Eicosapentaenoic acid inhibits cell growth and triacylglycerol secretion in McA-RH7777 rat hepatoma cultures

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The plasma triacylglycerol-decreasing effect of fish-oil fatty acids was studied *in vitro* by using the rapidly growing cultured rat hepatoma cell line McA-RH7777. Cells were exposed to albumin-complexed eicosapentaenoic acid ($C_{20:5n-3}$; EPA), to oleic acid ($C_{18:1n-9}$; OA), or to albumin alone. Cell growth was similar in albumin- and OA-supplemented cultures, but EPA treatment inhibited growth. As estimated by [^{14}C]glycerol incorporation, OA stimulated both net triacylglycerol synthesis and secretion over control levels in a dose-dependent manner. EPA stimulated triacylglycerol synthesis in similar fashion to OA, but paradoxically decreased net triacylglycerol secretion and led to exaggerated intracellular accumulation of radiolabelled triacylglycerol. The EPA and OA effects were additive at low concentrations of total fatty acid, but at higher fatty acid concentrations OA appeared to negate some effects of EPA. Chemical analysis of albumin- and OA-treated cultures revealed OA-dominant profiles for both cellular and medium triacylglycerol-associated fatty acids. In contrast, EPA was the principal fatty acid in cellular triacylglycerol of EPA-supplemented cultures, whereas medium triacylglycerol from these cultures contained very little EPA. We conclude that McA-RH7777 hepatoma cells readily synthesize EPA-containing triacylglycerol molecules, but they have variable capacity for secreting them. We consider potential mechanisms to account for the effects of EPA in this system.

INTRODUCTION

The $n-3$ or $\omega-3$ series of polyunsaturated fatty acids (PUFAs) exert pleiotropic effects on biological systems. They have found widespread and often controversial application in medicine, ranging from alleviation of dermatological maladies [1] to the dietary and pharmacological management of diabetes mellitus [2], renal disorders [3], inflammatory bowel disease [4] and cardiovascular disease [5]. Human populations that regularly consume ocean fish exhibit low rates of cardiovascular disease, especially in combination with a diet low in saturated fat content [6]. Fish-oil fatty acids of the $n-3$ series may exert a protective influence beyond that associated with other types of polyunsaturates, as suggested by retrospective cohort analyses showing an inverse relationship between fish consumption and ischaemic heart disease [7]. Reported beneficial effects of fish-oil $n-3$ PUFAs include altered vascular tone, decreased coagulability of blood, and changes in plasma lipoprotein metabolism [5].

Authors have generally concluded that fish-oil $n-3$ PUFAs lower plasma triacylglycerol concentrations in many species, including humans [5,8–11]. The $n-3$ PUFAs effectively lower plasma triacylglycerol in normotriglyceridaemic [12] and in both transiently [13] and chronically [14,15] hypertriglyceridemic subjects. Turnover studies have shown that $n-3$ PUFAs decrease very-low-density lipoprotein (VLDL) production *in vivo* in animals [8,16] and humans [17,18]. Decreased VLDL production has also been documented *ex vivo* in perfused rat livers [19,20] and in primary cultures of rat hepatocytes [21,22].

The Morris hepatoma 7777 and its cultured derivative McA-RH7777 [23] have been used extensively for biochemical and morphological studies since 1968 (summarized in [24]). Both the solid tumour and the cultured cells actively synthesize and secrete plasma lipoproteins [25,26]. Previous work in our lab-

oratory has focused on the regulation and growth-relatedness of lipoprotein biogenesis in McA-RH7777 cells. We have observed that growing cells are considerably more responsive than confluent cells to inhibitors and stimulants of lipoprotein production, and that triacylglycerol synthesis and triacylglycerol secretion efficiency are differentially sensitive to diffusible factors and to changes in cell proximity [26]. Supplementation of growing McA-RH7777 cells with oleic acid (OA; $C_{18:1n-9}$) stimulates both triacylglycerol synthesis and VLDL-triacylglycerol secretion, with dose-dependent effects on the efficiency of VLDL-triacylglycerol secretion [24]. In contrast, linoleic acid ($C_{18:2n-6}$) is a potent inhibitor of both processes [24]. We therefore felt that McA-RH7777 cells might provide a sensitive model for investigating the influence of $n-3$ PUFAs on hepatocyte triacylglycerol synthesis and secretion in a rapidly growing system.

In this paper we report that the $n-3$ PUFA eicosapentaenoic acid (EPA; $C_{20:5n-3}$) selectively inhibits triacylglycerol secretion by McA-RH7777 cells in the context of sustained triacylglycerol synthesis, resulting in exaggerated intracellular triacylglycerol accumulation compared with cells given OA, and that this phenomenon is accompanied by inhibition of cell growth. This work was presented in preliminary form at the 60th Scientific Sessions of the American Heart Association [26a].

EXPERIMENTAL

Materials

Fetal-bovine serum (FBS) was purchased from Hazelton Laboratories (Lenexa, KS, U.S.A.) and heat-inactivated according to the supplier's recommendations before use. EPA (all-*cis*-5,8,11,14,17-eicosapentaenoic acid; ~90% pure), (OA octadec-9-enoic acid), BSA (Cohn fraction V) and BF_3 /methanol were purchased from Sigma. Cell-culture media and related supplies were purchased from Gibco. [$U-^{14}C$]Glycerol was ob-

Abbreviations used: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FBS, fetal-bovine serum; NEFA, non-esterified fatty acid; OA, oleic acid; PUFA, polyunsaturated fatty acid; TGFA, triacylglycerol-associated fatty acid; VLDL, very-low-density lipoprotein.

