Metabolism of 18:4n-3 (stearidonic acid) and 20:4n-3 in salmonid cells in culture and inhibition of the production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from 20:4n-6 (arachidonic acid).

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Abstract

Arachidonic acid (AA; 20:4n-6) is the precursor of a range of highly biologically active derivatives, collectively termed eicosanoids, including prostaglandins, thromboxanes, leukotrienes and lipoxins, that act as autocrine hormones regulating many physiological processes including haemostasis, reproduction, immune and inflammatory responses. Eicosapentaenoic (EPA; 20:5n-3) and dihomo-γ-linolenic (20:3n-6) acids modulate eicosanoid metabolism by both inhibiting the conversion of AA to eicosanoids whilst simultaneously being converted to eicosanoids with different, often attenuated, properties compared to their AA homologues. Eicosatetraenoic acid (20:4n-3) is a naturally occurring C_{20} polyunsaturated fatty acid (PUFA), present in fish oil at levels of around 1-2%, that has been suggested to be the active metabolite responsible for the anti-inflammatory effects of plant oils containing stearidonic acid (18:4n-3). However, the biochemical properties of 20:4n-3 in terms of cellular biology have rarely been investigated, partly due to difficulties in obtaining the fatty acid in high purity. In this paper, we describe methods for the medium scale laboratory preparation of high purity 20:4n-3, and investigate its metabolism in fish cell culture systems which normally contain significant amounts of n-3 PUFA. Thus the incorporation and metabolism of 18:4n-3 and 20:4n-3, and their distribution in phospholipid classes was studied in an established cell line from Atlantic salmon (*Salmo salar*) (AS), and the effects of 20:4n-3 on eicosanoid production studied in freshly isolated macrophages from rainbow trout (*Oncorhynchus mykiss*). Both 18:4n-3 and 20:4n-3 were preferentially esterified into phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in contrast with the accumulation of AA in phosphatidylinositol. Incorporated 18:4n-3 was readily converted to 20:4n-3, and both fatty acids were further desaturated and elongated to EPA and 22:5n-3 but not 22:6n-3. Supplementation with 20:4n-3 decreased the conversion of AA into prostaglandins, as demonstrated by the decreased levels of $PGF_{2\alpha}$ produced in trout macrophages supplemented with 20:4n-3 and AA compared to cells supplemented with AA alone. In addition, 20:4n-3 was converted into eicosanoids in fish cells as indicated by the presence of $\Delta^{17,18}$ 12-HETE, $\Delta^{17,18}$ PGE_1 and $\Delta^{17,18}$ $PGF_{1\alpha}$ in extracts from rainbow trout macrophages incubated with 20:4n-3.

Introduction

Eicosanoids are produced by virtually all tissues and are, by definition, derivatives of C_{20} polyunsaturated fatty acids (PUFA) that include prostaglandins, thromboxanes, prostacyclins, leukotrienes, and lipoxins, and serve as autocrine hormones controlling and regulating many physiological processes including haemostasis (blood clotting), reproduction, immune and inflammatory responses (Johnson et al. 1983; Lands 1993). The enzymic pathway producing eicosanoids is often called the "arachidonic acid cascade" since arachidonic acid (AA; 20:4n-6) is the principal precursor in mammals (Horrobin 1983). However, other C_{20} PUFA such as dihomo-γ-linolenic acid (20:3n-6) and eicosapentaenoic acid (EPA; 20:5n-3) are known to modulate eicosanoid metabolism by both inhibiting the conversion of AA to eicosanoids whilst simultaneously being converted to eicosanoids with different, often attenuated, properties compared to their AA homologues (Willis 1981; Crawford 1983; Horrobin 1983; Weber 1990). Eicosatetraenoic acid (20:4n-3) is another naturally occurring C_{20} PUFA capable of forming eicosanoids, and has been termed "ω3 arachidonic acid", although this is perhaps confusing as it is actually the n-3 series equivalent of 20:3n-6. However, despite the well known interactions between 20:3n-6, EPA and AA in eicosanoid metabolism, research on 20:4n-3 is sparse, notable exceptions being the early studies of Sprecher and coworkers (Oliw et al. 1986a,b; Careaga and Sprecher 1987) and the more recent study of Croset et al. (1999), all establishing that 20:4n-3 can modulate eicosanoid production in mammalian cell systems. The relative neglect of 20:4n-3 is partly due to its rarity in natural oils making it difficult to obtain and expensive. Marine fish oils can contain minor quantities (around 1-2%) of 20:4n-3 and also larger quantities (up to 4-5%) of stearidonic acid (18:4n-3), the immediate biosynthetic precursor of 20:4n-3 (Ackman 1980). However, 18:4n-3 is also found in the seed oils of some plants, particularly of the Boraginaceae species (Moine et al. 1992), with some containing very high levels, with up to 21% being reported in *Echium asperrimum* (Guil-Guerrero et al. 2001). Echium oil is thus similar to borage oil except that the levels of 18:4n-3 can exceed those of its n-6 series equivalent,18:3n-6 (γ-linolenic acid), and thus may be expected to have somewhat similar therapeutic activity in humans (Guichardant et al. 1993).

Fish do not appear to be fundamentally different to mammals with respect to eicosanoid production, with virtually all tissues between them producing the same wide range of eicosanoids in a large range of freshwater and marine fish (Tocher 1995; 2003). Thus, although both AA and EPA serve as eicosanoid precursors in fish tissues, AA is the preferred substrate despite the preponderance of EPA in fish phospholipids. It has also been established in fish cell cultures that

EPA and 20:3n-6 competitively depress the production of eicosanoids from AA (Bell et al. 1994). The effect of 20:4n-3 on eicosanoid metabolism is unstudied in fish.

Currently there is considerable interest in the metabolism of PUFA in fish, particularly major aquacultured species such as salmonids, including Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). This is because much aquaculture, including salmonid culture, is dependent upon wild capture fisheries for the provision of fish meals and oils that have traditionally been the basis of pelleted feeds (Sargent and Tacon 1999). However, stagnation in industrial fisheries, along with the increased demand for fish oils, has dictated that alternatives to fish oil must be found if aquaculture is to continue to expand and supply more of the global demand for fish (Barlow 2000). The only sustainable alternative to fish oils are plant oils which are rich in C_{18} PUFA, but few of these are rich in n-3 PUFA, a notable exception being linseed oil, rich in 18:3n-3 (Padley et al. 1986). The lack of oils rich in n-3 PUFA indicates that oils such as echium, rich in 18:4n-3, may be particularly useful.

The present study aims to test two primary hypotheses. Firstly, that 20:4n-3 will have a modulatory effect on eicosanoid metabolism in fish through both inhibiting eicosanoid production from the predominant precursor fatty acid, AA, and also by being converted to its own eicosanoid derivatives. Secondly, that 18:4n-3 (and thus 18:4n-3-rich plant oils) will potentially have similar modulatory effects by being converted to, and thus increasing the cellular content of, 20:4n-3. Lastly, as high purity 20:4n-3 is extremely expensive and it's supply erratic, indeed it was unavailable commercially at the time of this study, methods were investigated for the laboratory production of medium scale amounts of 20:4n-3.

Material and methods

Preparation of high purity 20:4n-3

a) Medium scale chromatography. Fatty acid ethyl esters, prepared by acid catalysed transesterification in ethanol of a conventional marine fish oil containing ~1% of 20:4n-3 (wt % of total fatty acids present) were used as starting material. Three grams of ethyl esters in acetonitrile were applied to a Biotage 40 Flash Cartridge System (Biotage UK Ltd), comprised of a column Flash 40M (135 grams KP-C18 HS TM, 35-70 mm, 60 Å, 500-550 m^2/g, 18% carbon load), end capped column, diameter 40 mm, length 15 cm, packing weight 90 g and 100 ml volume. The column was eluted with acetonitrile under nitrogen at a pressure of 0.6 bar at 28^oC, the first 360 ml of eluate discarded and the subsequent eluate collected in 20 ml fractions and analysed by GC. The 20:4n-3-ethyl ester enriched fraction (120 mg) contained 20:4n-3 at 12% (wt % of the total fatty acids present) along with other fatty acids including 20:4n-6 (13.3%), 20:5n-3 (1.2%), 22:5n-3

(18.1%) and 22:6n-3 (3.8%). The ethyl ester fraction was further purified by semi-preparative HPLC (Aveldano et al. 1983) using a Beckman Ultrasphere ODSA column (C18, 25x1cm, 5m particle size) and a Waters 501 HPLC pump, with acetonitrile/water (85:15, v/v) as eluant at a flow rate of 7 ml/min at 20^{o}C giving a pressure of approximately 1400 psi., and UV detection at 215 nm. Three mg batches of the total ethyl ester mixture in a acetonitrile (50mg/ml) were applied to the column using a 100 ml injection loop. Fractions were collected according to increase in UV absorbance (peak elution) and the ethyl ester of 20:4n-3 was collected at a retention time of 25.2 min

b) Partial Synthesis. Partially synthesis of 20:4n-3 was achieved by two successive C-1 extensions of 18:4n-3 through conversion to fatty alcohols, then mesylates, chain elongated nitriles and finally methyl esters. The process is very briefly described below. The starting material was an oil consisting of 18:4n-3 and 20:5n-3 in approximately equal amounts, produced by fractional distillation of a conventional marine fish oil (Croda Universal Ltd., Hull, England).

