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Incorporation and metabolism of (n-3) and (n-6) polyunsaturated fatty acids in phospholipid classes in cultured rainbow trout (*Salmo gairdneri*) cells

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Abstract

The incorporation and metabolism of various (n-3) and (n-6) polyunsaturated fatty acids supplemented to the culture medium was investigated in the rainbow trout cell line, RTG-2. The distribution, and the occurrence and relative extent of further desaturation and elongation of the incorporated acids was determined in individual phospholipid classes by analysis of the fatty acid compositions. RTG-2 cells exhibited Δ6 and Δ5 desaturase activities whereas Δ4 desaturase activity was almost totally absent. The percentage of precursor acids was greatest in the phosphatidic acid/cardiolipin fraction (PA/CL), suggesting a role for possibly PA in the initial incorporation of these acids into the phospholipid pool. The compositional data indicated that individual intermediates and products of the desaturation pathways were associated with specific phospholipid classes probably via mechanisms depending upon the specificities of the acylating enzymes. The composition of phosphatidylinositol (PI) and the tightly controlled mechanisms for generating/maintaining it are consistent with a role for this phospholipid in providing precursor fatty acid for eicosanoid synthesis.

Abbreviations: CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid(s); SM, sphingomyelin.

Introduction

Fish tissue lipids are characterized by relatively high levels of polyunsaturated fatty acids (PUFA), generally higher than those found in corresponding mammalian systems (Ackman 1980; Padley *et al.* 1986; Henderson and Tocher 1987). In particular, (n-3)PUFA tend to predominate in many fish species rather than the (n-6)PUFA which predominate in the terrestrial environment (Ackman 1980; Padley *et al.* 1986; Henderson and Tocher 1987). Rainbow trout (*Salmo gairdneri*) is an extensively studied freshwater species, and it was found that 18:3(n-3) had an essential role in their nutrition, whereas a significant requirement for 18:2(n-6) has yet to be defined (Watanabe 1982). Feeding studies with ^{14}C-labelled 18:3(n-3) showed that 70% of the incorporated radioactivity was present as 22:6(n-3), confirming that rainbow trout were able to fully desaturate and chain elongate 18:3(n-3) (Owen *et al.* 1975).

Recently, we have shown that an established cell line from rainbow trout (RTG-2) grown in medium containing fetal calf serum (FCS) as the only lipid input had a PUFA composition reflecting almost exactly the composition of the serum (Tocher *et al.*

1988). Total (n-6)PUFA at over 16% were 2–3 fold higher, and total (n-3)PUFA at 6% were 5–6 fold lower than values for trout tissues. Subsequently, we investigated the incorporation and further metabolism by RTG-2 cells of a variety of (n-3) and (n-6)PUFA supplemented to the culture medium (Tocher *et al.* 1989). The data showed that RTG-2 cells could only convert 18:3(n-3) and 18:2(n-6) through to 20:5(n-3) and 20:4(n-6), respectively, and that 22:6(n-3) could not be produced from either 18:3(n-3) or 20:5(n-3) (Tocher *et al.* 1989). This implied that RTG-2 cells only expressed Δ6 and Δ5 desaturase activities and not Δ4 desaturase activity.

In the previous study only total lipid fatty acid compositions were studied (Tocher *et al.* 1989). However, phospholipids accounted for 60–67% of the total lipid, and there is currently considerable interest in the precise distribution of different fatty acids between the individual phospholipid classes in normally (n-3)PUFA-rich systems (Bell *et al.* 1983, 1985; Tocher and Sargent 1984, 1986; Tocher *et al.* 1985; Tocher and Harvie 1988). The mechanisms of generation of the observed phospholipid class fatty acid distributions are also under investigation (Voss and Sprecher 1988).

The present paper describes the incorporation of specific (n-3) and (n-6)PUFA into individual phospholipid classes in RTG-2 cells. The occurrence and extent of further metabolism of the incorporated PUFA via desaturation and chain elongation was determined from the compositional data.

In general, all fish cells cultured in mammalian sera have a fatty acid composition that is grossly altered (Tocher *et al.* 1988). For some purposes, *e.g.* eicosanoid metabolism, this would limit their use as an experimental model for fish *in vivo*. Therefore, as a practical application of the incorporation studies, two fish oil concentrates were evaluated as to their use as routine supplements for fish cell culture via their ability to restore the fatty acids of the RTG-2 cells to a normal composition.