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tained from Amersham. Mixtures of fatty acid methyl esters, including those of EPA and docosahexaenoic acid (DHA; $C_{22:6n-3}$), were kindly provided by Dr. William Elliott. Other chemicals and reagents were of the highest grade commercially available.

Preparation of fatty acid-BSA complexes

BSA was rendered free of fatty acid by the method of Chen [27]. Non-esterified fatty acids (NEFA) were complexed with treated BSA at a molar ratio of 4:1 by co-sonication in phosphate-buffered saline (10 mM-sodium phosphate/0.15 M-NaCl, pH 7.4) under a N_2 atmosphere to prevent peroxidation. As additional precautions we stored unopened ampoules of fatty acid at -30°C in a vacuum desiccator in the dark; only freshly opened ampoules were used for each experiment, and we discarded the unused contents of opened ampoules; and fatty acid-BSA complexes were kept under N_2 in darkness until the time of addition to the medium.

Cell growth and labelling

The rat hepatoma cell line McA-RH7777 was obtained from the American Type Culture Collection (CRL 1601) and maintained in basal medium (Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM-glutamine, 100 IU of penicillin/ml and 100 μg of streptomycin/ml) at 37°C in a humidified atmosphere with 5% CO_2 . It was previously established in our laboratory that McA-RH7777 cells maintain significantly greater rates of growth and protein synthesis, as well as increased protein secretion, in medium containing FBS than in serum-deficient medium; moreover, the specific effects of fatty acid supplementation are not affected by the presence of bovine lipoprotein components or other undefined elements present in FBS [24]. We therefore exposed the cells to supplemental fatty acids and performed all labelling studies in medium containing FBS. Because EPA is resistant to peroxidation in aqueous solution [28], since commercial antioxidants may perturb lipoprotein structure or function [29], and because we empirically established that our medium formulation prevents peroxidation of supplemental fatty acids (see the Results section), no exogenous antioxidants were added to medium other than the mixture provided by FBS (i.e. vitamin E, transferrin and ceruloplasmin, in addition to other minor species).

Cells were plated at a density of $(1-2) \times 10^4$ cells/ cm^2 . After 2 days of growth in basal medium, the medium was replaced with fresh basal medium supplemented with fatty acid-BSA complex, as either EPA or OA at the final concentrations of supplemental fatty acid indicated in the Figures and their legends. Control cultures received an equivalent dose of fatty-acid-free BSA subjected to mock conditions of sonication, storage and handling. Cell counts were determined during the growth period with a haemocytometer; there was an excellent correlation between cell number and total cell protein per dish ($r^2 = 0.947$).

For labelling experiments, cells were first exposed to fatty acid-BSA complex or to BSA alone for 24 h, and then labelled with [^{14}C]glycerol (1 μCi /dish) for an additional 24 h without otherwise changing the incubation conditions. Cells and media were harvested and analysed separately. Triacylglycerols were separated from total lipid extracts by t.l.c. [30], and [^{14}C]glycerol incorporation was determined by liquid-scintillation counting. Cell protein mass was measured by the method of Lowry *et al.* [31].

Assay for fatty acid peroxidation products

Samples of medium (10 ml each) and cell lysates (equivalent to approx. 10^7 cells) from growing cultures supplemented for 48 h with OA-BSA complex, EPA-BSA complex or BSA alone were

adjusted to pH 3.0 with HCl and extracted five times with n-hexane. These extracts were dried over Na_2SO_4 and concentrated to a final volume of 0.1 ml by partial evaporation under N_2 . U.v. spectra were obtained from 200 to 500 nm in a Beckman model UV5230 scanning spectrophotometer.

Chemical analysis

For preparative-scale experiments without radiolabelling, cells were plated at a density of $(1-2) \times 10^4$ cells/ cm^2 and grown in 75 cm^2 Corning Costar culture flasks for 2 days in 10 ml of basal medium. This medium was replaced with fresh basal medium supplemented with OA-BSA complex, EPA-BSA complex, mixtures of the two, or with BSA alone, and then grown for an additional 2 days. Total lipid was extracted by the Folch method, total triacylglycerols were purified by preparative t.l.c., and triacylglycerol-associated fatty acids (TGFA) were analysed by trans-esterification and g.l.c. as previously described [30]. Individual fatty acid methyl ester species were identified by retention time as compared with standards composed of a saturated series (C_{16} , C_{18} , C_{20} , C_{22} , C_{24}), an $n-9$ monounsaturated series (C_{16} , C_{18} , C_{20} , C_{22} , C_{24}), a mixture of long-chain saturated and unsaturated species ($C_{18:0}$, $C_{18:1n-7}$, $C_{18:2n-6}$, $C_{18:3n-3}$, $C_{20:4n-6}$), and a mixture of fatty acid methyl esters containing the methyl esters of EPA and DHA.

Data and statistical analysis

Data were tabulated and descriptive statistics (mean and S.D.) calculated according to standard formulae. Differences between groups were analysed by Student's *t* test for unpaired samples.