The fatty acid mixture in dry diethyl ether was refluxed for 1 h with lithium aluminium hydride under argon. After cooling, saturated ammonium chloride solution was added and the ether phase separated, the aqueous phase extracted twice with ether. The combined ether fractions were dried over $MgSO_4$, filtered and evaporated on a rotary evaporator to yield the mixed fatty alcohols. This product was dissolved in dry dichloromethane and methanesulphonyl chloride and dry pyridine added and the mixture stirred overnight in the dark under argon. The solvent was removed on a rotary evaporator and diethylether and 2M HCl added, and the mixture shaken and separated. The extracted ether phase was dried over $MgSO_4$, filtered, evaporated, and the crude product purified by flash column chromatography using an *iso*-hexane/diethylether gradient to yield a homogeneous mixture of the mesylates. The purified mesylates were stirred at 90^{o}C overnight with potassium cyanide in dry dimethylformamide, under argon. The cooled reaction mixture was poured on to water and the resultant mixture extracted six times with ether. The combined ether extracts were dried over $MgSO_4$, filtered, evaporated, and the crude product purified by flash column chromatography to yield a homogeneous mixture of the C-1 chain extended nitriles. The purified nitriles (C_{19} and C_{21}) were stirred at room temperature with dry HCl in methanol under argon in the dark. The methanol was removed by rotary evaporation, water and *iso*-hexane added to the residue, and the resultant mixture stirred at room temperature under argon for 2 hours. The *iso*-hexane phase was separated, the aqueous phase extracted with *iso*-hexane, and the combined hexane fractions dried over $MgSO_4$, filtered and evaporated. The crude product was purified by flash column chromatography to yield a homogeneous mixture of the C-1 chain extended methyl esters .

The reaction sequence described above was repeated to give the C-2 chain extended

methyl esters. The esters were chromatographically homogeneous on thin-layer chromatography (TLC) but were readily separated by HPLC, as described above for ethyl esters other than the retention time was 19.1 min for 20:4n-3 methyl ester. The mass ions, fragmentation patterns and chromatographic properties (retention times) of the purified esters were determined by GC/MS (CI- and EI+) as described previously (Ghioni et al. 1999) and compared with known standards. Proton NMR spectra recorded in deuterated chloroform at 600 MHz: methyl (Z)8,(Z)11,(Z)14,(Z)17-eicosatetraenoate showed the following NMR signals: d 0.96(3H, t, J=7.5Hz), 1.26-1.37(6H, om*), 1.59-1.64(2H, qn*), 2.03-2.09(4H, om*), 2.29(2H,t, J=7.6Hz), 2.79-2.84(6H,om*), 3.65(3H,s) and 5.27-5.43(8H, om*) (where *om = overlapping multiplets, i.e. couplings not measurable due to high overlap and/or second order spectrum).

Cell culture systems

The Atlantic salmon (*Salmo salar*) cell line (AS) as described previously (Nicholson and Byrne 1973), was maintained at 22°C in Leibovitz L-15 medium supplemented with 10 mM HEPES buffer, 2 mM glutamine, antibiotics (50 IU/ml penicillin and 50 mg/ml streptomycin) and 10% fetal bovine serum (FBS).

Rainbow trout (*Oncorhynchus mykiss*) macrophages were obtained as follows. The head kidney was freshly isolated from fish (approx. 50 g each) anaesthetised with benzocaine, bled from the caudal vein and sacrificed according to protocols approved by the British Home Office. The tissue, in 10 ml L-15 as above and containing 10 units heparin, was gently mashed through a gauze (100 μm mesh), the resultant cell suspension layered on 50 ml 51-34 Percoll gradients in Dulbecco's modified Eagle medium (DMEM), and centrifuged at 400g for 25 min. The macrophage layer at the interface was collected and centrifuged at 2000 rpm for 7 min. The pellet (approx. 4 x 10^7 cells) was resuspended in serum-free L-15 medium and seeded into two 25 cm^2 tissue culture flasks, the macrophages left to adhere for 3 hours at 22^{o}C, the excess cells removed by washing 3 times with phosphate buffered saline (PBS), and the culture finally incubated in 5 ml L-15 containing 5% FBS. Lipopolysaccharide (LPS)-stimulated cells were incubated with 25 mg/ml LPS (E.coli Serotype 026:B6) and the medium collected after 24 hours for prostaglandin analysis.

Supplementation of cells with PUFA

Polyunsaturated fatty acids were added to actively growing AS cells or fresh preparations of trout macrophages, in L-15 media supplemented with reduced FCS (2%), either as complexes with fatty acid free-bovine serum albumin (FAF-BSA) or as methyl esters, to give a final concentration of 25 μM. Earlier studies had shown no difference between supplementations with methyl esters or BSA-

bound free acids (Ghioni et al. 1999). Cultures were incubated with fatty acids for 4 days at 22°C. Fatty acid/BSA complexes were prepared by stirring 10 mg of fatty acid for 10 min in 100 μl 0.1 M KOH and for further 45 min after the addition of 5 ml of 50 mg/ml FAF-BSA in PBS at 20°C. The fatty acid/BSA complexes were filtered (0.22 μm Millex GV) into sterile vials.

Lipid extraction

Medium was removed by aspiration, cells washed with 5 ml PBS and then dissociated with 0.05% trypsin/ 0.02% EDTA solution. Cells were harvested in 3 ml Hanks' balanced salt solution (HBSS), centrifuged at 300g for 5 min at 4°C, and washed with 5 ml HBSS containing 1% FAF-BSA resuspended in 5 ml HBSS. After centrifuging, the washed cell pellet was extracted with 5 ml ice-cold chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, according to Folch et al. (1957). Lipid was determined gravimetrically and resuspended in chloroform/methanol (as above) at a concentration of 10 or 100 mg/ml and stored at -20°C under argon.

Lipid analysis

Separation and quantification of lipid classes was performed by high-performance thin-layer chromatography (HPTLC), followed by calibrated scanning densitometry (Henderson and Tocher 1992). Approximately 10 μg (1 μl) of lipid extract was loaded as a 2 mm streak and the plate developed to two-thirds distance with methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.) (Vitiello and Zanetta 1978). After desiccation, the plate was fully developed with hexane/diethyl ether/acetic acid (80:20:2, by vol.). Plates were charred at 160°C for 15 min after spraying with 3% aqueous cupric acetate containing 8% phosphoric acid. Densitometric analysis was performed on a Shimadzu CS-9000 dual wavelength scanner and DR-13 recorder.

To determine fatty acid composition of total lipids, approximately 1 mg of lipid extract was transmethylated overnight in 2 ml of 1% sulphuric acid in methanol and 1 ml of toluene at 50°C and under nitrogen (Christie 1982). Fatty acid methyl esters (FAME) were extracted and purified as described previously (Tocher and Harvie 1988). Purified FAME were dissolved in hexane containing 0.05% BHT to a final concentration of 2 $mg.ml^{-1}$. Phospholipid classes were separated as described by Tocher et al. (1995). Total lipid extract were applied as streaks (1 mg per cm) on TLC plates and the plate fully developed with methyl acetate/isopropanol/chloroform/ methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.). Phospholipids were visualized with 0.1%

2',7'-dichlorofluorescein (DCF) in 97% methanol containing 0.05% BHT and the corresponding silica scraped. FAME of individual phospholipid classes were prepared by transmethylation in methanolic sulphuric acid as above, directly on the silica without prior elution of the lipids as described by Christie (1982). Analysis of FAME was performed by gas chromatography in a Fisons GC8000 gas chromatograph (Crawley, UK) equipped with a fused-silica capillary column (30m x 0.32 mm i.d., CP Wax 52 CB, Chrompack, UK) using hydrogen as carrier gas. Temperature programming was from 50 to 150°C at 35°C/min and to 225°C at 2.5°C/min. Individual FAME were identified by comparison with known standards and published data (Ackman 1980; Tocher and Harvie 1988).

Eicosanoid extraction and analysis

Supernatants from macrophage or AS cultures were obtained after 4 days of culture and stored at -20°C prior to analysis. The samples, acidified to pH 3.0 with a 20% v/v solution of acetic acid in water, were applied to Sep-Pak Vac 3 cc (200 mg) tC18 Cartridges (Waters Ltd., Elstree, U.K.), preconditioned with methanol and acid water (pH 3.0). The cartridges were washed with acid water and heptane before eicosanoids were eluted using heptane/ethyl acetate/methanol (40:50:10 by vol.). Separation and analysis of eicosanoids was by gas chromatography-mass spectrometry (GC-MS) using a Fisons GC8000 gas chromatograph coupled to an MD800 mass spectrometer (Fisons Instruments, Crawley, U.K.) with helium as carrier gas (50 KPa) and equipped with a DB1701 capillary column (J&W 22-0732, 30 m, 0.25 mm i.d., J. & W. Scientific, Folsom, CA, U.S.A.), with temperature programming of 216°C for 1 min, then to 260°C at 9°C/min, and to 300°C at 2°C/min with the final temperature held for 6 min.