Materials and methods

Cells and medium

The rainbow trout (*Salmo gairdneri*) gonad cell line (RTG-2) (Wolf and Quimby 1962) was obtained from Flow Laboratories and was maintained in Glasgow's modification of Eagle's medium containing 16 mM Tris-HCl buffer (Trizma, pH 7.4), 0.3% sodium bicarbonate, 10% tryptose phosphate broth, antibiotics (50 $I.U.ml^{-1}$ penicillin and 50 $\mu g.ml^{-1}$ streptomycin) and either 10% or 2% FCS.

(n-3) and (n-6)PUFA supplements

PUFA supplements were added to the RTG-2 cultures as bovine serum albumin (BSA) complexes prepared essentially as described by Spector and Hoak (1969). To minimise autoxidation all procedures were performed at room temperature and under a stream of N_2. The resultant solutions were filter sterilized through 0.2 μm filters (Flowpore D26, Flow Laboratories) prior to assay and use. The fatty acid concentrations were determined by addition of an internal standard (17:0) to an aliquot and GC analyses as described previously (Tocher *et al.* 1989). The protein concentrations were determined by the method of Lowry *et al.* (1951). The PUFA concentrations were generally in the range 2.0–2.8 mM (BSA = 0.25 mM) giving PUFA:BSA ratios of approximately 8–11:1. The PUFA/BSA mixtures were stored in brown vials under N_2 at −25°C between procedures.

Preparation of fish oil concentrates

Two fish oil concentrates (FOC) were prepared for supplementation to the cells. FOC 1 was prepared by low temperature urea adduction (Christie 1982) of a commercial fish oil and FOC 2 was prepared *via* phospholipase A_2 treatment of cod roe (Tocher *et al.* 1986) followed by urea adduction. The precise details have been described elsewhere (Tocher *et al.* 1989). The oils were added as fatty acid-BSA complexes prepared as described earlier. FOC 1 con-

tained 37% 20:5(n-3), 14% 22:6(n-3), 6% 18:4(n-3) and a total (n-3)PUFA content of 62% whereas FOC 2 contained 28% 20:5(n-3), 65% 22:6(n-3) and a total (n-3)PUFA content of 94%. The full fatty acid compositions were given in detail previously (Tocher *et al.* 1989).

Cell growth and incubation conditions

For routine cultivation of cells the cultures were grown in sealed 75 cm^2 flasks (Gibco-Nunc Ltd., Paisley, U.K.) in 20 ml medium containing 10% FCS. Cells were harvested for analysis or further subculture within 24 h of achieving confluence, which was usually after 5–7 days. For incubation with PUFA, cells were subcultured into 175 cm^2 flasks in 50 ml medium containing 2% FCS and supplemented with the appropriate volume of a specific PUFA/BSA mixture. Control flasks received the same volume of the fatty acid-free BSA solution. All incubations were performed at 22°C.

Cell harvesting and lipid extraction

The cells were harvested and washed extensively as described previously (Tocher *et al.* 1988). Total lipid was then extracted from the cells essentially according to Folch *et al.* (1957) and as described in detail previously (Tocher *et al.* 1988).

Phospholipid class separation

Total lipid (approx 2 mg) was applied in 7.5 cm streaks to thin-layer chromatography (TLC) plates that had been prerun in diethylether and activated at 110°C for 1 h. The plates were developed in methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9,v/v/v/v/v) to separate phospholipids and other polar lipid classes, with the neutral lipids running at the solvent front (Vitiello and Zanetta 1978). The phospholipid classes were visualized under u.v. after a brief spray with 2′,7′ dichlorofluorescein and scraped into test tubes. All solvents contained 0.05% butylated hydroxytoluene (BHT) as antioxidant.

Fatty acid analysis

The phospholipid classes were subjected to acid-catalysed transmethylation directly on the silica gel according to the method of Christie (1982). Fatty acid methyl esters were extracted and purified as described previously (Tocher *et al.* 1989). Methyl esters dissolved in hexane containing 0.05% BHT were analyzed using a Packard 436 gas chromatograph (Chrompack U.K., London) equipped with a chemically bonded CP Wax 52CB fused silica capillary column (50 m × 0.34 mm i.d.) (Chrompack U.K., London), on-column injection and using H_2 as carrier gas with a biphasic thermal gradient form 50°C to 225°C. Individual methyl esters were identified as described previously (Tocher and Harvie 1988) and quantified using a Shimadzu CR-3A recording integrator. Results are means of duplicate experiments with the individual samples analysed in triplicate; ranges are omitted for clarity but differences between duplicates were generally less than 5% of the mean except for minor components below 2% where the variation could be greater than 10%.