RESULTS

EPA inhibits growth of McA-RH7777 cells

The growth of McA-RH7777 cells in basal medium has been well characterized in our laboratory [26], as well as by other investigators [23,32]. It has also previously been documented that addition of OA to basal medium does not affect cell growth rates, uptake of labelled methionine, protein synthesis, or protein secretion efficiency by McA-RH7777 cells [24]. Nevertheless, because cultured cells tolerate some fatty acids better than others, we initially assessed how McA-RH7777 cells respond to EPA under standard supplementation conditions. Surprisingly, when exposed to EPA at a concentration typically used for fatty acid supplementation *in vitro* (330 μM , about 100 $\mu\text{g}/\text{ml}$), these cells grew poorly and were easily detached from the culture dish surface. We therefore systematically compared the growth rates of McA-RH7777 cultures treated with 330 μM -EPA-BSA complex, 330 μM -OA-BSA complex, or with BSA alone. Fig. 1(a) depicts the results of a large experiment of this type (performed on this scale in order to eliminate sources of inter-experimental variability), in which simultaneously seeded cultures were harvested in triplicate for each treatment at each time point. Cultures treated with OA-BSA complex or with BSA alone showed essentially identical rates of growth. In contrast, growth of McA-RH7777 cells was completely inhibited by the presence of EPA-BSA complex at this concentration.

Since OA had no demonstrable effect on cell growth, we then examined in a separate large experiment the effects of varying [EPA] in the presence of OA such that the overall concentration of supplemental fatty acid remained constant at 330 μM (Fig. 1b). The growth-inhibiting effect of EPA was related directly to the concentration of EPA contained in the EPA-OA mixtures. As the EPA:OA ratio was increased from 1:3 to 1:1 to 3:1, we observed a monotonic decrease in the rate of cell growth, beginning at the time of fatty acid supplementation.

Fig. 1. Growth rates of McA-RH7777 cells supplemented with fatty acids

Hepatoma cells were seeded at $(1-2) \times 10^4$ cells/cm² and grown for 1 day in basal medium. Fresh medium was then added and supplemented with BSA alone or with NEFA at the indicated concentrations, pre-complexed to BSA at a molar ratio of 4:1. Growth was then monitored for a further 2 days. Each panel represents a single experiment, with results plotted as means $\pm$ 1 s.d. from triplicate dishes harvested for each treatment at each time point. (a) Cultures were supplemented with BSA alone ($\square$) or complexed with 330 μ M-EPA ($\blacklozenge$) or with 330 μ M-OA ($\blacksquare$). The difference at 72 h between BSA- and OA-supplemented cells was not significant; differences at 72 h were significant for EPA- versus BSA- and for EPA- versus OA-supplemented cells ($P \leq 0.004$). (b) Cultures were supplemented with BSA alone ($\square$) or complexed with 1:3 ($\blacklozenge$), 1:1 ($\blacksquare$) or 3:1 ($\diamond$) molar-ratio mixtures of EPA and OA such that the total supplemental fatty acid concentration was 330 μ M. Differences between any two growth rates were significant at 72 h ($P \leq 0.05$).

Because polyunsaturated fatty acids may undergo peroxidation to generate potentially cytotoxic products, in early experiments we examined concentrated n-hexane extracts of both media and cell lysates from cultures grown with and without fatty acid supplementation for the presence of conjugated dienes by u.v. scanning spectrophotometry. Of the many accepted techniques for evaluating lipid peroxidation, we chose this as a convenient and reliable way to detect early yet persistent products of the peroxidation process [33-35], with an estimated sensitivity in the low nanomolar range in our 100-fold-concentrated extracts [36,37], and hence in the picomolar range with respect to the samples before extraction. Except for a minor peak at 205 nm representing EPA in the EPA-treated cultures, all the extracts gave essentially identical absorption spectra. Specifically, none of the extracts contained detectable material with an absorption maximum between 230 and 240 nm that would suggest the presence of conjugated dienes. Since we found no spectrophotometric evidence of fatty acid peroxidation in our own system, we conclude that the antioxidant mixture contributed to the medium by FBS is effective, and we consider it highly unlikely that the growth-inhibitory effects of EPA on McA-RH7777 cultures reflect free-radical-mediated cell injury.

Fig. 2. Effects of fatty acid supplementation on net triacylglycerol secretion and cellular triacylglycerol accumulation in McA-RH7777 cells

Hepatoma cells were seeded at $(1-2) \times 10^4$ cells/cm² and grown for 2 days in basal medium. Fresh medium was then added and supplemented with BSA alone or with the concentrations of NEFA indicated on the abscissa. Cells were grown for 24 h with supplementation before the introduction of 1 μ Ci of [¹⁴C]glycerol per dish, and labelling was carried out for a further 24 h. Cultures were harvested, and media and cell lysates were analysed individually. Data were expressed as net c.p.m. of [¹⁴C]triacylglycerol/ μ g of cell protein for each sample, and are presented as means $\pm$ 1 s.d. of triplicate dishes. Abbreviation: TG, triacylglycerol. (a) Net triacylglycerol secretion in McA-RH7777 cultures. The differences between any two data points were significant at $P \leq 0.009$, except for 33 μ M-OA versus BSA. (b) Net cellular triacylglycerol accumulation in McA-RH7777 cells. The differences between any two data points were significant at $P \leq 0.05$, except for 33 μ M-EPA versus BSA. (c) Ratio of net secreted to net retained triacylglycerol in McA-RH7777 cultures. The differences between any two data points were significant at $P \leq 0.04$, except for supplementation with either fatty acid at 33 μ M versus BSA, or 83 μ M-OA versus BSA.