Eicosanoids were analyzed using chemical ionization (CI^-) with ammonia as reagent gas and electron ionization (EI^+) at 70 eV. Samples for GC-MS (EI^+) analysis, obtained from pooled preparations (1.8 x 10^8 cells), were MOX derivatised (50µl acetonitrile and 300µl 3% methoxyamine HCl solution, at room temperature for 1 hour), treated with 0.5ml diazomethane for 30 min and with 200 µl BSTFA overnight. For CI^- analysis, deuterated prostaglandin standards (1 ng $dPGF_{2\alpha}$ and 1 ng $dPGE_2$) were added to the acidified samples (each obtained from approx. 2-3 x 10^7 cells) before extraction. Each sample was sequentially derivatised as follows: MOX (as above); PFB (20 µl 10% DIPEA and 40 µl 10% pentafluorobenzxylbromide in acetonitrile at room temperature for 30 min) and TMS (10 µl anhydrous dimethylformamide and 20 µl BSTFA contaning 1% TMCS, at room temperature for 30 min). Dried residual were transferred to autosampler vials in 20 µl dodecane for GC/MS analysis.

Quantitation of mass ions in CI^- mode (SIM, selected ion mode) was performed using the following parameters: interface 300°C, source 200°C, emission current 350 mA, methane electron energy 70 eV, electron multiplier 750 V.

Materials

Arachidonic acid (20:4n-6) and eicosapentaenoic acid (20:5n-3), both 99% pure, stearidonic acid (18:4n-3) > 98% pure, Leibovitz L-15 Medium, DMEM, HBSS, PBS, glutamine, antibiotics, HEPES buffer, FAF-BSA, and trypsin/EDTA 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 (Fisher Scientific UK, Loughborough, England).

Statistical analysis

Results are reported as means ± SD (n = 3) unless otherwise stated. All statistical analyses were performed using a statistical computer package (Prism 3.0). Differences between mean values were determined by one-way ANOVA followed where appropriarte by Tukey's multiple comparison test. Variance homogeneity was checked with Bartlett's test and non-homogeneous data were arcsin transformed prior to further statistical analysis. Differences were reported as significant if $p<0.05$ (Zar 1984).

Results

Effect of 18:3n-3 and 20:4n-3 on lipid class composition of AS cells

Supplementation of AS cells with 18:4n-3 and 20:4n-3 had generally similar effects on lipid class compositions in that both resulted in increased proportions of triacylglycerol (TAG) and decreased proportions of cholesterol (Table 1). The increased TAG was quantitatively greater and accompanied by increased steryl esters in cells supplemented with 18:4n-3 resulting in increased total neutral lipids with decreased proportions of all polar lipids other than phosphatidylcholine (PC). The proportion of PC was increased in cells supplemented with 20:4n-3 resulting in a higher proportion of total polar lipids (Table 1).

Effect of 18:3n-3 and 20:4n-3 on fatty acid composition of total lipid of AS cells

Supplementation of AS cells with both 25 μM 18:4n-3 and 20:4n-3 resulted in increased proportions of total n-3 PUFA, 18:4n-3, 20:4n-3, 20:5n-3, 22:4n-3 and 22:5n-3 but not 22:6n-3 (Table 2). The relative proportions of n-6 PUFA were slightly decreased but there was no significant effect on the level of AA in total lipid by supplementation with either 18:4n-3 or 20:4n-3. In both cases, the increased n-3 PUFA was balanced by decreased proportions of monoenoic acids, predominantly 18:1n-9, whereas the levels of saturated fatty acids were relatively less affected.

Distribution of 18:4n-3 and 20:4n-3 in phospholipid classes

There was virtually no 20:4n-3 and only very small amounts of 18:4n-3 present in phospholipid classes of unsupplemented (control) AS cells (Tables 3 & 4). Supplementation of AS cells with either 18:4n-3 or 204n-3 increased the proportions of total n-3 PUFA in all phospholipid classes and the increase was consistently greater in cells supplemented with 20:4n-3 compared to 18:4n-3. The increase in total n-3 PUFA was lowest in phosphatidylinositol (PI), the class in which AA was concentrated and, indeed, the relative proportion of AA was increased in PI but decreased in the other phospholipid classes in response to supplementation with 18:4n-3 and 20:4n-3 (Tables 3 & 4). As with total lipid, supplementation of AS cells with both 18:4n-3 and 20:4n-3 resulted in increased proportions of 18:4n-3, 20:4n-3, 20:5n-3, 22:4n-3 and 22:5n-3 in all phospholipid classes. The increased levels of 18:4n-3 and 20:4n-3 were greatest in PC, whereas the increased levels of 20:5n-3 and 22:5n-3 were greatest in phosphatidylethanolamine (PE) and phosphatidylserine (PS), respectively. Although the relative amount of 22:6n-3 was not increased in total lipid, it was increased in PS and PE, at least in cells supplemented with 18:4n-3. As with total lipid, the increased n-3 PUFA was balanced primarily by decreased proportions of monoenes, predominantly 18:1n-9, although n-9 PUFA also decreased as did n-6 PUFA in all phospholipid classes other than PI. Most interestingly, the proportion of AA decreased in all phospholipid classes, particularly in cells supplemented with 20:4n-3, except PI where the percentage of AA was significantly increased in cells supplemenmted with either 18:4n-3 or 20:4n-3. In contrast to total lipid, the relative proportions of saturated fatty acids increased in AS cells supplemented with 18:4n-3 and 20:4n-3 in all phospholipid classes except PC.

Eicosanoid production after supplementation with 20:4n-3

It was outwith the scope of this project to fully characterise the range of eicosanoids produced

by the fish cell culture systems used in the study. Rather the focus was to determine if specific eicosanoid products of 20:4n-3 could be identified in the cell culture systems after supplementation with 20:4n-3. Previously, it had been shown that the predominant eicosanoid produced by rainbow trout head kidney macrophages was 12-hydroxyeicosatetraenoic acid (12-HETE), the product of 12-lipoxygenase activity on AA (Pettitt et al., 1989, 1991). Furthermore, the characteristic ions for 12-HETE in EI^+ GC-MS analysis are known (m/z 406, 391, 375, 316, 295 and 73 with 295 as the base peak) which enabled the characteristic ions for the 12-lipoxygenase product of 20:4n-3 to be calculated. Therefore, we decided to look for the 12-lipoxygenase products in trout macrophages and AS cells incubated with 25 μM 20:4n-3. Total ion chromatograms (TIC) from electron ionisation (EI^+) GC-MS analysis of eicosanoid extracts from rainbow trout macrophages (and AS cells) incubated with 20:4n-3 showed a peak (peak X in Fig.1A) with ions at m/z 406, 391, 375, 354, 316, 297 and 73 with the base peak at 297 (Fig.2) indicating that this peak was $\Delta^{17,18}$ dehydro-12-HETE. The EI^+ GC-MS analysis of eicosanoid extracts from cells incubated with 20:4n-3 gave no peak corresponding to 12-HETE, that is, with characteristic ions at m/z 406, 391, 375, 316 and a base peak at 295, although it was the predominant hydroxy fatty acid in cells supplemented with AA. The EPA derivative, 12-hydroxypentaenoic acid (12-HEPE) (m/z 404, 389, 314, 295) was found in eicosanoid extracts from cells supplemented with 20:5n-3 (data not shown). However, EI^+ GC-MS enables specific masses, characteristic of eicosanoids, to be scanned for. Thus, confirmation of the presence of $\Delta^{17,18}$ dehydro-12-HETE was obtained by scanning eicosanoid extracts of cells supplemented with 20:4n-3 for mass 297, with a clear peak corresponding to $\Delta^{17,18}$ dehydro-12-HETE being observed (Fig. 1C). Similarly, scanning for mass 295 did not give any peaks with the characteristic spectra of 12-HETE. Analysis of extracts from macrophages supplemented with 20:4n-3 by negative ion electrospray MS showed traces of m/z 319 (characteristic of HETE), m/z 317 (HEPE), 335 (dihydroxyeicosatetraenoic acid; DHETE) and 333 (dihydroxyeicosapentaenoic acid; DHEPE). These may represent products of, say, 5-lipoxygenase (HETE and HEPE) and 5-and 12-lipoxygenase combined (DHETE and DHEPE). However, the amount of material in these peaks was too low to give diagnostic spectra. However, for comparison, macrophages supplemented with AA showed the same m/z characteristic of the three classes of hydroxy fatty acids, while macrophages supplemented with 20:5n-3 only showed m/z 317 (HEPE) and 333 (DHEPE) in negative ion electrospray MS. These results demonstrated that the *in vitro* cell systems showed 12-lipoxygenase activity and generated 12-hydroxy fatty acids for all three fatty acids, 20:4n-6, 20:5n-3 and 20:4n-3 along with other hydroxy and dihydroxy fatty acids.

The TIC from EI^+ GC-MS analyses of trout macrophages supplemented with 20:4n-3 also

showed traces of another eicosanoid (peak Y, Fig.1A) eluting with a similar retention time to that for the main isomer in an authentic PGE_2 standard (Fig.1B) and with a similar spectrum (m/z 524, 508, 449, 418, 366, 295, 225, 73). A mass scan for 508 in EI^+ GC-MS clearly showed that the PGE_2-like eicosanoid in macrophages supplemented with 20:4n-3 actually eluted just before the authentic PGE_2 standard isomer (Fig.1B and D). The PGE_2-like eicosanoid could be another PGE_2 isomer, but its elution profile strongly suggests that it represents the $\Delta^{17,18}$–dehydro-PGE_1 product of 20:4n-3, as described by Oliw et al. (1986a). No samples from either of the cell systems showed traces of clearly identifiable PGE or PGF in EI^+, including cells incubated with AA or EPA. If present, their concentration must have been below the detection limit of this technique and/or the strong ionisation used destroyed their characteristic ions.