Materials

GMEM, sodium bicarbonate, tryptose phosphate broth, antibiotics and FCS were from Flow Laboratories, Rickmansworth, U.K. All PUFA (approx. 99% pure), fatty acid-free BSA, Trizma and BHT were from Sigma Chemical Co. Ltd., Poole, U.K. TLC plates (20 × 20 cm × 0.25 mm) precoated with silica gel 60 were obtained from Merck, Darmstadt, FRG. All solvents were HPLC grade and were obtained from Rathburn Chemicals, Walkerburn, U.K.

Table 1. Fatty acid composition[a] of phospholipid classes from RTG-2 cells cultured in medium containing 10% FCS.

Fatty acid	PC	PE	PS	PI	PA/CL	SM
14:0	1.8	0.3	0.2	0.2	1.3	6.2
15:0	0.1	0.3	0.1	0.3	0.4	0.9
16:0	17.3	3.1	6.6	4.3	5.6	20.4
17:0	0.1	0.4	0.2	T	T	0.7
18:0	4.4	1.7	19.7	15.2	3.4	11.5
20:0	0.1	0.1	0.3	0.1	0.1	0.9
Total saturates	23.8	5.9	27.1	20.2	10.8	42.2[d]
16:1[b]	9.3	7.0	2.3	1.9	17.7	5.7
18:1(n-9)	49.0	47.5	26.8	29.0	38.1	15.7
18:1(n-7)	4.3	4.7	3.2	3.0	3.2	1.8
20:1(n-9)	2.2	3.1	3.9	2.9	3.3	2.0
22:1	0.1	–	–	0.1	0.1	3.2
24:1	1.1	–	0.1	0.1	–	17.3
Total monoenes	66.0	62.3	36.3	37.0	62.4	45.7
18:2(n-9)	2.2	3.0	1.8	0.8	0.4	0.5
20:2(n-9)	3.2	4.0	6.6	9.6	6.9	0.6
Total (n-9)PUFA	5.4	7.0	8.4	10.4	7.3	1.1
18:2(n-6)	0.8	1.1	0.6	0.4	3.5	0.5
18:3(n-6)	0.1	0.2	0.1	0.1	T	0.5
20:2(n-6)[c]	0.4	1.9	1.0	6.8	1.0	0.2
20:3(n-6)	0.5	1.0	1.8	3.0	3.5	0.6
20:4(n-6)	0.6	7.3	2.9	17.0	3.1	0.5
22:4(n-6)	0.1	0.9	2.5	0.4	0.4	–
22:5(n-6)	–	0.3	0.7	0.1	0.2	–
Total (n-6)PUFA	2.5	12.7	9.6	27.8	11.7	2.3
18:3(n-3)	0.1	0.2	0.3	0.1	0.3	0.2
18:4(n-3)	0.2	0.2	0.2	0.2	0.3	T
20:5(n-3)	0.1	0.9	0.2	0.2	0.3	0.1
22:5(n-3)	0.3	2.6	6.6	1.2	1.3	0.2
22:6(n-3)	0.2	3.1	7.6	1.3	3.1	0.6
Total (n-3)PUFA	0.9	7.0	14.9	3.0	5.3	1.1
Total PUFA	8.8	26.7	32.9	41.2	24.3	4.5
(n-3)/(n-6)	0.4	0.6	1.6	0.1	0.5	0.5
Total unidentified	1.4	5.1	3.7	1.6	2.5	7.6

[a]Results are expressed as a percentage of weight; [b]predominantly (n-7) isomer; [c]includes trace amounts of 20:3(n-9); [d]includes 1.0% 22:0 and 0.6% 24:0; T, trace; –, not detected.

Results

Fatty acid composition of phospholipid classes from RTG-2 cells cultured in medium containing 10% FCS

RTG-2 cells cultured in the presence of 10% FCS exhibit a degree of EFA deficiency as defined by the presence of (n-9)PUFA, predominantly 20:2(n-9) but also 18:2(n-9) (Table 1). The more usual (n-9) PUFA, 20:3(n-9), could not be consistently resolved from 20:2(n-6) and is included in that peak in the data. However, on the occasions it was possible to resolve two peaks, 20:3(n-9) was always a minor portion. The major fatty acid group in all classes was the monoenes, predominantly 18:1(n-9) (Table 1). In all the subsequent PUFA supplementation experiments it were mainly the levels of the (n-9)

Table 2. Dimethyl acetal content[a] of PC and PE.

