 1-^{14}C-labelled isotopes, thereby possibly affecting the amount initially taken up by the cells. In addition, the 1-^{14}C-labelled isotopes lose the radiolabel after one round of β-oxidation whereas the U-^{14}C-labelled isotopes will not, also affecting the relative recoveries of radioactivity from the differently labelled isotopes. Therefore, some caution is required in directly comparing the data in Table 3. Previously, we have shown in several fish cell lines that there was little difference between the recovery of radioactivity when incubated with the 1-^{14}C-labelled isotopes of 18:2n-6, 18:3n-3, 20:4n-6 and 20:5n-3 (10).

The pattern of incorporation of the PUFA into lipid classes was very similar to that reported

previously in AS, RTG-2 and TF cell lines (10). In that earlier study, 86-94%, and 96-98% of radioactivity from C_{18} PUFA and C_{20} PUFA, respectively, were recovered in polar lipids compared with figures of 88-92% for C_{18} PUFA and 92-98% for C_{20} PUFA obtained with SAF-1 cells in the present study (10). The preferential incorporation of C_{18} PUFA into PC was also observed in the earlier study as was the preferencial incorporation of 20:4n-6 into PI and 20:5n-3 into PE (10). The present study has shown that the pattern obtained with 20:3n-6 was basically as expected for a C_{20} PUFA in general, but particularly similar to 20:4n-6, whereas the incorporation of 20:4n-3 was more similar to that for C_{18} PUFA. However, there was no major difference between the freshwater fish cell lines, AS and RTG-2, and the marine fish cell lines, TF and SAF-1, in the incorporation of PUFA into lipid classes. These results appear to indicate that the ability of the cells to metabolize, or not, the fatty acids via desaturation and elongation does not greatly influence their uptake, retention or distribution in cell lipids, at least at the low concentrations used in the present study.

The primary aim of the present study was to determine if the deficiency in the desaturase/elongase pathway, established in C_{18-20} elongase in TF cells (11), was identical in another marine fish cell line. The results obtained using the direct substrates for each individual enzymic activity in the pathway enabled the deficient step in SAF-1 cells to be identified. The fact that 43.2% and 66.1% of added [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3, respectively, were desaturated showed that SAF-1 cells expressed a highly active Δ6 desaturase. In addition, 19% of the direct substrate for C_{18-20} elongase, [U-^{14}C]18:4n-3, was elongated. In contrast, only 2.5% and 0.7% of added [1-^{14}C]20:3n-6 and [U-^{14}C]20:4n-3 were recovered as Δ5 desaturation products. Almost 7% and 11% of [1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3, respectively, were C_{20-22} elongated. In summary, the apparent activities of Δ6 desaturase, C_{18-20} elongase and C_{20-22} elongase were approximately 94-fold, 27-fold and 16-fold greater than that for Δ5 desaturase towards their respective n-3 polyunsaturated fatty acid substrates. These results showed that, in

contrast to turbot cells, fatty acyl Δ5 desaturation was the step with the lowest activity in the pathway biosynthesizing 20:5n-3 (or 20:4n-6) in the sea bream cells.

It was noteworthy that the Δ5 desaturase activity in SAF-1 cells was particularly ineffective towards the n-3 substrate. This was unexpected as desaturases in general are normally more active towards n-3 fatty acids in mammals (32,33) and fish cells (10,11,34). Furthermore, the other enzymes in the pathway, Δ6 desaturase and the C_{18-20} and C_{20-22} elongases, showed greater activity towards n-3PUFA in SAF-1 cells.

Deficiencies in fatty acyl desaturase activities have precedents in terrestrial carnivores such as cats in which Δ6 and Δ5 desaturase activities may both be very low (35-38). It has been hypothesized that this situation may be an evolutionary adaptation to carnivorous diets rich in preformed C_{20} and C_{22} HUFA. This may be parallelled in fish because, as described above, the diets of the generally more herbivorous/omnivorous freshwater fish are rich in C_{18} PUFA and do not contain much C_{20} or C_{22} HUFA whereas marine fish which are predominantly carnivorous consume diets rich in 20:5n-3 and 22:6n-3 (30,31). Therefore, the differences in the fatty acid desaturase/elongase pathways between fish species may be an evolutionary response to dietary differences. However, this cannot explain the why turbot should have low C_{18-20} elongase and sea bream low Δ5 desaturase.