Gas-chromatographic conditions were optimised as described in the methods to obtain a clear chromatographic separation of the three PGF standards commercially available, in order to use chemical ionisation (CI^-) GC-MS to monitor their molecular ions at specific retention times. A mixture of $PGF_{1\alpha}$, $PGF_{2\alpha}$ and $PGF_{3\alpha}$ was resolved in CI^- GC-MS giving three peaks at 21.09 min, 21.43 min and 21.96 min (Fig.3A). The mass ions for these peaks identified them as $PGF_{2\alpha}$, (m/z 569.4) (Fig.4A), $PGF_{3\alpha}$, (m/z 567.4) and $PGF_{1\alpha}$, (m/z 571.4), respectively. Under the same conditions, samples extracted from cells incubated with 25 μM 20:4n-3 gave a peak at 22.3 min, just after where $PGF_{1\alpha}$ would elute (Fig.3B). The m/z signal of 569.4 for the molecular ion for the peak at 22.3 min was identical to that for $PGF_{2\alpha}$ indicating that it was the $PGF_{2\alpha}$ isomer from 20:4n-3, $\Delta^{17,18}$ –dehydro-$PGF_{1\alpha}$ (Fig.4B). Unfortunately there is no standard commercially available for this compound, but the elution properties of the additional signal at 22.3 min, compared to the other three isomers, would be in accordance with its molecular structure. The presence of a double peak for each PGE isomer (syn and anti isomers produced by reaction with MOX) made CI^- MS as described above for PGF less reliable for measuring PGEs as total chromatographic separation could not be achieved.

Given the good chromatographic separation of PGF isomers and the ability to positively resolve $PGF_{2\alpha}$ from the other PGF isomers, we compared levels of $PGF_{2\alpha}$ in activated macrophages supplemented with 20:4n-3 in relation to controls (unsupplemented), to macrophages supplemented with AA alone and in experiments in which both 20:4n-3 and AA were administered together. Figure 5 summarises the results of two typical experiments. Although there is some variability between experiments, the results indicated that a considerable increase in the production of $PGF_{2\alpha}$ occurred in cells supplemented with AA alone. A reduction of $PGF_{2\alpha}$ was observed when 20:4n-3 was supplemented to cells in conjunction with AA. The levels of $PGF_{1\alpha}$ and PGE_3 were too low to

be detected even in CI^+ SIM.

Discussion

With respect to our specified aims, both of the primary hypotheses which we set out to test were proved correct. Thus, the present study has shown that 20:4n-3 does have effects on eicosanoid metabolism in fish cells, through both inhibiting eicosanoid production from AA and being converted to eicosanoids itself, and that 18:4n-3 can serve as a precursor for 20:4n-3 in salmonids. Specifically, we have shown that incubation of Atlantic salmon cells or trout macrophages with either 18:4n-3 or 20:4n-3 both increased the cellular content of 20:4n-3 and that $PGF_{2\alpha}$ production in macrophages incubated with 20:4n-3 and AA was reduced compared to macrophages supplemented with AA alone. Furthermore, 20:4n-3 was converted by cyclooxygenases to $\Delta^{17,18}$-PGE_1 and $\Delta^{17,18}$-$PGF_{1\alpha}$ in trout macrophages and by 12-lipoxygenase to $\Delta^{17,18}$-12-HETE in both macrophages and Atlantic salmon cells.

In comparison to mammals, there is not a great deal of data on eicosanoids in fish, but what is known suggests that the metabolism and roles of eicosanoids in fish and mammals is generally similar. Certainly, the above data are consistent with previous studies in mammals. Early work had shown that 20:4n-3 was a poor substrate for platelet cyclooxygenase (Needleman et al. 1981), but exogenously added 20:4n-3 was converted by the 12-lipoxygenase in human platelets to the 12-hydroxy derivative, but no lipoxygenase products were formed from 18:4n-3 (Careaga and Sprecher 1987). However, ram seminal vesicle microsomes converted exogenous 20:4n-3 to Δ^{17}-PGE_1 and Δ^{17}-$PGF_{1\alpha}$ but, no eicosanoid products were produced from 18:4n-3 (Oliw et al. 1986a). Subsequently, the same authors showed that ram seminal fluid contained Δ^{17}-PGE_1 produced from endogenous 20:4n-3, presumably present as a result of desaturation and elongation of the copious 18:3n-3 in the natural diet of sheep (Oliw et al. 1986b). Samel et al. (1987) confirmed the presence of a cyclooxygenase derivative of 20:4n-3 in ram seminal fluid and the ability of homogenates to convert exogenous 20:4n-3 to 5,6-dihydro-PGE_3 (identical to $\Delta^{17,18}PGE_1$). Thus, the same eicosanoid products of 20:4n-3 were identified in the salmonid cells incubated with 20:4n-3 as had been observed in mammalian systems. In addition, monkey seminal vesicles have also been shown to convert 20:4n-3, EPA and 18:3n-3 to their corresponding ω4,ω3-diols (Oliw and Sprecher 1991).

There are few data on the inhibition of eicosanoid synthesis from AA by 20:4n-3 in mammalian systems. However, one study has reported that 20:4n-3 inhibited PGH synthetase activity and reduced the production of 2-series prostaglandins from AA in human platelets and

endothelial cells (Croset et al. 1999). In the present study, we did not examine the direct effect of 18:4n-3 supplementation on eicosanoid synthesis, but the results strongly suggested that 18:4n-3 would have modulatory effects through conversion to 20:4n-3 and this has been shown in mammalian systems. The 5-lipoxygenase was inhibited, and leukotriene synthesis decreased, in human leukocytes incubated with 18:4n-3, with the inhibition being comparable to that observed with EPA (Guichardant et al. 1993). Incubation of MC/9 mouse mast cells with 18:4n-3 suppressed the production of leukotriene B_4 (LTB_4), LTC_4 and 5-HETE (Ishihara et al. 1998). Similarly, the level of PGE_2 in splenocytes was reduced in mice by feeding diets containing 10% 18:4n-3 or EPA or 18:3n-3 compared to mice fed an 18:2n-6-rich safflower oil diet (Ishihara et al. 2002). The authors also reported that the levels of 20:4n-3, EPA and DHA in plasma and splenocytes were increased in mice fed the diet containing 18:4n-3 (Ishihara et al. 2002).

The above study showed that dietary 18:4n-3 was converted to 20:4n-3 and EPA in mice. In rats fed diets containing 1% 18:4n-3 ethyl ester, the levels of liver and plasma 18:4n-3 were very low, but 20:4n-3 was found in phospholipids and the level of EPA was twice as high compared to rats fed 1% 18:3n-3 (Yamazaki et al. 1992). Similarly, when rats were fed blackcurrant oil containing 2.6% 18:4n-3 (and 12.9% 18:3n-3), the levels of EPA and 22:5n-3, but not DHA, were increased in liver plasma membranes, but the levels of 18:4n-3 and 20:4n-3 were not reported and presumably not detected (Barzanti et al. 1995).

The above results from *in vivo* studies were also reflected in *in vitro* mammalian cell studies. Exogenously added 18:4n-3 was incorporated into NIH-3T3 cells and elongated to 20:4n-3, and desaturated to EPA and 22:5n-3, but not DHA (Cantrill et al. 1993). Similarly, the level of EPA, but not 22:5n-3 or DHA, was increased in human endothelial cells incubated with 20:4n-3 (Croset et al. 1999). Equivalent results were obtained in the present study, where incorporated 18:4n-3 was readily converted to 20:4n-3, and both 18:4n-3 and 20:4n-3 were further desaturated and elongated to EPA and 22:5n-3, but not 22:6n-3. In an earlier study, we had shown that supplementation of AS cells with 18:4n-3 increased the levels of 18:4n-3, 20:4n-3 and EPA, but not 22:5n-3 or DHA, in total lipid (Tocher and Dick 1990a). More recently a radioisotope study showed that in AS cells incubated with [U-^{14}C]18:4n-3, radioactivity was recovered predominantly in EPA followed by 20:4n-3 and 22:5n-3 with only a small amount recovered in DHA (Ghioni et al. 1999). Similarly, [U-^{14}C]20:4n-3 was predominantly converted to EPA with small amounts of radioactivity recovered in 22:4n-3 and 22:5n-3, with only very little radioactivity recovered as DHA (Ghioni et al. 1999).