Phospholipid class	Dimethyl acetals				
	16:0	18:0	18:1(n-9)	18:1(n-7)	Total
PC	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	–	0.4
PE	2.7 ± 1.2	1.4 ± 0.6	6.9 ± 2.4	2.3 ± 0.8	13.3

[a]Percentage of total peak area from GC traces; Results are expressed as means ± SD (n = 12).

PUFA and 18:1(n-9) that decreased in response to the increasing (n-3) and (n-6)PUFA content. In addition to the very high 18:1(n-9), PC also had a high percentage of 16:0 and only 3.4% total (n-3) and (n-6)PUFA. PE had a very low percentage of saturates and moderate percentages of PUFA. The percentage of 18:1(n-9) was lower in PS but saturates, especially 18:0, were higher and C22 PUFA accounted for 71% of the total (n-3) and (n-6) PUFA in this class. PI also had relatively high 18:0 and the highest percentages of (n-6)PUFA, particularly 20:4(n-6), and low (n-3)PUFA. The PA/CL composition was characterized by relatively low saturates, a high percentage of 16:1 and moderate PUFA levels. SM had the highest percentages of saturates including 16:0, 14:0 and long chain (greater than C20), a large percentage of 24:1 and less than 5% total PUFA. Plasmalogens were present in both the PC and especially PE as evinced by the presence of dimethyl acetals on the GC traces (Table 2). These levels were relatively unaffected by PUFA supplementation at the concentrations used and consequently all the fatty acid compositional data were calculated excluding the dimethyl acetals.

Incorporation and metabolism of 18:3(n-3) and 18:2(n-6)

Supplementation of the cell cultures with 20 μM 18:3(n-3) or 18:2(n-6) for 3 days greatly increased the percentages of total (n-3) and (n-6)PUFA, respectively, in all the phospholipid classes (Tables 3 and 4). Culture with 20 μM 18:3(n-3) for 7 days further increased the total (n-3)PUFA whereas culture with 20 μM 18:2(n-6) for the longer period resulted in reduced percentages of total (n-6)PUFA in some phospholipid classes. However, increasing the concentration of the supplemented acids to 50 μM resulted in the highest percentages of total PUFA in all phospholipid classes except PA/CL (Tables 3 and 4). In general, the percentages of total (n-6) PUFA exceeded those of total (n-3)PUFA in all three culture conditions and in all classes, mainly due to the much higher percentages of 18:2(n-6). With 18:3(n-3), the levels of 18:3(n-3), 18:4(n-3) and 20:5(n-3) were increased in all classes along with their respective elongation products 20:3(n-3), 20:4(n-3) and 22:5(n-3) (Table 3). In particular, 20:5(n-3) was increased by increasing time of culture and concentration of 18:3(n-3). However, with 18:2(n-6) supplementation, the percentages of 18:2(n-6) and 18:3(n-6) were increased in all classes along with their respective elongation products 20:2(n-6) and 20:3(n-6) whereas the percentages of 20:4(n-6) were relatively unaffected or reduced (Table 4). In general, PA/CL contained the highest percentages of the supplemented C18 acids, whereas PE had the highest percentages of C20(n-3) PUFA and PI had the highest percentages of C20 (n-6)PUFA. The percentages of 22:6(n-3) were generally reduced by supplementation with 18:3(n-3) Table 3), and 22:5(n-6) was not produced in significant amounts in response to 18:2(n-6) supplementation (Table 4). However, the greatest proportion of total (n-3)PUFA as C22 acids was found in PS (Table 3).

Incorporation and metabolism of 20:5(n-3) and 22:6(n-3)

Supplementation with 20:5(n-3) and 22:6(n-3) both generally increased the total (n-3)PUFA content in

Table 3. (n-3)PUFA composition[a] of phospholipid classes from RTG-2 cells cultured in medium supplemented with 18:3(n-3).

