Effect of chronic incubation of CaCo-2 cells with eicosapentaenoic acid (20:5, *n*−3) and oleic acid (18:1, *n*−9) on triacylglycerol production

Trine RANHEIM,* Ane GEDDE-DAHL,† Arild C. RUSTAN† and Christian A. DREVON*‡

*Section for Dietary Research, Institute for Nutrition Research, and †Department of Pharmacology, Institute of Pharmacy, University of Oslo, Oslo, Norway

CaCo-2 monolayers, cultured for 1 week after reaching confluence, were incubated with micellar solutions of fatty acids for up to 7 days. These conditioned cells were incubated acutely (5 h) with eicosapentaenoic acid and oleic acid, and the levels of cell-associated and secreted triacylglycerol were determined. With acute addition of oleic acid, both cell-associated and secreted triacylglycerol were decreased in cells chronically exposed to eicosapentaenoic acid. This effect was observed after as little as 2 days of chronic incubation with eicosapentaenoic acid. A further decrease was found when these cells were incubated acutely with eicosapentaenoic acid, regardless of which radioisotopes were used to label precursors in the incubation media. The secretion of both labelled and total triacylglycerol and apolipoprotein B was reduced approximately 50% in cells incubated chronically with eicosapentaenoic acid. The amounts of triacylglycerol and apolipoprotein B within the cells were not decreased by chronic exposure to eicosapentaenoic acid. Our data indicate that CaCo-2 cells chronically incubated with eicosapentaenoic acid secrete significantly less triacylglycerol than cells incubated chronically with oleic acid. When eicosapentaenoic acid was also included acutely, triacylglycerol secretion was reduced even more. We conclude that chronic exposure of eicosapentaenoic acid to this intestinal cell type reduces the rate of chylomicron secretion and may help explain the decreased postprandial lipaemia observed in humans taking fish oil supplements.

INTRODUCTION

The interest in *n*−3 fatty acids from seafood and their implication for coronary heart disease was aroused by observations in Greenland Eskimos suggesting that the high intake of these polyunsaturated fatty acids by the Eskimos was associated with their low mortality rate from coronary heart disease [1,2]. One of the striking effects of dietary supplementation with *n*−3 fatty acids is the lowering of plasma concentrations of triacylglycerol. In addition, fish oil supplementation may alter apolipoprotein synthesis and secretion and possibly increase high-density lipoprotein levels [3–7].

In 1980 Harris and Connor [8] found a reduction in postprandial lipaemia in subjects consuming an acute fish oil load in a diet rich in salmon oil, compared with that observed when a control test meal was given during the control diet phase. Harris et al. [9] and Weintraub et al. [10] also found a significant decrease in postprandial lipaemia after fish oil feeding and showed that it was a function of background diet not the fat given acutely in the test meal load. Weintraub et al. [10] suggested that the hypochylomicronaemia may have been secondary to reduced very-low-density lipoprotein levels making more lipoprotein lipase available for the chylomicron clearance. On the other hand, Murthy et al. [11] reported that *n*−3 fatty acids decreased triacylglycerol synthesis dramatically in CaCo-2 cells, and Hall et al. [12] reported that CaCo-2 cells incubated for 1 week with *n*−3 fatty acids secreted less triacylglycerol than control cells. Thus evidence exists supporting both increased clearance and decreased production of chylomicrons in CaCo-2 cells.

Previous data from our laboratory indicated that, contrary to the findings of Murthy et al. [11], eicosapentaenoic acid did not reduce formation of triacylglycerol in CaCo-2 cells during short-term (up to 24 h) incubations [13,14]. These findings were in accordance with the observation that postprandial levels of triacylglycerol were no different when subjects consumed a fish oil test meal rather than a control meal while on their normal diets [9].

Therefore in the present study, we attempted to mimic the effect of chronic *in vivo* exposure of intestinal cells to *n*−3 fatty acids on chylomicron formation.

MATERIALS AND METHODS

Chemicals

[1,2,3-^{3}H]Glycerol (200 Ci/mol), D-[1-^{14}C]mannitol (45–55 Ci/mol), [1-^{14}C]eicosa-5,8,11,14,17-pentaenoic acid (52 Ci/mol), [1-^{14}C]oleic acid (58 Ci/mol) and ^3H_2O (18 Ci/mol) were obtained from du Pont–NEN Products, Boston, MA, U.S.A. Eicosapentaenoic acid, oleic acid and sodium taurocholate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. T.l.c. plates (silica gel F 1500) were from Schleicher and Schuell, Dassel, Germany.