Therefore it is clear that, whether fed in the diet in *in vivo* studies or supplemented to cells *in vitro*, both 18:4n-3 and 20:4n-3 are readily converted to EPA. As a result, although our postulated hypotheses were proved, the mechanism whereby 20:4n-3 could exert a modulatory effect on

eicosanoid metabolism is not fully clear. Inhibition of eicosanoid production from AA could be due to competitive inhibition by either 20:4n-3 itself or by EPA produced by desaturation of incorporated 20:4n-3. However, it is also clear that 20:4n-3 serves as a fatty acid substrate for cyclooxygenase and lipoxygenase enzymes and it has been demonstrated that the 20:4n-3-derived eicosanoids have unique biological activities. For example, the 20:4n-3-derived prostaglandin 5,6-dihydro-PGE_3 (or $\Delta^{17,18}$-dehydro-PGE_1) had 14 times less activity as a uterine stimulant than PGE_1 while retaining 75% of the anti-aggregatory potency of PGE_1 (Samel et al. 1987).

The phospholipid source of precursor PUFA is an intriguing aspect of eicosanoid metabolism in fish as the vast majority of species have membrane phosphoglycerides containing a large excess of EPA relative to AA (Tocher 1995). Despite this, AA is still the primary precursor of eicosanoids in fish where it is concentrated in PI, and thus it has often been speculated that PI may be the source of AA for eicosanoid production in fish (Tocher 1995). Hence our interest in the phosphoglyceride distribution of incorporated 18:4n-3 and 20:4n-3 in the present study. Again PI appeared to be unique among the phospholipid classes, as the proportion of AA increased in PI along with increased levels of n-3 PUFA in response to supplementation with 18:4n-3 and 20:4n-3. This was in contrast to the other phospholipid classes in which the levels of AA and total n-6 PUFA decreased upon supplementation with n-3 PUFA. As the level of AA in total lipid was unchanged by supplementation with the n-3PUFA, it appeared that AA was, in effect, redistributed among the phospholipid classes.

Although the increased levels of 18:4n-3 and 20:4n-3 were greatest in PC, and increased levels of 20:5n-3 and 22:5n-3 were greatest in PE and PS, respectively, all the n-3 PUFA were distributed among PC, PE and PS with the most striking feature being their relative exclusion from PI. Slightly different results were obtained in an earlier study where the incorporation of 18:4n-3 into AS cells was determined after a longer incubation period of 7 days (Tocher and Dick 1990b). However, in that earlier study, although the greatest proportion of 18:4n-3 was found in PC and phosphatidic acid (PA), and the highest level of 20:4n-3, derived from the 18:4n-3, in PA, the levels of 18:4n-3 and 20:4n-3 were low in PI as observed in the present study (Tocher and Dick 1990b). The results from other studies are also consistent with the present data. Exogenously added 20:4n-3 was primarily incorporated into PC, PI and PE in human endothelial cells, but did not affect AA levels in short-term incubations of 24h (Croset et al. 1999). Both [U-^{14}C]18:4n-3 and [U-^{14}C]20:4n-3 were predominantly incorporated into PC in sea bream cells (Tocher and Ghioni 1999). Exogenously added 18:4n-3 was incorporated into NIH-3T3 cells at the expense of monounsaturated fatty acids, especially 18:1n-9, rather than n-6 PUFA (Cantrill et al. 1993). In the present study, incorporation of both 18:4n-3 and 20:4n-3 into total lipid of AS cells was also mainly

at the expense of monoenes, predominantly 18:1n-9.

Thus, the results from the present study are generally consistent with previous data and, in particular, suggest a potentially important role for PI in eicosanoid metabolism in fish cells. However, this is circumstantial, and there is no direct evidence from the present study, or any previous study, to support a unique role for PI in eicosanoid metabolism in fish, and it appears that no single phosphoglyceride class is the sole supplier of eicosanoid precursor in fish (Tocher 1995). Therefore, whether the specificity for AA as a precursor for eicosanoid production in fish is the result of an AA-specific phospholipase A_2 or from the specificity of the cyclooxygenase and lipoxygenase enzymes, and whether phospholipid class distribution plays any part, is unresolved.

The above results and discussion clearly suggest that 20:4n-3 could have significant modulatory effects on eicosanoid metabolism, and thus inflammatory responses, in fish and that consumption of diets including plant oils containing high levels of 18:4n-3, as in echium oil, could have beneficial effects. However, further studies of the effects of 20:4n-3 *in vivo* are required. Oils containing high levels of 20:4n-3 are not available at the moment but could be in the future in the form of single cell oil from *Mortierella alpina* mutants defective in Δ5 desaturase activity (Kawashima et al. 1997). In the present study, we required to produce high purity 20:4n-3 ourselves due to the lack of commercially available product, and although 20:4n-3 is now available the cost is prohibitive for *in vivo* trials. The methods we describe here to prepare 20:4n-3 could be scaled up to produce gram amounts of material particularly if a lower grade of purity was required as in dietary trials.

Marine fish oil was used as starting material for both the chromatographic and partial synthetic production of 20:4n-3 as it contains 1-2% of 20:4n-3. Fractionation of 3 g quantities of ethyl esters of total fatty acids from a conventional marine fish oil by preparative reverse phase liquid chromatography readily yielded a fraction substantially enriched in 20:4n-3, and also in 20:4n-6 and 22:5n-3, these two fatty acids also being minor constituents of fish oil. The large scale fraction of ethyl esters enriched in 20:4n-3, 20:4n-6 and 22:5n-3 greatly facilitated the subsequent isolation of the pure ethyl ester of 20:4n-3 by semi-preparative HPLC, albeit in mg amounts only, due to the close retention times of 20:4n-3 and 20:4n-6 causing peak overlap at higher column loadings. The semi-preparative HPLC also yielded pure 22:5n-3.

For the partial synthesis of 20:4n-3, a readily available commercial fraction of marine fish oil containing essentially only 18:4n-3 and 20:5n-3, in equal quantities, was used as starting material. This fatty acid mixture was chosen rather than pure 18:4n-3 (which is readily available) on cost grounds as the mixture is far cheaper and available in bulk which would facilitate the production of larger amounts of 20:4n-3. The mixture did not affect final purity, as the mixed

elongated products could be easily separated by preparative HPLC to yield pure products. Thus, 18:4n-3 and 20:5n-3 were simultaneously converted to 20:4n-3 and 22:5n-3, with the option of further conversion of these to 22:4n-3 and 24:5n-3, respectively, thus generating a range of potentially useful fatty acids and intermediates, radiolabelled if need be, for metabolic studies.

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Legends to Figures

Fig.1. Eicosanoid analyses by negative ion (EI^+) GC-MS. (A) Total ion chromatogram (TIC) of an eicosanoid extract from rainbow trout macrophages incubated for 4 days with 25 μM 20:4n-3. (B) TIC of an authentic PGE_2 standard (note more than one isomer present). (C) Mass scan for m/z 297, the base peak for $\Delta^{17,18}$ dehydro-12-HETE, the 12-lipoxygenase metabolite of 20:4n-3. (D) Mass scan for m/z 508, the base peak for PGE_2 showing a peak running slightly behind the main authentic PGE_2 isomer, probably representing the PG product of 20:4n-3, $\Delta^{17,18}$–dehydro-PGE_1, a PGE_2 isomer.

Fig.2. Negative ion (EI^+) GC-MS spectrum of peak Y (Fig.1A), showing the ions characteristic for $\Delta^{17,18}$ dehydro-12-HETE.

Fig.3. Gas-chromatography of PGF isomers. The figure shows the portion of the GC trace where PGF isomers elute. The GC conditions were optimised as described in the methods to obtain a clear chromatographic separation of the different isomers, in order to use CI^- MS to monitor their molecular ions at specific retention times. (A) Separation of a mixture of commercially available $PGF_{1\alpha}$, $PGF_{2\alpha}$ and $PGF_{3\alpha}$ standards. The three peaks at 21.09 min, 21.43 min and 21.96 min were identified as $PGF_{2\alpha}$, (m/z 569.4), $PGF_{3\alpha}$, (m/z 567.4) and $PGF_{1\alpha}$, (m/z 571.4), respectively. (B) Chromatography, under the same conditions as above, of an eicosanoid extract from rainbow trout head kidney macrophages incubated with 25μM 20:4n-3 as described in the Methods section. This gave a major peak at 22.3 min, just after where $PGF_{1\alpha}$ would elute.

Fig.4. CI^- MS spectra of standard $PGF_{2\alpha}$ (A) and the major peak at 22.3 in the eicosanoid extract from rainbow trout head kidney macrophages incubated with 20:4n-3 (B). The m/z signal of 569.4 for the molecular ion for the peak at 22.3 min was identical to that for the standard $PGF_{2\alpha}$ indicating that it was the $PGF_{2\alpha}$ isomer from 20:4n-3, $\Delta^{17,18}$ –dehydro-$PGF_{1\alpha}$.

Fig.5. Effect of 20:4n-3 on the production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by rainbow trout kidney head macrophages. Using the chromatographic conditions as in Fig.2. for separation and quantification, levels of $PGF_{2\alpha}$ were compared in activated macrophages with no supplemented fatty acids (control), with macrophages supplemented with either 20:4n-3 or

20:4n-6 alone and in experiments in which both 20:4n-3 and 20:4n-6 were administered together.

Table 1. Effect of supplementation with 18:4n-3 and 20:4n-3 on the lipid class composition of Atlantic salmon cells.