In both cases studied in marine fish, Δ5 desaturase in SAF-1 cells and C_{18-20} elongase in TF cells, it should be noted that there is not a complete lack of the enzyme activity responsible for the deficiency in the pathway. This is now also known to be the case in cats. In early studies, a complete lack of Δ6 desaturation enzymatic activity in felines was originally reported in the literature (35-38), although recent and more sensitive studies conducted with deuterated fatty acids, showed that a low level of Δ6 desaturase is present in the cat (39).

The present study provides good evidence that SAF-1 cells have a deficiency in fatty acyl Δ5 desaturase activity in contrast to turbot cells in which C_{18-20} elongase is deficient. Both of

these relative deficiencies in the fatty acid desaturation/elongation pathway are entirely consistent with the known EFA requirements of marine fish. However, it is important to determine if this is a reflection of the situation *in vivo* and that the cell lines are a model for the whole animal. Studies with mammalian cell lines have shown that three out of six expressed little Δ6 desaturase activity, Δ5 desaturase activity appeared to be absent in four cell lines and only one cell line expressed any desaturation activity beyond Δ5 (40). An apparent loss of stearoyl-CoA Δ9 desaturase has been reported in cultured murine T lymphocytes (41). Therefore, experiments with stable isotopes, similar to those performed by other authors in felines (39) and humans (42,43) are required to confirm the deficiencies in fatty acid desaturation and elongation in marine fish *in vivo*.

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Table 1. Lipid content and lipid class composition (percentage of total lipid) of SAF-1 cells grown at 22°C in medium supplemented with 10% fetal calf serum.

Lipid	Content
Total lipid content	
μg lipid /10^6 cells	59.8 ± 4.7
μg lipid /mg protein	135 ± 7
Lipid class composition	
Phosphatidylcholine	29.8 ± 0.8
Phosphatidylethanolamine	19.0 ± 0.6
Phosphatidylserine	6.3 ± 0.2
Phosphatidylinositol	7.3 ± 0.2
Phosphatidic acid/cardiolipin	4.5 ± 0.2
Sphingomyelin	2.4 ± 0.3
Sulfatides/cerebrosides	4.9 ± 0.3
Total polar lipids	74.2 ± 1.7
Total neutral lipids	25.8 ± 1.7
Cholesterol	20.3 ± 0.7
Triacylglycerol	1.7 ± 0.4
Steryl ester	3.8 ± 2.0
Free fatty acids	t

Results are means ± S.D. (n=3); t, trace value (< 0.05%).

Table 2. Fatty acid compositions (percentage of weight) total lipid from SAF-1 cells grown at 22ºC in medium supplemented with 10% fetal bovine serum.

Fatty acid	Percentage
14:0	1.0 ± 0.1
15:0	0.2 ± 0.0
16:0	15.0 ± 0.8
17:0	0.5 ± 0.2
18:0	8.5 ± 0.2
Total saturated	25.2 ± 1.1
16:1(n-9)	3.9 ± 0.5
16:1(n-7)	4.6 ± 0.2
18:1(n-9)	25.2 ± 1.0
18:1(n-7)	7.0 ± 0.3
20:1(n-9)	t
20:1(n-7)	0.1 ± 0.1
24:1	0.6 ± 0.1
Total monounsaturated	41.4 ± 1.6
18:2(n-9)	9.0 ± 0.9
20:2(n-9)	0.4 ± 0.2
20:3(n-9)	0.1 ± 0.1
C22(n-9)	0.5 ± 0.4
Total (n-9) PUFA	10.0 ± 1.0
18:2(n-6)	1.2 ± 0.2
18:3(n-6)	1.2 ± 0.2
20:2(n-6)	0.1 ± 0.1
20:3(n-6)	1.6 ± 0.1
20:4(n-6)	5.4 ± 0.5
Total (n-6) PUFA	9.5 ± 0.6
18:4(n-3)	0.1 ± 0.1
20:4(n-3)	0.2 ± 0.2
20:5(n-3)	1.4 ± 0.9
22:5(n-3)	3.5 ± 0.1
22:6(n-3)	5.5 ± 0.7
Total (n-3) PUFA	10.7 ± 1.7
Total dimethylacetals	1.7 ± 1.5
Total PUFA	30.2 ± 1.5
Total unidentified	1.5 ± 0.4