	PC			PE			PS			PI			PA/CL		
(n-3)PUFA	20/3	20/7	50/7	20/3	20/7	50/7	20/3	20/7	50/7	20/3	20/7	50/7	20/3	20/7	50/7
18:3	5.5	4.0	1.6	3.5	2.5	1.1	2.0	1.1	0.7	1.4	0.9	0.6	13.0	6.3	13.1
18:4	2.1	1.0	0.7	0.9	0.4	0.6	0.8	0.4	0.3	0.5	0.3	0.3	2.0	0.5	1.5
20:3	0.4	1.3	0.5	0.5	1.3	1.7	0.4	1.1	0.6	0.3	0.8	0.4	0.9	0.8	1.8
20:4	1.3	2.0	1.6	1.8	2.7	0.7	1.9	4.7	4.3	0.9	1.3	0.8	2.1	1.9	9.2
20:5	1.8	4.1	6.2	7.6	13.6	25.9	2.2	3.6	8.0	1.9	3.2	5.1	1.0	14.7	6.9
22:5	0.3	0.5	0.7	2.3	2.2	4.0	4.3	4.8	10.3	1.1	3.1	3.1	0.9	0.8	1.6
22:6	0.2	0:2	0.2	2.5	1.1	1.2	4.0	2.2	3.2	1.0	1.3	0.7	2.3	11.1	1.3
Total	11.6	13.1	11.5	19.1	23.8	35.9	15.6	17.9	27.4	7.1	10.9	11.0	22.2	36.1	35.4

[a]Results are expressed as a percentage of the total fatty acids; 20/3, incubation with 20 μM 18:3(n-3) for 3 days; 20/7, incubation with 20 μM 18:3(n-3) for 7 days; 50/7, incubation with 50 μM 18:3(n-3) for 7 days.

Table 4. (n-6)PUFA composition of phospholipid classes from RTG-2 cells cultured in medium supplemented with 18:2(n-6).

	PC			PE			PS			PI			PA/CL		
(n-6)PUFA	20/3	20/7	50/7	20/3	20/7	50/7	20/3	20/7	50/7	20/3	20/7	50/7	20/3	20/7	50/7
18:2	15.7	10.6	33.4	18.5	14.4	31.5	6.5	7.3	9.2	6.0	5.2	12.7	24.3	32.2	31.0
18:3	1.0	0.5	2.5	1.1	0.5	1.8	0.5	0.3	0.9	0.4	0.3	0.7	0.6	0.3	0.9
20:2	1.5	2.0	3.4	2.7	2.5	4.0	1.5	2.1	3.2	5.4	6.7	3.0	4.3	4.1	5.5
20:3	2.1	1.7	3.3	3.2	3.5	4.5	4.2	7.3	5.7	8.4	10.6	7.8	4.4	4.0	2.9
20:4	0.8	0.5	1.4	6.7	3.5	8.2	1.7	1.7	2.3	14.5	9.8	14.4	3.2	3.7	1.8
22:2	0.1	–	0.2	0.2	–	0.9	0.5	–	0.2	0.3	–	–	–	–	–
22:3	T	0.2	T	0.2	T	0.4	0.1	–	–	–	–	–	–	–	–
22:4	0.1	0.1	0.1	0.9	3.5	1.0	2.0	0.9	2.1	0.4	0.2	0.2	0.3	0.2	0.2
22:5	T	–	–	0.3	0.1	0.3	0.5	–	0.5	0.1	–	–	–	–	–
Total	21.3	15.6	44.3	33.8	26.8	52.6	17.5	19.6	24.1	35.5	32.8	38.8	37.1	44.5	42.3

Data presented as described in Table 3; T, trace; –, not detected.

all phospholipid classes, although PC was least affected (Tables 5 and 6). With PC and PA/CL the levels of total (n-3)PUFA were less than those attained with 18:3(n-3) (Table 3). However, the levels of total (n-3)PUFA attained in PE, PS and PI were greater than those attained with 18:3(n-3) supplementation. The increased (n-3)PUFA was due solely to increased 22:6(n-3) when it itself was supplemented (Table 6) and to both 20:5(n-3) and 22:5(n-3) when 20:5(n-3) was supplemented (Table 5). The elongation of 20:5(n-3) was most evident in PS, which also contained the highest percentages of 22:6(n-3) in both cases. Shorter chain and less unsaturated species were also detected in most cases but retroconversion was not a major route for either acid.

Incorporation and metabolism of 18:3(n-6), 20:3 (n-6) and 20:4(n-6)

Supplementation with 18:3(n-6) increased the total percentage of (n-6)PUFA in all phospholipid classes (Table 7) and to a greater degree than 18:2(n-6) (Table 4). The incorporated 18:3(n-6) was significantly elongated to 20:3(n-6) in all phospholipid classes and in PS, PI and especially PE the percentages of 20:4(n-6) were also increased. Limited

Table 5. (n-3)PUFA composition[a] of phospholipid classes from RTG-2 cells cultured in medium supplemented with 20:5(n-3).