Lipid class	Control	18:4n-3	20:4n-3
Phosphatidylcholine	26.8 ± 0.8 [b]	27.3 ± 0.9 [b]	30.4 ± 1.2 [a]
Phosphatidylethanolamine	19.8 ± 0.8 [a]	17.6 ± 0.5 [b]	19.9 ± 0.9 [a]
Phosphatidylserine	6.3 ± 0.2 [a]	5.3 ± 0.1 [b]	6.4 ± 0.2 [a]
Phosphatidylinositol	7.8 ± 0.2 [a]	7.0 ± 0.3 [b]	7.7 ± 0.3 [a]
PG/PA/CL	3.0 ± 0.1 [b]	3.1 ± 0.1 [b]	3.8 ± 0.2 [a]
Sphingomyelin	2.8 ± 0.5	2.1 ± 0.5	2.8 ± 0.5
Total polar lipids	66.6 ± 0.2 [b]	62.4 ± 1.3 [c]	70.9 ± 2.0 [a]
Total neutral lipids	33.4 ± 0.2 [b]	37.6 ± 1.3 [a]	29.1 ± 2.0 [c]
Cholesterol	26.3 ± 0.8 [a]	19.1 ± 0.4 [b]	19.5 ± 1.1 [b]
Triacylglycerol	6.5 ± 0.5 [c]	15.1 ± 1.3 [a]	9.2 ± 1.0 [b]
Steryl esters	0.6 ± 0.4 [b]	3.4 ± 1.3 [a]	0.3 ± 0.1 [b]

Results are means ± SD (n=3). Mean values with different superscript letters within a row are significantly different ($P < 0.05$) as determined by one-way ANOVA followed where appropriate by Tukey's multiple comparison test. CL, cardiolipin; PA, phosphatidic acid; PG, phosphatidylglycerol.

Table 2. Effect of supplementation with 18:4n-3 and 20:4n-3 on the fatty acid composition of total lipid from Atlantic salmon cells.

	Control	18:4n-3	20:4n-3
14:0	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
16:0	9.4 ± 0.4 [a]	8.0 ± 0.2 [b]	9.4 ± 0.7 [a]
18:0	5.8 ± 0.2 [c]	10.2 ± 0.4 [a]	7.6 ± 0.8 [b]
Total saturates[1]	16.1 ± 0.6 [b]	19.2 ± 0.6 [a]	17.8 ± 1.5 [ab]
16:1n-9	3.3 ± 0.2 [a]	2.1 ± 0.2 [b]	2.2 ± 0.2 [b]
16:1n-7	2.0 ± 0.1 [a]	1.1 ± 0.0 [b]	1.2 ± 0.1 [b]
18:1n-9	39.9 ± 1.0 [a]	22.6 ± 1.0 [b]	20.4 ± 1.8 [b]
18:1n-7	4.5 ± 0.1 [a]	3.2 ± 0.1 [b]	2.9 ± 0.3 [b]
20:1n-9	0.6 ± 0.1 [ab]	0.9 ± 0.2 [a]	0.4 ± 0.1 [b]
Total monoenes[2]	51.6 ± 1.2 [a]	30.9 ± 1.1 [b]	27.9 ± 2.6 [b]
18:2n-9	1.3 ± 0.2 [a]	0.6 ± 0.0 [b]	0.7 ± 0.2 [b]
20:2n-9	0.5 ± 0.1 [a]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]
20:3n-9	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Total n-9 PUFA	2.5 ± 0.2 [a]	1.1 ± 0.4 [b]	1.2 ± 0.4 [b]
18:2n-6	1.2 ± 0.0	1.1 ± 0.1	1.0 ± 0.1
20:3n-6	1.2 ± 0.1 [a]	1.1 ± 0.2 [a]	0.3 ± 0.4 [b]
20:4n-6	2.9 ± 0.1	2.9 ± 0.1	2.6 ± 0.3
22:4n-6	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.2
Total n-6 PUFA[3]	6.2 ± 0.5 [b]	5.5 ± 0.1 [b]	4.1 ± 0.4 [a]
18:4n-3	0.0 ± 0.0 [c]	11.5 ± 0.8 [a]	2.2 ± 0.3 [b]
20:4n-3	0.0 ± 0.0 [c]	4.8 ± 0.5 [b]	12.0 ± 0.7 [a]
20:5n-3	0.8 ± 0.1 [c]	4.6 ± 0.4 [b]	7.1 ± 1.2 [a]
22:4n-3	0.1 ± 0.2 [c]	0.5 ± 0.1 [b]	1.4 ± 0.1 [a]
22:5n-3	1.6 ± 0.0 [b]	2.0 ± 0.2 [b]	2.9 ± 0.4 [a]
22:6n-3	3.8 ± 0.4	3.2 ± 0.2	3.1 ± 0.5
Total n-3 PUFA[4]	6.3 ± 0.5 [b]	27.1 ± 0.8 [a]	28.6 ± 2.6 [a]
Total PUFA	16.3 ± 0.5 [b]	36.3 ± 1.1 [a]	36.1 ± 3.2 [a]
Total DMA	5.1 ± 0.5	4.3 ± 0.5	4.5 ± 0.7

Results are means ± SD (n=3). Mean values with different superscript letters within a row are significantly different ($P < 0.05$) as determined by one-way ANOVA followed where appropriate by Tukey's multiple comparison test. DMA, dimethylacetals; PUFA, polyunsaturated fatty acids. [1], Totals include 15:0, 17:0 and 20:0 and 22:0; [2], Totals include 17:1, and 24:1; [3], Totals include 18:3n-6, 20:2n-6, 22:2n-6 and 22:5n-6; [4], Totals include 18:3n-3 and 20:3n-3.

Table 3. Effect of supplementation with 18:4n-3 and 20:4n-3 on the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine in Atlantic salmon (AS) cells.