Micellar solutions of fatty acids

Sodium salt solutions of the fatty acids, eicosapentaenoic acid and oleic acid (2 and 6 mM), were mixed with sodium taurocholate (20 mM) and dissolved in chloroform/methanol (2:1, (v/v). The organic solvent was removed by rotary evaporation at room temperature. The resulting lipid film was dispersed in serum-free Dulbecco's modified Eagle's medium (DMEM) and the micellar solution containing fatty acid and sodium taurocholate was sonicated at 37 °C for 5 min using an ultrasonic cleaner, Branson B-220. Only optically clear micellar solutions were used. The micellar solutions were diluted tenfold in serum-

Abbreviations used: FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium.

‡ To whom correspondence should be addressed.

free DMEM to final concentrations of 0.2 and 0.6 mM fatty acids and 12 mM sodium taurocholate [14].

Cell culture

CaCo-2 cells were utilized to find an *in vitro* model for studying the regulation of lipid metabolism in the intestine [15]. In spite of the fact that these cells probably lack monoacylglycerophosphate acyltransferase [16] and intestinal fatty acid-binding protein [17], they have the capacity to secrete nascent lipoproteins from the basolateral side [18], and intestinal low-density lipoprotein receptor expression is regulated by luminal sterol flux [19]. Monolayer cultures of CaCo-2 cells were obtained from American Type Culture Collection, Rockville, MD, U.S.A., at passage no. 17 and grown in 75 cm^2 plastic flasks (Costar, Cambridge, MA, U.S.A.) at 37 °C in air and 5% CO_2 in DMEM (4.5 g/l glucose and 3.7 g/l $NaHCO_3$) (Flow Laboratories, Irvine, Ayrshire, U.K.). The medium was supplemented with 5 and 20% fetal calf serum (FCS) (Gibco, Paisley, U.K.), L-glutamine (2 mM), insulin (10 μg/ml) (Sigma), penicillin (50 units/ml), streptomycin (50 μg/ml) and 1% non-essential amino acids (Flow Laboratories). Grown under these conditions, the doubling time of the CaCo-2 cells was approximately 70 h. The culture medium was changed every other day, and the day before an experiment. For subculture the medium was removed, and the cells were detached from the culture flasks with 0.25% trypsin (Difco Laboratories, Detroit, MI, U.S.A) in Ca^{2+}-, Mg^{2+}-free PBS containing 0.2 g/l EDTA. Culture medium with FCS was added to stop trypsinization. Cells were suspended and seeded at approx. 4×10^4 cells/cm^2 in new flasks according to the method of Mohrmann et al. [20]. Cells were grown to confluence in 1.5 ml of complete DMEM containing approx. 10^6 cells plated on the apical side of presoaked membrane filters of 3.0 μm pore size and 24.5 mm diameter (Transwell-COL; Costar). We used this large pore size to allow chylomicron particles to diffuse freely through the membrane [13]. The lower well contained 2.6 ml of complete DMEM. Monolayers were harvested 2 weeks after reaching confluence. Cell viability, as evaluated by the Trypan Blue exclusion test, was never less than 90%. The cells were mycoplasma-negative as determined with Hoechst 33258 [21]. To evaluate the barrier properties of the cell monolayers, [^{14}C]mannitol was added to the apical side of the monolayers [14]. In the present study we observed that 1.7% or less of added radioactivity diffused from the apical to the basolateral side within 5 h of incubation.