EPA inhibits secretion, but not synthesis, of triacylglycerols

Net synthesis and secretion of triacylglycerols was assessed by measuring the incorporation of radiolabelled glycerol into triacylglycerol in McA-RH7777 cultures supplemented with OA or with EPA (Fig. 2). OA supplementation stimulated net triacylglycerol secretion in a dose-dependent fashion compared

Fig. 3. Fatty acyl composition of medium and cellular triacylglycerols of supplemented McA-RH7777 cultures

Cells were seeded at a density of $(1-2) \times 10^4$ cells/cm² and grown in 75 cm² flasks for 2 days in 10 ml of basal medium. This medium was replaced with fresh basal medium supplemented with 4:1-molar-ratio complexes of OA-BSA or EPA-BSA, mixtures of the two, or with BSA alone, and then cultures were grown for a further 2 days. Cultures were harvested, and media and cell lysates were analysed individually. Data are expressed as percentage composition by individual fatty acyl species, with residues representing less than 5% of the total not shown. (a) Composition of TGFA in cultures supplemented with either BSA alone or with 330 μ M-OA. (b) Composition of TGFA in cultures supplemented with either 165 μ M- or 330 μ M-EPA. (c) Composition of TGFA in cultures cosupplemented with 330 μ M total NEFA, consisting of EPA plus OA in molar ratios of 2:1 or 1:2.

with control cultures. In contrast, cell cultures treated with EPA displayed a dose-dependent inhibition of triacylglycerol secretion (Fig. 2a). This inhibition was apparent as low as 33 μ M-EPA. Triacylglycerol secretion was decreased to 41% of the control (BSA) value at 165 μ M-EPA and to 21% of the value obtained with an equivalent concentration of supplemental OA.

The appearance of [¹⁴C]glycerol in cellular triacylglycerol was also measured in these cultures. Cultures treated with either OA or EPA displayed a dose-dependent net accumulation of labelled cellular triacylglycerol compared with control cultures (Fig. 2b). However, at 165 μ M supplemental NEFA, EPA-treated cultures showed a mean of 70% more cellular [¹⁴C]triacylglycerol than did cultures exposed to OA, and cells treated with 165 μ M-EPA accumulated 10-fold more labelled triacylglycerol than control

cells. Consequently, both the ratio of medium to cellular [¹⁴C]triacylglycerol (Fig. 2c) and the efficiency of net triacylglycerol secretion (i.e. the ratio of medium to total labelled triacylglycerol) were significantly decreased in EPA-treated cultures compared with OA-treated or control cultures at supplemental NEFA concentrations above 33 μ M. Despite these striking contrasts, there were no significant differences between EPA- and OA-supplemented cultures with respect to total synthesis of triacylglycerol (i.e. the sum of labelled triacylglycerol in medium and cells; $P = 0.38$).

The inhibition of triacylglycerol secretion by EPA could be rescued by co-supplementation with OA, at least at OA-dominant mole fractions. In a series of mixing experiments, total supplemental NEFA was varied from 330 μ M to 1320 μ M, and at any given level of total supplemental NEFA the mole fraction of EPA was varied from 0.1 to 0.9. Net triacylglycerol synthesis and secretion were assessed as for Fig. 2. At EPA mole fractions of 0.5 or less, there was no significant inhibition of triacylglycerol secretion compared with cells given an equivalent supplemental concentration of OA only, regardless of the level of total supplemental NEFA. However, at EPA mole fractions greater than 0.5, the degree of inhibition of triacylglycerol secretion resembled that of cells supplemented with an equivalent dose of EPA alone (results not shown).

Fatty acyl composition of triacylglycerols

The observations presented so far suggest that EPA causes some post-synthetic derangement in hepatoma triacylglycerol metabolism, either at the level of assembling triacylglycerol molecules into lipoprotein particles or during the secretion of these particles from the cell. Although our labelling data point to an exaggerated intracellular accumulation of newly synthesized triacylglycerol in EPA-supplemented cultures, they do not distinguish among the many possible chemical forms of the accumulated triacylglycerol. We therefore determined the fatty acyl composition of total triacylglycerol isolated from non-labelled media and cell lysates (Fig. 3). Under control conditions 22% of medium TGFA was OA; medium-chain saturated fatty acids comprised about 30%, and the remainder were mostly medium- and long-chain saturated and unsaturated species. Control cellular triacylglycerol was composed of 52% OA, 17% palmitate, and smaller amounts of other species (Fig. 3a). Cultures supplemented with 330 μ M-oleic acid displayed a TGFA composition resembling that of control cultures, except for a greater proportion of OA in both medium and cells (Fig. 3a). The medium triacylglycerols contained 29% OA and lesser amounts of other medium-chain and long-chain fatty acids; cellular triacylglycerols contained 62% OA, with a variety of fatty acids making up the remainder.