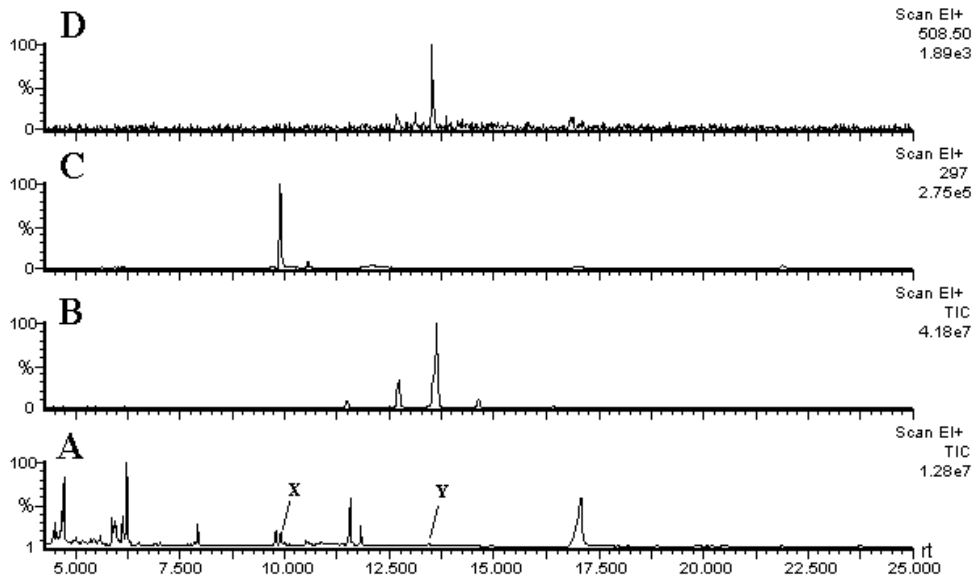
	Phosphatidylcholine			Phosphatidylethanolamine		
	Control	18:4n-3	20:4n-3	Control	18:4n-3	20:4n-3
14:0	1.6 ± 0.1 [a]	1.1 ± 0.1 [b]	1.6 ± 0.1 [a]	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.0
16:0	16.7 ± 0.3	14.6 ± 0.1	15.9 ± 3.5	3.2 ± 0.8	2.5 ± 0.1	3.4 ± 0.2
18:0	4.9 ± 0.2 [b]	8.5 ± 0.2 [a]	5.2 ± 0.3 [b]	3.6 ± 0.8 [c]	9.2 ± 0.1 [a]	6.9 ± 0.5 [b]
Total saturates[1]	23.5 ± 0.5	24.6 ± 0.3	23.3 ± 3.5	7.2 ± 1.7 [b]	12.1 ± 0.2 [a]	10.7 ± 0.7 [a]
16:1n-9	4.7 ± 0.2 [a]	2.7 ± 0.1 [b]	4.1 ± 0.8 [a]	3.6 ± 0.7 [a]	2.3 ± 0.5 [b]	1.9 ± 0.1 [b]
16:1n-7	3.0 ± 0.1	1.7 ± 0.1	2.7 ± 0.9	1.4 ± 0.4	1.1 ± 0.9	0.6 ± 0.0
18:1n-9	53.4 ± 0.4 [a]	33.4 ± 0.8 [b]	30.3 ± 0.8 [c]	37.1 ± 5.7 [a]	18.4 ± 1.9 [b]	17.7 ± 0.8 [b]
18:1n-7	4.8 ± 0.1 [a]	4.0 ± 0.3 [b]	3.7 ± 0.1 [b]	5.4 ± 1.8	4.0 ± 0.4	3.7 ± 0.0
20:1n-9	0.4 ± 0.1 [a]	0.0 ± 0.1 [b]	0.0 ± 0.0 [b]	0.7 ± 0.1 [a]	0.0 ± 0.0 [c]	0.2 ± 0.0 [b]
Total monoenes[2]	67.5 ± 0.2 [a]	43.0 ± 1.0 [b]	41.9 ± 2.2 [b]	49.8 ± 8.7 [a]	26.9 ± 2.9 [b]	25.1 ± 0.4 [b]
18:2n-6	1.2 ± 0.1 [a]	0.9 ± 0.0 [b]	0.8 ± 0.0 [b]	1.0 ± 0.2	1.2 ± 0.1	0.9 ± 0.1
18:3n-6	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.2	0.3 ± 0.0	0.2 ± 0.0
20:2n-6	0.2 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.3	0.3 ± 0.2	0.3 ± 0.2
20:3n-6	0.8 ± 0.0 [a]	0.5 ± 0.0 [b]	0.4 ± 0.0 [b]	0.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.0
20:4n-6	0.9 ± 0.1 [a]	0.8 ± 0.0 [ab]	0.7 ± 0.0 [b]	4.9 ± 0.4 [a]	3.4 ± 0.2 [b]	2.6 ± 0.2 [c]
22:2n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.1 [a]	0.1 ± 0.1 [b]	0.1 ± 0.1 [b]
22:4n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
22:5n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.1
Total n-6 PUFA	3.2 ± 0.1 [a]	2.3 ± 0.1 [b]	2.1 ± 0.1 [b]	8.0 ± 0.7 [a]	6.3 ± 0.3 [b]	5.1 ± 0.3 [c]
18:3n-3	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
18:4n-3	0.1 ± 0.0 [c]	14.7 ± 1.1 [a]	3.1 ± 0.3 [b]	0.2 ± 0.2 [c]	5.6 ± 0.7 [a]	0.7 ± 0.0 [b]
20:4n-3	0.0 ± 0.0 [c]	5.0 ± 0.5 [b]	13.0 ± 0.8 [a]	0.0 ± 0.0 [c]	6.0 ± 0.6 [b]	12.9 ± 0.5 [a]
20:5n-3	0.7 ± 0.1 [c]	4.8 ± 0.3 [b]	9.7 ± 0.9 [a]	1.7 ± 0.2 [b]	9.4 ± 0.2 [a]	10.0 ± 0.7 [a]
22:4n-3	0.0 ± 0.0 [c]	0.3 ± 0.0 [b]	0.8 ± 0.1 [a]	0.0 ± 0.0 [c]	0.4 ± 0.1 [b]	1.6 ± 0.2 [a]
22:5n-3	0.5 ± 0.0 [c]	0.7 ± 0.0 [b]	1.3 ± 0.0 [a]	2.5 ± 0.1 [c]	3.6 ± 0.2 [b]	4.9 ± 0.2 [a]
22:6n-3	0.7 ± 0.1	0.6 ± 0.3	0.5 ± 0.1	4.5 ± 0.2 [b]	5.7 ± 0.2 [a]	4.5 ± 0.0 [b]
Total n-3 PUFA[3]	2.1 ± 0.2 [c]	26.1 ± 0.8 [b]	28.6 ± 0.7 [a]	9.0 ± 0.4 [c]	30.6 ± 1.1 [b]	34.6 ± 0.5 [a]
Total n-9 PUFA	1.6 ± 0.2 [a]	0.7 ± 0.1 [b]	0.7 ± 0.1 [b]	3.2 ± 0.5 [a]	1.1 ± 0.3 [b]	1.2 ± 0.5 [b]
Total PUFA	6.9 ± 0.6 [c]	29.1 ± 1.1 [b]	31.4 ± 0.6 [a]	20.2 ± 1.9 [c]	38.0 ± 1.5 [b]	40.9 ± 2.4 [a]
Total DMA	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	17.5 ± 10.9	15.5 ± 1.2	16.6 ± 2.0

Results are means ± SD (n=3). Mean values with different superscript letters within a row for each lipid class are significantly different ($P < 0.05$) as determined by one-way ANOVA followed where appropriate by Tukey's multiple comparison test. DMA, dimethylacetals; PUFA, polyunsaturated fatty acids. [1], Totals include 15:0, 17:0, 20:0 and 22:0; [2], Totals include 17:1, and 24:1; [3], Totals include 20:3n-3.

Table 4. Effect of supplementation with 18:4n-3 and 20:4n-3 on the fatty acid composition of phosphatidylserine and phosphatidylinositol in Atlantic salmon (AS) cells.

	Phosphatidylserine			Phosphatidylinositol		
	Control	18:4n-3	20:4n-3	Control	18:4n-3	20:4n-3
14:0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
16:0	7.3 ± 0.3 [a]	4.7 ± 1.1 [b]	5.8 ± 0.7 [ab]	4.0 ± 0.4 [a]	2.5 ± 0.4 [b]	3.4 ± 0.2 [a]
18:0	22.0 ± 1.5 [b]	34.5 ± 1.3 [a]	32.4 ± 1.0 [a]	15.0 ± 1.0 [c]	31.2 ± 1.2 [a]	27.7 ± 0.9 [b]
Total saturates[1]	30.2 ± 1.0 [b]	40.0 ± 0.2 [a]	39.0 ± 0.4 [a]	19.3 ± 1.3 [c]	34.1 ± 0.8 [a]	31.4 ± 0.5 [b]
16:1n-9	1.4 ± 0.0 [a]	0.4 ± 0.1 [b]	0.5 ± 0.1 [b]	1.8 ± 0.1 [a]	0.6 ± 0.1 [b]	0.8 ± 0.1 [b]
16:1n-7	0.4 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.7 ± 0.1 [a]	0.2 ± 0.0 [b]	0.3 ± 0.0 [b]
18:1n-9	32.5 ± 0.9 [a]	15.8 ± 2.2 [b]	13.0 ± 0.3 [b]	34.5 ± 0.8 [a]	14.0 ± 1.5 [b]	14.8 ± 0.5 [b]
18:1n-7	3.9 ± 0.7 [a]	2.4 ± 0.1 [b]	2.7 ± 0.2 [b]	4.9 ± 0.3 [a]	3.3 ± 0.4 [b]	2.8 ± 0.2 [b]
20:1n-9	0.8 ± 0.2 [a]	0.3 ± 0.2 [b]	0.4 ± 0.1 [b]	0.8 ± 0.1 [a]	0.1 ± 0.1 [b]	0.2 ± 0.1 [b]
Total monoenes[2]	39.8 ± 1.7 [a]	19.4 ± 2.4 [b]	16.9 ± 0.5 [b]	43.5 ± 1.0 [a]	18.5 ± 1.5 [b]	19.4 ± 0.6 [b]
18:2n-6	1.2 ± 0.2 [a]	0.8 ± 0.1 [ab]	0.5 ± 0.3 [b]	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
20:2n-6	1.2 ± 1.0 [a]	0.1 ± 0.2 [b]	0.1 ± 0.2 [b]	0.4 ± 0.7	0.2 ± 0.0	0.1 ± 0.1
20:3n-6	2.2 ± 0.0	2.3 ± 0.1	2.0 ± 0.1	3.2 ± 0.0 [b]	4.1 ± 0.2 [a]	3.2 ± 0.2 [b]
20:4n-6	1.3 ± 0.2 [a]	1.1 ± 0.1 [ab]	0.8 ± 0.0 [b]	13.6 ± 0.2 [b]	19.7 ± 1.4 [a]	18.8 ± 0.7 [a]
22:2n-6	1.6 ± 1.1 [a]	0.5 ± 0.3 [b]	0.5 ± 0.4 [b]	0.8 ± 0.3 [a]	0.3 ± 0.2 [b]	0.3 ± 0.3 [b]
22:4n-6	0.5 ± 0.2	0.6 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
22:5n-6	0.2 ± 0.1	0.0 ± 0.0	0.3 ± 0.2	0.6 ± 0.3	0.3 ± 0.1	0.2 ± 0.1
Total n-6 PUFA[3]	8.2 ± 0.6 [a]	5.4 ± 0.3 [b]	4.6 ± 0.2 [b]	19.8 ± 0.8 [b]	25.7 ± 1.4 [a]	23.5 ± 0.8 [a]
18:3n-3	0.1 ± 0.1	0.1 ± 0.2	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.1
18:4n-3	0.2 ± 0.1 [b]	2.2 ± 0.3 [a]	0.2 ± 0.2 [b]	0.2 ± 0.1 [b]	1.2 ± 0.2 [a]	0.3 ± 0.0 [b]
20:4n-3	0.0 ± 0.1 [c]	4.9 ± 0.7 [b]	11.7 ± 0.2 [a]	0.1 ± 0.1 [c]	3.1 ± 0.3 [b]	5.7 ± 0.5 [a]
20:5n-3	0.5 ± 0.1 [b]	2.6 ± 0.3 [a]	2.5 ± 0.2 [a]	0.4 ± 0.1 [c]	2.4 ± 0.1 [b]	4.2 ± 0.3 [a]
22:4n-3	0.2 ± 0.3 [b]	0.1 ± 0.1 [b]	1.0 ± 0.1 [a]	0.1 ± 0.2 [b]	0.3 ± 0.1 [b]	0.7 ± 0.1 [a]
22:5n-3	5.2 ± 0.4 [b]	8.7 ± 0.9 [a]	9.8 ± 0.6 [a]	3.8 ± 0.4 [b]	4.4 ± 0.3 [b]	5.6 ± 0.5 [a]
22:6n-3	6.9 ± 0.7 [b]	10.1 ± 1.1 [a]	8.5 ± 0.2 [ab]	4.3 ± 0.7	4.7 ± 0.3	4.0 ± 0.1
Total n-3 PUFA[4]	13.2 ± 1.0 [c]	28.6 ± 3.1 [b]	33.7 ± 1.2 [a]	9.5 ± 1.1 [c]	16.8 ± 0.7 [b]	20.6 ± 1.0 [a]
Total n-9 PUFA	3.3 ± 0.7 [a]	1.1 ± 0.2 [b]	1.1 ± 0.3 [b]	3.6 ± 0.3 [a]	1.5 ± 0.4 [b]	1.3 ± 0.4 [b]
Total PUFA	24.7 ± 0.6 [b]	35.1 ± 3.1 [a]	39.4 ± 1.2 [a]	32.9 ± 1.6 [b]	44.0 ± 2.7 [a]	45.4 ± 1.9 [a]