Results are means ± S.D. (n=3).
PUFA, polyunsaturated fatty acids.

Table 3. Recovery of radioactivity in total lipid from SAF-1 cells incubated with ^{14}C - polyunsaturated fatty acids (PUFA).

	Recovery	
^{14}C - PUFA	(pmol / million cells)	(pmol / mg protein)
[1-^{14}C]18:2n-6	3.82 ± 0.94^{b}	1.14 ± 0.32^{b}
[1-^{14}C]18:3n-3	3.71 ± 0.99^{b}	1.22 ± 0.12^{b}
[U-^{14}C]18:4n-3	1.36 ± 0.31^{c}	0.44 ± 0.10^{c}
[1-^{14}C]20:3n-6	2.43 ± 0.60^{bc}	0.76 ± 0.10^{bc}
[1-^{14}C]20:4n-6	3.14 ± 0.64^{bc}	1.18 ± 0.02^{b}
[U-^{14}C]20:4n-3	1.95 ± 0.03^{bc}	0.63 ± 0.01^{bc}
[1-^{14}C]20:5n-3	6.53 ± 1.10^{a}	2.21 ± 0.46^{a}

Results are means ± SD (n = 3). Data were subjected to one-way analysis of variance followed, where appropriate, by Tukey's multiple comparison test. Means in the same column with different superscript letters are significantly different ($p < 0.05$).

Table 4. Recovery of radioactivity in lipid classes from SAF-1 cells incubated with ^{14}C - polyunsaturated fatty acids (PUFA).

	^{14}C - polyunsaturated fatty acid added		
Lipid class	18:2n-6	18:3n-3	18:4n-3
PC	51.1 ± 1.1^{c}	51.1 ± 0.8^{c}	70.5 ± 1.8^{a}
PE	18.1 ± 0.5^{e}	20.4 ± 0.9^{de}	10.2 ± 1.0^{f}
PS	5.3 ± 0.5^{b}	4.1 ± 0.3^{c}	1.9 ± 0.2^{d}
PI	5.1 ± 0.1^{de}	5.3 ± 0.5^{d}	3.0 ± 0.2^{e}
PA/CL	12.8 ± 0.3^{a}	8.9 ± 0.5^{c}	2.4 ± 0.2^{d}
TP	92.4 ± 0.2^{bc}	89.8 ± 0.3^{cd}	87.9 ± 3.2^{cd}
TN	7.6 ± 0.2^{ab}	10.2 ± 0.3^{a}	12.1 ± 3.2^{a}

	^{14}C - polyunsaturated fatty acid added			
Lipid class	20:3n-6	20:4n-6	20:4n-3	20:5n-3
PC	35.5 ± 0.9^{d}	11.9 ± 1.4^{f}	61.4 ± 2.0^{b}	20.8 ± 1.1^{e}
PE	26.6 ± 0.5^{c}	43.0 ± 1.6^{b}	22.0 ± 0.9^{d}	63.2 ± 1.6^{a}
PS	6.7 ± 0.3^{a}	2.2 ± 0.2^{d}	1.9 ± 0.2^{d}	2.6 ± 0.1^{d}
PI	15.0 ± 1.4^{b}	32.8 ± 1.3^{a}	4.6 ± 0.2^{de}	8.8 ± 0.3^{c}
PA/CL	10.8 ± 0.1^{b}	8.1 ± 0.8^{c}	2.4 ± 0.3^{d}	2.8 ± 0.1^{d}
TP	94.7 ± 0.2^{b}	98.1 ± 0.1^{ab}	92.2 ± 2.9^{bd}	98.1 ± 0.2^{ab}
TN	5.3 ± 0.2^{bc}	1.9 ± 0.1^{c}	7.8 ± 2.9^{ab}	1.9 ± 0.2^{c}