The pattern of TGFA composition in cultures receiving either 165 μ M- or 330 μ M-EPA was markedly different from that of control or OA-supplemented cells (Fig. 3b). Medium TGFA (TGFA_m) from 165 μ M- or 330 μ M-EPA-supplemented cells contained predominantly palmitate (26 and 22% respectively) and OA (14 and 21% respectively), whereas EPA and its chain-elongated metabolite DHA made up only a small fraction of recovered fatty acyl residues in medium triacylglycerols (6 and 3% EPA and 4 and 2% DHA respectively). In contrast, cellular TGFA (TGFA_c) from both 165 μ M- and 330 μ M-EPA-treated cells contained mainly EPA (33 and 37% respectively) and DHA (24 and 13% respectively).

We also determined the TGFA composition for cultures treated with EPA-dominant (supplemental EPA:OA molar ratio = 2:1; designated medium C) and OA-dominant (supplemental EPA:OA = 1:2; designated medium D) mixtures at an overall supplemental fatty acid concentration of 330 μ M (Fig. 3c).

The TGFA from cultures treated with the EPA-dominant medium C resembled that from cultures treated with EPA alone, but had a greater percentage of OA in both medium and cellular triacylglycerol. The cellular triacylglycerol from medium C cultures, like those treated with EPA alone, contained large proportions of EPA (18%) and DHA (22%); but, unlike cells treated with EPA alone, those supplemented with the medium C mixture had predominantly OA (38%) in TGFA_c. Cells treated with the OA-dominant medium D behaved more like cells treated with OA alone. TGFA_c from medium D cultures contained a larger proportion of OA (56%) than the cultures treated with medium C, and EPA and DHA made relatively small contributions to TGFA_c in medium D cultures (5 and 10% respectively). Despite their differences in TGFA_c composition, however, neither cultures exposed to medium C nor those exposed to medium D were found to have substantial proportions of *n*-3 fatty acids in TGFA_m. TGFA_m from the cultures exposed to medium C contained predominantly OA (16%) and palmitate (11%), but little EPA (5%) or DHA (2%). Likewise, TGFA_m from cultures receiving medium D contained predominantly OA (27%) but little EPA (4%) or DHA (6%).

It would be easy to interpret these findings, were it not for the fact that in this series of experiments unlabelled medium triacylglycerols probably represent a mixture of triacylglycerol molecules derived from FBS and those secreted *de novo* by hepatoma cells. We have explored several potential approaches toward resolving this mixture. We initially considered simply subtracting the TGFA species present in non-conditioned basal medium from the corresponding levels in conditioned medium. However, this would give an inaccurate estimate of net TGFA secretion: FBS-derived triacylglycerols, especially molecules from lipoprotein particles damaged during the requisite freeze-thawing and heat inactivation of FBS, are a ready substrate for rapidly growing cells, and therefore cannot be considered to rest undisturbed in the medium throughout the incubation period.

We have also tested alternative medium formulations for maintaining McA-RH7777 cultures, including decreasing FBS levels, the use of serum-free medium optimized for hepatocyte culture [38,39], and the use of lipoprotein-deficient serum instead of whole FBS [24]. Regardless of whether supplemental fatty acids are present or not, we find that these formulations either generally inhibit cell growth and protein secretion as compared with the basal medium described here, or they significantly alter the spectrum of radiolabelled lipoprotein particles secreted by McA-RH7777 cells; moreover, each poses the risk of depleting the medium of antioxidants normally provided by whole FBS.

Having found no practical solution for resolving the TGFA mixture of conditioned media into exogenous and endogenous components without perturbing the culture system as a whole, we offer instead a logical argument based on the initial fatty acid content of medium, inspection of Fig. 3, and some simple calculations.

First, lipid analysis of FBS revealed a total fatty acid pool (free and esterified) potentially available in basal medium as substrate to the cells of about 225 μM , ~26% of which is OA, as shown by fatty acid methyl ester analysis (results not shown). Therefore, total OA was roughly 60 μM in cultures supplemented either with BSA or with EPA alone, 225 μM or 390 μM respectively in cultures supplemented with 165 μM - or 330 μM -OA alone, and 170 μM and 280 μM respectively in cultures supplemented with 2:1 and 1:2 molar mixtures of EPA:OA at a combined level of 330 μM . Because the purest EPA available to us at the time of these experiments was ~90% EPA, the [EPA] added to our cultures was actually ~150, ~300, ~200 and ~100 μM in cultures designated above as receiving 165 μM -EPA (= medium A), 330 μM -EPA (= medium B), 2:1 EPA:OA, 330 μM total

(= medium C), and 1:2 EPA:OA, 330 μM total (= medium D) respectively. Thus the true initial molar ratios of EPA:OA potentially available to cells in EPA-supplemented media A through D were approx. 2.5, 5.0, 1.2 and 0.4 respectively.