(n-3)PUFA	PC	PE	PS	PI	PA/CL
18:3	0.2	0.1	0.1	0.2	0.4
18:4	0.2	0.1	0.1	0.2	0.2
20:3	T	–	–	–	–
20:4	0.2	0.2	0.2	0.2	0.7
20:5	8.7	16.6	3.9	5.6	10.2
22:5	2.7	12.0	21.5	6.2	6.7
22:6	0.3	2.0	3.0	0.6	2.2
Total	12.3	31.0	28.8	13.0	20.4

[a]Results are expressed as a percentage of the total fatty acids. Cells were incubated with 20 μM fatty acid for 6 days; T, trace; –, not detected.

Table 6. (n-3)PUFA composition of phospholipid classes from RTG-2 cells cultured in medium supplemented with 22:6(n-3).

(n-3)PUFA	PC	PE	PS	PI	PA/CL
18:3	0.1	0.2	0.2	0.4	0.4
18:4	0.1	0.2	0.1	1.6	0.2
20:3	–	–	–	–	–
20:4	–	0.1	0.1	0.2	0.4
20:5	0.1	0.2	0.3	1.9	0.9
22:5	0.4	1.3	1.9	1.1	1.0
22:6	5.6	28.3	30.5	12.3	16.8
Total	6.3	32.2	33.1	17.5	19.7

Incubation conditions and data presented as in Table 5; –, not detected.

desaturation of 20:3(n-6) to 20:4(n-6) was also evident when 20:3(n-6) itself was supplemented but mainly confined to PE (Table 8). Large increases in 20:4(n-6) levels were only obtained when 20:4(n-6) itself was supplemented (Table 9). Further elongation of 20:4(n-6) to 22:4(n-6) was observed predominantly in PS and PE when all three (n-6)PUFA were supplemented, but to the greatest extent when 20:4(n-6) was supplemented. Further desaturation to 22:5(n-6) was not observed with any supplement. Although shorter chain and less unsaturated species were observed in all three cases, retroconversion does not appear to be a major route for these acids. As observed with 20:5(n-3) and 22:6(n-3) supplements and total (n-3)PUFA levels, the total percentage of (n-6)PUFA was increased in PE, PS and PI but not PC and PA/CL by 20:3(n-6) and 20:4(n-6) to a greater extent than by 18:2(n-6).

Table 7. (n-6)PUFA composition of phospholipid classes from RTG-2 cells cultured in medium supplemented with 18:3(n-6).

(n-6)PUFA	PC	PE	PS	PI	PA/CL
18:2	0.7	1.0	0.3	0.3	2.6
18:3	17.2	11.4	3.9	3.5	10.6
20:2	0.2	0.6	0.3	1.3	0.5
20:3	12.5	13.8	15.5	17.0	30.3
20:4	3.2	18.1	5.5	23.2	1.2
22:2	–	0.1	–	–	–
22:3	0.5	0.9	0.2	0.3	0.5
22:4	0.4	1.8	4.4	0.7	0.7
22:5	–	0.3	0.6	–	0.3
Total	34.6	48.0	30.7	46.3	51.6

Incubation conditions and data presented as in Table 5; –, not detected.

Table 8. (n-6)PUFA composition of phospholipid classes from RTG-2 cells cultured in medium supplemented with 20:3(n-6).

(n-6)PUFA	PC	PE	PS	PI	PA/CL
18:2	0.4	0.6	0.3	0.2	1.1
18:3	0.5	0.4	0.2	0.1	0.3
20:2	0.4	1.5	0.6	3.2	0.7
20:3	7.9	11.1	17.2	15.4	16.6
20:4	1.4	12.1	4.2	17.3	4.0
22:2	–	0.3	0.8	–	0.1
22:3	0.3	0.7	0.2	–	0.3
22:4	0.3	1.6	4.1	1.0	0.6
22:5	–	0.1	0.3	T	T
Total (n-6)	11.2	28.4	27.9	37.2	23.7

Incubation conditions and data presented as in Table 5; T, trace; –, not detected.

Supplementation with fish oil concentrates (FOC)

FOC 1 and FOC 2 both increased the total (n-3) PUFA contents of the RTG-2 cells to similar final values which were less than the totals obtained with either pure 20:5(n-3) or 22:6(n-3) (Table 10). With FOC 1, 20:5(n-3), 22:5(n-3) and 22:6(n-3) were all

increased in each phospholipid class and 18:4(n-3) was increased in PA/CL. With FOC 2 the increase was most prominent in 22:6(n-3), although 20:5(n-3) and 22:5(n-3) were also increased although to a lesser extent than with FOC 1.