Results are expressed as percentages of total radioactivity recovered and are means ± SD (n = 3). Data were subjected to one-way analysis of variance followed, where appropriate, by Tukey's multiple comparison test. Means for each individual lipid class with different superscript letters are significantly different ($p < 0.05$).

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

Table 5. Metabolism of ^{14}C - polyunsaturated fatty acids (PUFA) by desaturation and elongation in SAF-1 cells.

	^{14}C - polyunsaturated fatty acid added		
Fatty acid	18:2n-6	20:3n-6	20:4n-6
18:2n-6	56.0 ± 1.6	n.d.	n.d.
20:2n-6	0.8 ± 0.1	n.d.	n.d.
18:3n-6	35.1 ± 1.8	n.d.	n.d.
20:3n-6	3.8 ± 0.3	84.1 ± 0.7	n.d.
22:3n-6	t	13.4 ± 0.8	n.d.
20:4n-6	2.5 ± 0.5	2.0 ± 0.2	93.4 ± 1.9
22:4n-6	1.8 ± 0.3	0.5 ± 0.1	5.5 ± 1.9
24:4n-6	n.d.	n.d.	1.1 ± 0.2
22:5n-6	n.d.	t	t

	^{14}C - polyunsaturated fatty acid added			
Fatty acid	18:3n-3	18:4n-3	20:4n-3	20:5n-3
18:3n-3	32.1 ± 1.0	n.d.	n.d.	n.d.
20:3n-3	1.9 ± 0.3	n.d.	n.d.	n.d.
18:4n-3	40.9 ± 0.9	81.0 ± 2.0	8.3 ± 1.4	n.d.
20:4n-3	20.7 ± 0.9	13.2 ± 1.4	85.8 ± 1.4	n.d.
22:4n-3	4.5 ± 0.3	5.1 ± 0.2	5.2 ± 1.7	n.d.
18:5n-3	n.d.	n.d.	n.d.	n.d.
20:5n-3	t	0.7 ± 0.5	0.7 ± 0.4	89.0 ± 0.7
22:5n-3	n.d.	n.d.	n.d.	9.9 ± 0.8
24:5n-3	n.d.	n.d.	n.d.	0.6 ± 0.1
22:6n-3	n.d.	n.d.	n.d.	0.4 ± 0.0

Results are expressed as percentages of total radioactivity recovered and are means ± SD (n = 3). n.d., not detected; t, trace (< 0.05%).

Table 6. Products of desaturase and elongase activities in SAF-1 cells incubated with various ^{14}C-labelled polyunsaturated fatty acids

Fatty acid added	Δ6 desaturase products	C_{18-20} elongase products	Δ5 desaturase products	C_{20-22} elongase products
[1-^{14}C]18:2n-6	43.2 ± 1.5	8.9 ± 0.5	4.3 ± 0.8	1.8 ± 0.3
[1-^{14}C]18:3n-3	66.1 ± 1.1	27.1 ± 0.6	t	4.5 ± 0.3
[U-^{14}C]18:4n-3	-	19.0 ± 2.1	0.7 ± 0.5	5.1 ± 0.2
[1-^{14}C]20:3n-6	-	-	2.5 ± 0.2	13.9 ± 0.7
[U-^{14}C]20:4n-3	-	-	0.7 ± 0.4	5.2 ± 1.7
[1-^{14}C]20:4n-6	-	-	-	6.6 ± 1.9
[1-^{14}C]20:5n-3	-	-	-	10.9 ± 0.8

Results are presented as percentages of total radioactivity recovered and are means ± SD (n=3). 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, 20:4n-6 and 22:4n-6). t, trace (< 0.05%).