Next we consider intracellular TGFA (TGFA_c) composition. Since the cells proliferate rapidly, we assume that TGFA_c originate by incorporation of both hydrolysed and supplemented NEFA molecules from medium into triacylglycerol *de novo*, rather than pools of non-hydrolysed FBS-derived triacylglycerol molecules taken up directly from the medium and re-secreted. For comparison with the EPA:OA molar ratios presented in the media (preceding paragraph), from Fig. 3 molar ratios of *n*-3 PUFA:OA in TGFA_c of EPA-supplemented cultures receiving media A–D were estimated at 4.7, 2.7, 1.1 and 0.3 respectively. The discrepancy between the first and second of these numbers and the corresponding initial EPA:OA ratios presented in medium A (= 2.5) and medium B (= 5.0) argues that EPA and OA are not utilized strictly in proportion to their availability for some aspect of triacylglycerol production, either synthesis or net secretion, at least in EPA-rich environments. At one extreme, if certain TGFA species are not preferentially retained within cells (rather than being secreted), it would appear that cells exposed to medium A are about twice as likely to incorporate the *n*-3 PUFAs as OA into triacylglycerol, only about half as likely in medium B, but almost equally likely in media C and D (i.e. as normalized by the corresponding EPA:OA ratio available in the medium). On the other hand, if there is no bias regarding incorporation of OA versus EPA into triacylglycerol, there must be large differences in the relative retention rates for OA- versus EPA- or DHA-containing triacylglycerol molecules in cultures given medium A or B.

Third, we consider the origin of TGFA species in the medium (TGFA_m). It is possible that the 2-day-conditioned TGFA_m profile represents only residual TGFA from the FBS. However, because both NEFA and esterified fatty acids in the medium pool must be considered potential substrates for the cells, and, since we know independently that the cells actively synthesize and secrete triacylglycerol even under basal conditions, this possibility is unlikely. On the other hand, if FBS-TGFAs are preferentially utilized as substrate by McA-RH7777 cells, the TGFA_m of 48 h-conditioned medium could represent either only newly synthesized TGFA (produced roughly in proportion to available substrate), or a mixture of TGFA produced *de novo* and FBS-derived TGFA. We therefore consider the last two possibilities as separate hypotheses, and interpret the data of Fig. 3 in light of each. Previous work has established *in vivo*, in organ perfusion and in cultured cells that the contribution to net triacylglycerol synthesis of fatty acid synthesis *de novo* or of re-uptake and catabolism of newly secreted triacylglycerol-rich lipoproteins is negligible, especially in the context of NEFA supplementation [40,41]. We have therefore not considered these routes of fatty acid acquisition in the argument.

Hypothesis 1: over the period of incubation, McA-RH7777 cells take up all the exogenous TGFA originally present in FBS, so that at 48 h TGFA_m represent exclusively triacylglycerol molecules synthesized and secreted *de novo*. If this hypothesis is correct, then Fig. 3 indicates that EPA-supplemented McA-RH7777 cells secreted a greater proportion of total new triacylglycerol-associated OA (and palmitate and stearate) than of total new triacylglycerol-associated EPA or its metabolite-DHA in media A, B and C (this is intuitively appreciated by comparing relative heights of bars for a given TGFA species from medium versus cells under each condition of supplementation in Fig. 3). In medium D, however, greater proportions of total new triacylglycerol-associated EPA and DHA were secreted than of triacylglycerol-associated OA. Moreover, at 330 μM total sup-

plemental fatty acid, the efficiency of secreting newly synthesized triacylglycerol-associated EPA and DHA into the medium increased with the OA content of the supplement (Figs. 3b and 3c). If the stated hypothesis is correct, these results suggest that McA-RH7777 cells exposed to EPA-dominant mixtures of fatty acid preferentially secrete triacylglycerol molecules lacking *n*-3 PUFAs, but that substituting OA for EPA can over-ride and even perhaps reverse this preference in a dose-dependent fashion.

Hypothesis 2: TGFA_m at 48 h of incubation represent a mixture of FBS-derived TGFA_s and newly produced TGFA_s: moreover, either (a) newly produced TGFA_s are secreted in the same proportions as they are synthesized, or (b) some TGFA_s are secreted in preference to others. If (a) is true, then the relative proportions of new TGFA species appearing in the medium should be identical with those remaining in the cell, i.e. there is no bias in secretion. Since all the triacylglycerol-associated EPA and DHA must come from triacylglycerol production *de novo*, the amount of new triacylglycerol-associated OA relative to FBS-derived triacylglycerol-associated OA in the medium can be estimated from the ratio of triacylglycerol-associated OA to EPA + DHA in the cells, i.e.

$$(\text{new TG-OA})_m / (\text{TG-[n-3]})_m = (\text{TG-OA})_c / (\text{TG-[n-3]})_c$$

where m denotes medium, c denotes cells, and *n*-3 includes triacylglycerol-associated EPA and DHA. Using this proportionality, we estimate that new triacylglycerol-associated OA would account for 15% of all medium triacylglycerol-associated OA in medium A, 9% in medium B, 48% in medium C and 142% in medium D. Although the first two values seem reasonable and the third is possible, the fourth is neither. We have met a logical contradiction; therefore, part (a) of the hypothesis must be false for at least one condition of supplementation. In addition, although these estimated percentages of medium triacylglycerol-associated OA attributable to triacylglycerol production *de novo* do not allow us to say whether triacylglycerol-associated OA or EPA is preferentially secreted in the presence of EPA alone, the cells' ability to secrete triacylglycerol-associated EPA relative to OA at 330 μM total supplemental fatty acid appears to improve with OA co-supplementation, in a dose-dependent fashion. Thus part (b) of this hypothesis is true, i.e. under some conditions (most notably in medium D) McA-RH7777 cells may alter their efficiency of secreting EPA molecules relative to that of secreting OA molecules recently incorporated into triacylglycerol.