Discussion

Although the RTG-2 cells grown in 10% FCS are grossly deficient in (n-3)PUFA, the fatty acid distribution between the phospholipid classes still retains many of the features observed in fresh tissues. For instance high percentages of 16:0 in PC, 18:0 in PI and PS, 20:4(n-6) in PI and 14:0 in SM have been reported previously (Hazel 1979). However, 22:6 (n-3) accounts for greater than 32% of fatty acids in PC and PE, 25% in PS and 10% in PI in trout liver cells at 20°C (Hazel 1979) showing the extent of (n-3)PUFA deficiency in the RTG-2 cells in the present study. Although 18:1(n-9) and the (n-9) PUFA are the major fatty acids that are increased in response to the lack of (n-3)PUFA, especially 22:6, the whole spectrum of fatty acids is affected.

Table 9. (n-6)PUFA composition of phospholipid classes from RTG-2 cells cultured in medium supplemented with 20:4(n-6).

(n-6)PUFA	PC	PE	PS	PI	PA/CL
18:2	0.7	0.6	0.3	0.6	2.6
18:3	0.3	0.5	0.7	1.1	1.6
20:2	0.6	1.3	0.6	1.7	1.0
20:3	1.6	3.1	1.7	1.6	4.2
20:4	9.6	21.6	9.4	30.4	15.3
22:2	0.1	–	0.7	–	–
22:3	0.1	1.3	–	–	–
22:4	2.5	15.4	20.1	3.2	5.2
22:5	–	0.2	0.3	–	–
Total	15.5	44.0	33.8	38.6	29.9

Incubation conditions and data presented as in Table 5; –, not detected.

The greater incorporation of 18:2(n-6) compared to 18:3(n-3) into the RTG-2 cell phospholipids was of note as this was not observed when total lipid was examined (Tocher *et al.* 1989). However the data on total lipid were obtained on cultures incubated with 20 μM PUFA over 7 days, and at this combination the values for total (n-3) and total (n-6)PUFA were comparable in the present study. The reason for the greater incorporation of 18:2 (n-6) into RTG-2 cell phospholipids in short incubations or at high concentrations is not known at present. Significantly, the bulk of the 18:2(n-6) incorporated did not get further chain elongated or desaturated and remained as 18:2(n-6).

There did not appear to be any specificity with the Δ6 desaturase for either substrate as 18:3 (n-6)/20:3(n-6) were present at similar percentages compared with 18:4(n-3)/20:4(n-3). However, the Δ5 desaturase of RTG-2 cells had an order of preference (n-3) > (n-6) >> (n-9) based on the relative increases in the percentages of 20:5(n-3)

Table 10. (n-3)PUFA composition of phospholipid classes from RTG-2 cells cultured in medium supplemented with FOC 1 and FOC 2

	FOC 1					FOC 2				
(n-3) PUFA	PC	PE	PS	PI	PA/CL	PC	PE	PS	PI	PA/CL
18:3	0.1	0.1	0.1	0.1	0.6	0.1	0.1	0.1	0.1	0.4
18:4	0.1	0.1	0.1	0.2	4.9	0.1	0.4	–	0.1	0.4
20:3	–	–	–	–	–	–	–	–	–	0.2
20:4	0.3	0.3	0.4	–	1.8	0.1	0.1	–	–	0.5
20:5	3.3	12.7	2.1	1.8	4.4	1.7	4.9	0.8	0.9	2.3
22:5	0.8	5.3	11.8	2.9	2.9	0.9	3.6	6.0	2.3	2.5
22:6	0.7	5.6	11.4	2.0	4.6	2.7	16.0	23.2	5.8	12.2
Total	5.3	24.1	25.9	7.0	19.2	5.6	25.1	30.1	9.2	18.5

Data presented as for Table 5. Cells were incubated with oils for 6 days at a concentration of 25 μM total (n-3)PUFA; –, not detected.

and 20:4(n-6) produced upon 18:3(n-3) and 18:2(n-6) supplementation, respectively, and the almost total lack of 20:3(n-9). Interestingly, the degree of Δ5 desaturase activity as measured by 20:4(n-6) levels was generally greater when 18:3 (n-6) was supplemented than when the immediate precursor 20:3(n-6) was supplemented, and may be linked to the greater incorporation of 18:3(n-6). Certainly, the percentages of total (n-6)PUFA, including 20:3(n-6), were higher when 18:3(n-6) was supplemented, suggesting that the resultant higher percentages of 20:4(n-6) were due to a substrate concentration effect. The lack of Δ4 desaturase activity in RTG-2 cells, as previously reported (Tocher *et al.* 1989), is clearly apparent and emphasizes the main difference between these cells and rainbow trout.