Results are means ± SD (n=3). Mean values with different superscript letters within a row for each lipid class are significantly different ($P < 0.05$) as determined by one-way ANOVA followed where appropriate by Tukey's multiple comparison test. PUFA, polyunsaturated fatty acids. [1], Totals include 15:0, 17:0 and 20:0 and 22:0; [2], Totals include 17:1, and 24:1; [3], Totals include 18:3n-6; [4], Totals include 20:3n-3.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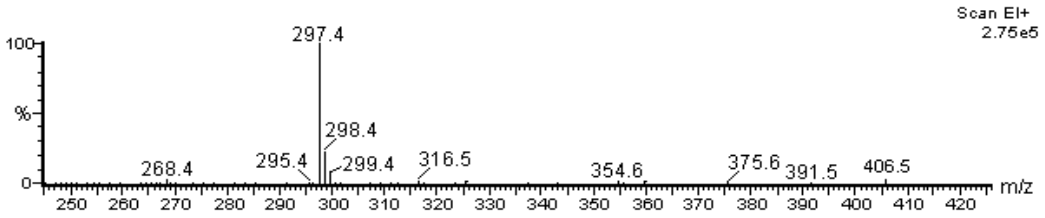
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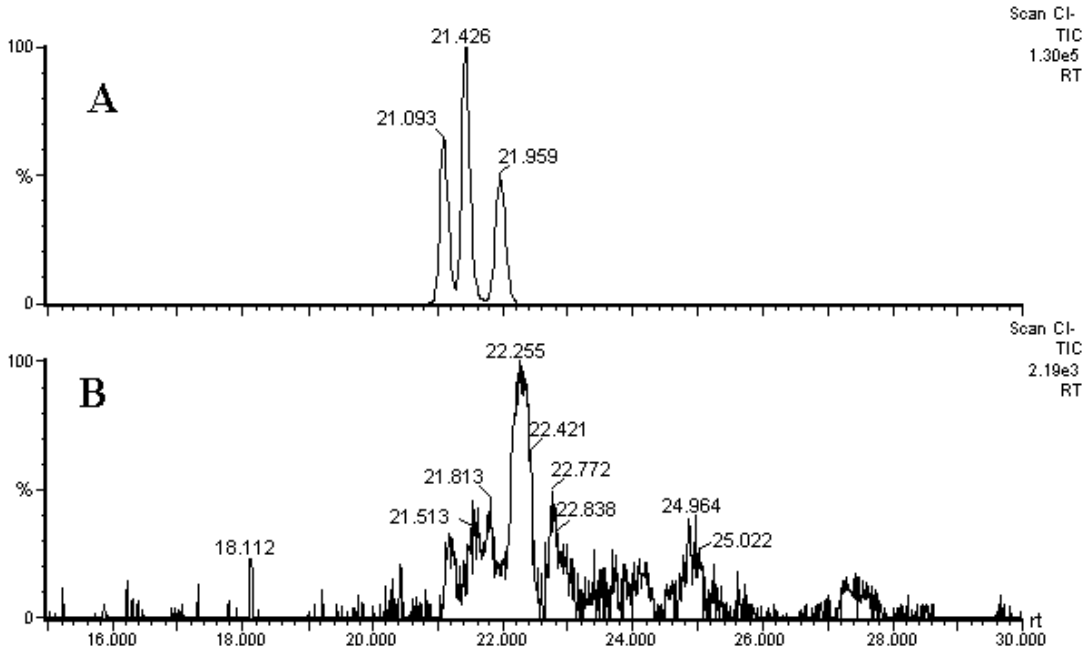
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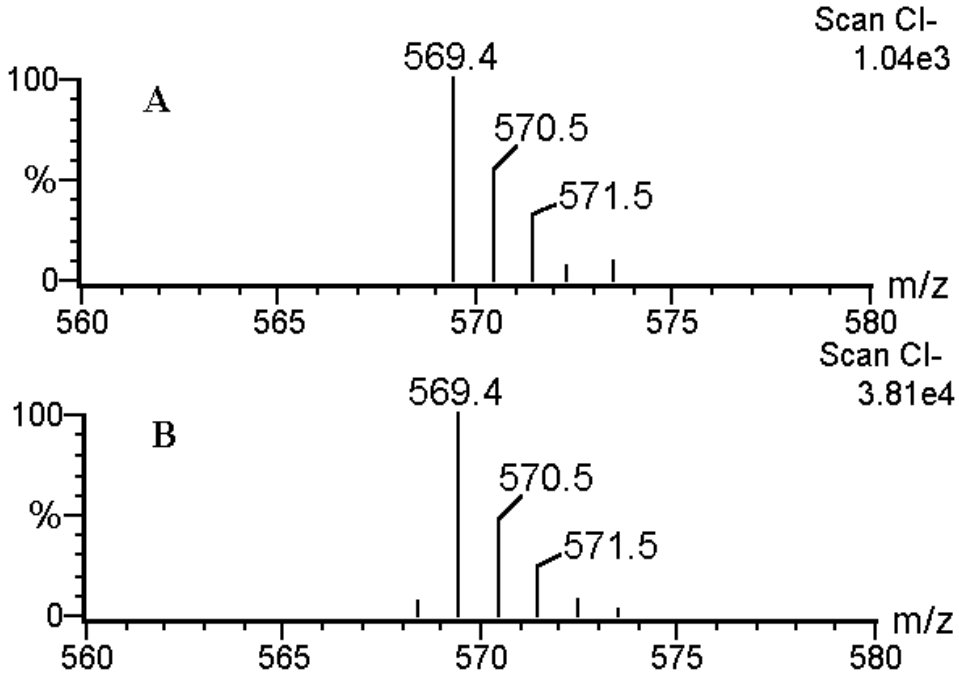
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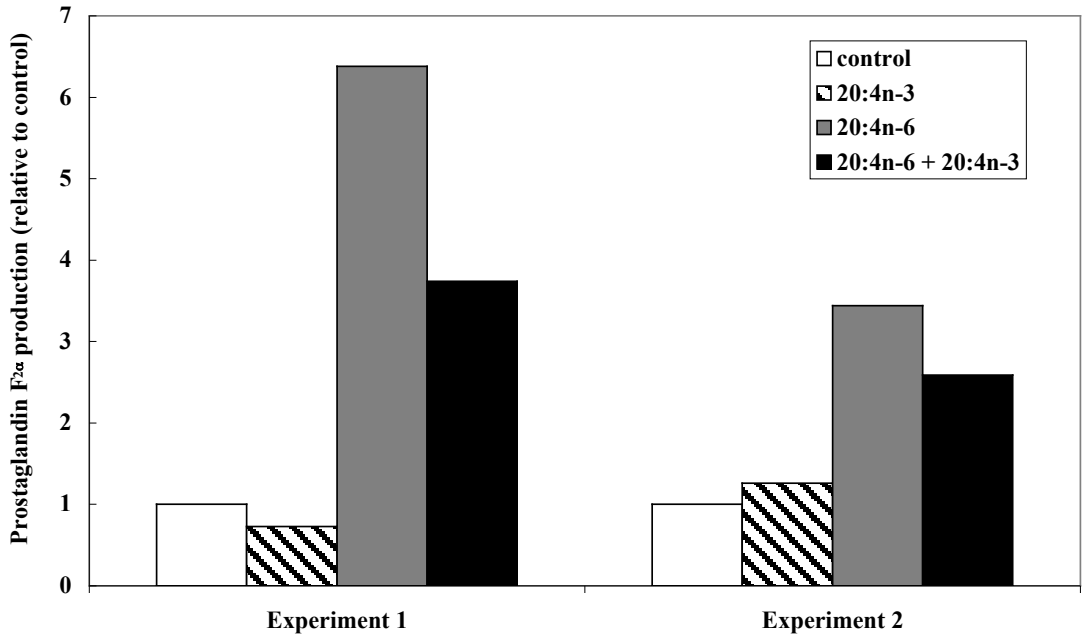
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1
2
3
4
5
6
7
control
20:4n-3
20:4n-6
20:4n-6 + 20:4n-3
Experiment 1
Experiment 2