From our own and other available data, we cannot say with certainty which hypothesis regarding TGFA_m is correct, although we favour the second. Nevertheless, regardless of which hypothesis one selects, the TGFA_c and the TGFA_m data of Fig. 3 together point to reluctant secretion by McA-RH7777 cells in an EPA-rich environment of EPA-containing triacylglycerol molecules relative to those containing OA, but with OA co-supplementation somehow relieving the impairment.

DISCUSSION

In the cultured rat hepatoma McA-RH7777, EPA stimulates triacylglycerol synthesis above basal levels to the same extent as does OA. OA stimulates net triacylglycerol secretion without affecting cell growth; in contrast, EPA is a potent inhibitor of both processes. EPA and its metabolite DHA are evidently suitable substrates for triacylglycerol synthesis in McA-RH7777 cultures, yet it appears that these cells have difficulty in secreting triacylglycerol molecules containing EPA or DHA unless simultaneously provided with a sufficient quantity of another species of fatty acid, such as OA.

It is generally agreed that EPA inhibits triacylglycerol production by hepatocyte systems, but no consensus has emerged about the mechanisms responsible. Some authors have inferred that *n*-3 PUFAs both suppress and serve as poor substrates for triacylglycerol synthesis [5,9,42-44]. Others, in agreement with our findings, have reported that EPA and OA comparably stimulate triacylglycerol synthesis in cultured human hepatoblastoma (HepG2) cells [45], in cultured rat hepatocytes [46] and in perfused monkey livers [47]. Moreover, in some triacylglycerol-producing systems *n*-3 PUFAs have proven excellent substrates for triacylglycerol synthesis [40,45,48,49]. In considering potential sources for such discrepancies, we should first caution that conditions of fatty acid supplementation used in experimental systems vary so widely that it is often difficult to compare just the technical aspects of a given study with others. Furthermore, our data and those of other investigators suggest that both quality and magnitude of EPA effects on triacylglycerol metabolism may be very sensitive to the level and composition of other fatty acids supplied to the cells, as well as to other nutritional and hormonal influences [50-52].

Previously we reported that both synthesis and secretion efficiency of triacylglycerol decline during McA-RH7777 growth in basal medium, through the action or depletion of one or more unidentified diffusible factors in the medium rather than through cell-cell contacts, but that triacylglycerol secretion efficiency is more responsive to these factors than is triacylglycerol synthesis [26]. In that study we also found that orotic acid, a potent hypotriglyceridaemic agent *in vivo* for rats, selectively inhibits triacylglycerol synthesis in growing McA-RH7777 cells without altering their triacylglycerol secretion efficiency. The results presented here argue that EPA affects McA-RH7777 cells in a manner converse to orotic acid, but similar to the putative growth-related factor(s). Taken together, our findings argue compellingly that triacylglycerol synthesis and triacylglycerol secretion are independently regulated processes. In support of this viewpoint, Otto *et al.* recently [50] concluded that the inhibitory effects of a fish-oil-containing diet on hepatic triacylglycerol secretion in rats are independent of its effects on triacylglycerol synthesis, and Martin *et al.* [40] determined that DHA need not inhibit triacylglycerol synthesis in cultured rat hepatocytes in order to decrease their rates of triacylglycerol secretion.

To the best of our knowledge, we are the first to report that EPA inhibits growth of hepatoma cells. There is, however, ample precedent for this finding in other cell types: Marine *n*-3 PUFAs have been found to depress growth and growth-related phenomena in other neoplasms [53-57], in vascular cells [58-60], and even in cultured normal human skin fibroblasts [61]. Although the mechanisms of growth inhibition are not known, changes in prostanoid metabolism in themselves are not thought to be responsible [58,59], nor is it likely that free-radical-mediated cell injury accounts for the anti-proliferative properties of EPA. In our system, careful handling of fatty acids during preparation of fatty acid:BSA complexes and the presence of FBS-supplied antioxidants in basal medium were sufficient to prevent detectable peroxidation of EPA. Moreover, no evidence of decreased cell viability (as a likely consequence of free-radical injury) has been found in cultured rat hepatocytes exposed to DHA [40] or in cultured CaCo-2 human intestinal cells exposed to EPA [62]; and Tisdale & Beck found that neither DHA nor linoleic acid (both at least as susceptible to peroxidation as EPA [28]) showed the growth-inhibiting activity of EPA [54].

We are also the first to suggest an association between the anti-proliferative and hypotriglyceridaemic effects of EPA. Such a connection should not be surprising, in light of reported parallel inhibitions of growth and other differentiated functions in cells