Superimposed upon the overall trends discussed above, the fatty acid compositions of individual phospholipid classes generated by the supplementations suggested that individual intermediates and products of the desaturation pathways were associated with specific phospholipid classes. For instance, PA/CL displayed the highest percentages of all the C18 PUFA and 20:3(n-6) when they were supplemented whereas PA/CL contained more average percentages of 20:5(n-3), 22:6(n-3) and 20:4(n-6) when they were supplemented. This suggests that PA and/or CL may play a role in the incorporation of precursor fatty acids into the phospholipid pool, which would be consistent with PA's central role in phosphoglyceride biosynthetic pathways (Harwood 1986).

PS tended to incorporate and/or accumulate C22 PUFA, especially 22:5(n-3) and 22:4(n-6). In control (10% FCS) cells and in cells supplemented with C18 and C20 PUFA the percentages of total C22 PUFA, predominantly 22:5(n-3) and 22:4(n-6), were always highest in PS. Furthermore the percentage of supplemented 22:6(n-3) itself was highest in PS. So, although Δ4 desaturase activity in RTG-2 cells is very low, it appears that PS is normally associated with C22 PUFA even if this part of the desaturation/elongation pathway was intact. Interestingly, PS also showed the highest percentages of 20:3(n-6), other than PI, in all (n-6)PUFA supplementations.

PE contained the highest percentages of 20:5(n-3) in control cells and cells supplemented with 18:3(n-3) and 20:5(n-3) and the highest percentages of 20:4(n-6), other than PI, when supplemented with (n-6)PUFA. In contrast to these situations, with PC there where no discernible patterns of PUFA incorporation. The PUFA and phospholipid specificities of the acylating enzymes involved and their role in the generation of these compositions are unknown but clearly central to the understanding of the patterns.

As with PS and PE, PI showed a distinct pattern of incorporation and accumulation in that the percentages of 20:4(n-6) and 20:3(n-6) were always highest in PI. The composition of PI, particularly the high level 20:4(n-6) is consistent with a role as a precursor pool for eicosanoid synthesis in normally (n-3)-rich fish tissues as postulated previously (Bell *et al.* 1983; Tocher and Sargent 1984). Metabolic studies showed that various trout tissues and thrombocytes produced cyclooxygenase products from endogenously (Christ and Van Dorp 1972) and exogenously (Kayama *et al.* 1985) added 20:4(n-6). Similarly 20:4(n-6) was metabolised via lipoxygenase to 12-hydroxyeicosatetraenoic acid in trout skin (German *et al.* 1985) and to mono- (mainly 12-hydroxy) (German *et al.* 1986) and trihydroxy derivatives in trout gill (German and Kinsella 1986). Although direct comparative studies are lacking in freshwater species, the available data have indicated that 20:4(n-6) is the major eicosanoid precursor in fish (Henderson and Tocher 1987) supporting a possible role for PI as a source of substrate. The results of the present study further support this hypothesis by indicating that precise and tightly controlled mechanisms exist for the maintenance of the 20:4(n-6) content of PI in fish cells.

It may be advantageous, certainly in some studies of lipid metabolism, for cultured fish cells to have a fatty acid composition more closely resembling that of actual fish tissues. Pure PUFA would be a relatively costly option and so two fish oil concentrates were examined in this study. FOC 2 adequately increased 20:5(n-3) and increased the 22:6 (n-3) level to a greater extent that FOC 1, as expected from the 22:6(n-3) content of the concentrates. However, the percentages of 22:6(n-3) at this con-

centration of supplement were lower than those found in trout liver phospholipids, especially PC (Hazel 1979). FOC 1 generated levels of 20:5(n-3) that were higher than those found in trout liver phospholipids at 20°C (Hazel 1979) and also very high 22:5(n-3) levels. Therefore although FOC 2 also increased 22:5(n-3) levels, it was able to generate an (n-3)PUFA composition more similar to trout tissues and so this high 22:6(n-3) with lower 20:5(n-3) concentrate would be better as a routine supplement.

Overall, the supplementation of (n-3) and (n-6)PUFA to cultured cells followed by analysis of the distribution of fatty acids between phospholipid classes has provided information on possible links between individual phospholipids and specific intermediates or products in the desaturation/elongation pathways. A central role is played by the acylating enzymes and the elucidation of their properties and, in particular, their fatty acid and phospholipid class specificities are vital to the understanding of this problem.

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