exposed to EPA [54,60]. However, it is understandable that this particular association was not heretofore recognized in other triacylglycerol producing model systems. Hepatocyte turnover *in vivo* is normally slow, and hepatocytes do not grow during the usual periods of liver perfusion *ex vivo*. Primary cultures of adult-rat hepatocytes grow sluggishly, with a doubling time under optimal culture conditions of about 5 days [63,64]; this is significantly longer than the usual periods chosen for exposure to EPA or DHA *in vitro* [40,42,46,65]. Other investigators using triacylglycerol-producing neoplastic cell types have examined the effects of *n*-3 PUFAs only in confluent or near-confluent cultures [45,62,66,67]. In contrast with these other systems, exponentially growing McA-RH7777 cultures exhibit a cell doubling time of only about 1 day [26]; and unlike HepG2 cells, which do not normally synthesize or secrete significant amounts of VLDL triacylglycerol in the absence of exogenous fatty acid [68,69], McA-RH7777 cells vigorously produce triacylglycerol-rich lipoproteins even under basal conditions. Since decreases in cell growth or in triacylglycerol production are most easily measured in systems displaying high basal rates of each, McA-RH7777 cultures provide an attractive caricature of the interplay between these phenomena. Because growth requires ongoing membrane synthesis, and because rapidly growing hepatoma cells more actively synthesize and secrete triacylglycerol-rich lipoproteins than do sub-confluent or confluent cultures, any functional consequences of structurally altered membrane lipids and/or nascent lipoprotein particles might be more apparent in this system than in other model hepatocytes.

With regard to possible mechanisms, in McA-RH7777 cultures it may be that growth and triacylglycerol secretion in the presence of EPA are causally related, if only in a trivial way: if VLDL secretion is generally driven by some mechanism coupled to cell growth, as our previous work implies [26], any factor that inhibits growth might be expected to inhibit triacylglycerol secretion in parallel. For example, were EPA to inhibit the synthesis or release of some autocrine hepatocytic mitogen (borrowing from suggestions by Sarris *et al.* [58]), McA-RH7777 cells might interrupt triacylglycerol secretion merely because they have stopped growing. Alternatively, growth and triacylglycerol secretion could be concurrent yet coincidental victims of EPA- or DHA-induced alterations in membrane phospholipid structure and function [62,65,70] or in pathways of signal transduction via second messengers [71]. The selective inhibition of triacylglycerol-rich lipoprotein secretion by *n*-3 PUFAs, without affecting secretion of other major protein products such as albumin [67], further suggests that organelle compartments thought to be specifically involved in plasma lipoprotein assembly and transport [72] might be particularly sensitive to such intracellular alterations.

Besides influencing the mechanisms governing triacylglycerol secretion in general, *n*-3 PUFAs apparently also perturb some intrinsic property of the triacylglycerol molecules into which they are incorporated. Both the TGFA analyses presented here and other groups' analyses of intracellular and secreted triacylglycerols obtained from cultured rat hepatocytes incubated with EPA or DHA [40,42] argue that under certain circumstances triacylglycerol molecules containing *n*-3 fatty acids are not readily secreted from the cell. Martin *et al.* [40] proposed that the intracellular partitioning of newly synthesized triacylglycerols between lipoprotein assembly and cytosolic accumulation favours retention of DHA-containing molecules. Our results support and extend their proposal by demonstrating that triacylglycerol partitioning responds to changing conditions of fatty acid supplementation, such that the balance between retention and secretion of triacylglycerol-associated EPA and DHA depends on the availability of other fatty acyl species.

At present we can only speculate about mechanisms that could account for such specific effects of *n*-3 PUFAs. One possibility could be that McA-RH7777 cells (and others in which *n*-3 PUFAs are suitable substrates) may incorporate one, two or three molecules of EPA or DHA into a particular molecule of triacylglycerol, depending on the relative abundance of different fatty acid species. In contrast, either packaging of triacylglycerols into nascent lipoprotein particles, or subsequent secretion of triacylglycerol-rich lipoproteins themselves, may favour molecules containing one or two fatty acyl species other than *n*-3 PUFAs. When the available fatty acid pool is EPA- or DHA-dominant, the likelihood of synthesizing a triacylglycerol molecule containing two or three *n*-3 PUFAs is high, yet these cannot be readily secreted; in addition to any general impairment of triacylglycerol secretion effected by changes in membrane structure and function or signal transduction, triacylglycerol-associated EPA and DHA are retained disproportionately by the cell. On the other hand, when the available pool is OA-dominant, newly synthesized triacylglycerol molecules containing at most one EPA or DHA acyl group would predominate, so that the apparent secretion efficiency of triacylglycerol-associated EPA and DHA is relatively preserved. This scenario is consistent with the notion of stereoselective triacylglycerol partitioning invoked by Martin *et al.* [40]. Moreover, if the computer-modelling studies of diacylglycerol conformation by Applegate & Glomset [73,74] may be extrapolated to triacylglycerol structure, the packing of EPA- or DHA-rich triacylglycerols into a nascent lipoprotein core should differ substantially from that of molecules containing few or no *n*-3 fatty acyl groups. These speculations, however reasonable, remain to be tested empirically.

As a final comment, it is not yet known whether our findings regarding McA-RH7777-cell growth are relevant to the effects of fish-oil fatty acids *in vivo*. We are hesitant to extrapolate the behaviour of hepatoma cells given an EPA-predominant fatty acid environment in culture to hepatic function in intact subjects for whom maximal plasma levels of EPA, as well as its proportion of the plasma NEFA pool, are considerably lower. Nevertheless, we are concerned that antiproliferative effects of *n*-3 PUFAs have been observed *in vivo* [58,59], and that fish-oil treatment is finding its way into paediatric use [75]. Until the effects of fish-oil *n*-3 PUFAs on hepatocyte proliferation are better understood, perhaps they should be administered cautiously in clinical situations where continued hepatic growth or regeneration is desirable.

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