Experimental procedures

Triacylglycerol synthesis and secretion were studied by two different designs. First, the cells were incubated for 7 days with either eicosapentaenoic acid or oleic acid (0.2 mM), then 5 h with 0.6 mM fatty acid (either eicosapentaenoic acid or oleic acid) to stimulate triacylglycerol synthesis. CaCo-2 monolayers, cultured for 1 week after confluence on filter membranes, were incubated with either eicosapentaenoic acid or oleic acid (0.2 mM) in DMEM supplemented with 5% FCS for 5 days, and then micellar fatty acid (either eicosapentaenoic acid or oleic acid; 0.2 mM) in serum-free (Ultroser G, 4%; Gibco) DMEM for 2 days. The duration of incubation with fatty acid (0.2 mM) was 7 days in total, in order to mimic the chronic intake situation. Thereafter, the cells were incubated in serum-free DMEM containing micellar fatty acids (either eicosapentaenoic acid or oleic acid; 0.6 mM) (concentrations and incubation periods are also indicated in the legends to Tables and Figures), [1,2,3-^{3}H]glycerol (13 μCi/ml), [1-^{14}C]eicosapentaenoic acid (1 μCi/ml), [1-^{14}C]oleic acid (1 μCi/ml) or ^3H_2O (9 mCi/ml). Radioisotopes were added to the upper chamber in the cell culture system. The cells were scraped off the filter membranes into PBS. Medium was collected from inside and outside of the tissue culture inserts and treated as further explained below.

In the second design, CaCo-2 cells were incubated in DMEM for 1, 2 or 3 days with either eicosapentaenoic acid or oleic acid (0.6 mM) to investigate when the inhibitory effect of eicosapentaenoic acid appeared. The fatty acids were added to the apical medium every day. In these experiments the apical medium was changed daily, whereas the basolateral medium was not changed during the fatty acid incubation period, thus accumulating all the chylomicrons produced. At given times the medium was collected, and the triacylglycerol-rich lipoproteins of density less than 1.006 g/ml were isolated by ultracentrifugation in a Sorvall TFT 45.6 fixed-angle rotor at 146500 *g* (38000 rev./min) for 20 h [22]. The top containing the chylomicron fraction was collected by tube slicing and further examined by analysis of lipid composition (see below) and apolipoprotein B. When CaCo-2 cells were incubated with [^{3}H]glycerol, we observed that approx. 90% of the secreted radioactive triacylglycerol was recovered in the basolateral fraction of $d < 1.006$ g/ml. The rate of triacyl[^{3}H]glycerol secretion was therefore used as an estimate of chylomicron secretion. A radioimmunoassay (Pharmacia Diagnostics AB, Uppsala, Sweden) was used for apolipoprotein B analysis.

Samples were taken for protein determination, using BSA as the reference protein [23].

Lipid extraction and t.l.c.

Lipids from cells and media were extracted with chloroform/methanol (2:1, v/v). The homogenized cell fraction was mixed with 20 vol. of chloroform/methanol (2:1, v/v) [24]. Then 4 vol. of 0.9% NaCl (pH 2) was added and the mixture allowed to separate into two phases. The organic phase was dried under a stream of nitrogen at 40 °C. To medium devoid of cellular debris was added 4 vol. of chloroform/methanol (2:1, v/v) and 2% serum as unlabelled carrier for the lipids. The water phase of the medium extract was re-extracted once with 4 vol. of chloroform/methanol (2:1 v/v), and the combined organic phases were treated further in the same way as for the cells. Residual lipid extract was redissolved in 200 μl of hexane and separated by t.l.c. using hexane/diethyl ether/acetic acid (80:20:1, by vol.) as developing solvent. In other experiments, where it was important to separate diacylglycerol and unesterified cholesterol, hexane/diethyl ether/acetic acid (65:35:1, by vol.) was used, followed by hexane as a second developing solvent. The various lipids were finally identified by iodine or 2′,7′-dichlorofluorescein (Sigma Chemical Co.), and the t.l.c. foils were cut into 8 ml of liquid-scintillation fluid and counted in a scintillation spectrometer (Packard Tri-Carb 1900 TR).

Mass measurements of triacylglycerol by g.l.c.

The fatty acid composition of triacylglycerol in CaCo-2 cells was determined as fatty acid methyl esters on a gas chromatograph (Shimadzu GC-14A, Kyoto, Japan), equipped with a polar capillary column (SGE BPX70; 0.33 mm internal diameter, 25 m length) and using helium as the carrier gas. The temperature was programmed to rise from 40 to 220 °C. The procedure for the transesterification reaction is described by Mason and Waller [25]. Triacylglycerol spots (separated by t.l.c. and visualized by fluorescein) were scraped into vials, 1.0 ml of benzene, 2 ml of

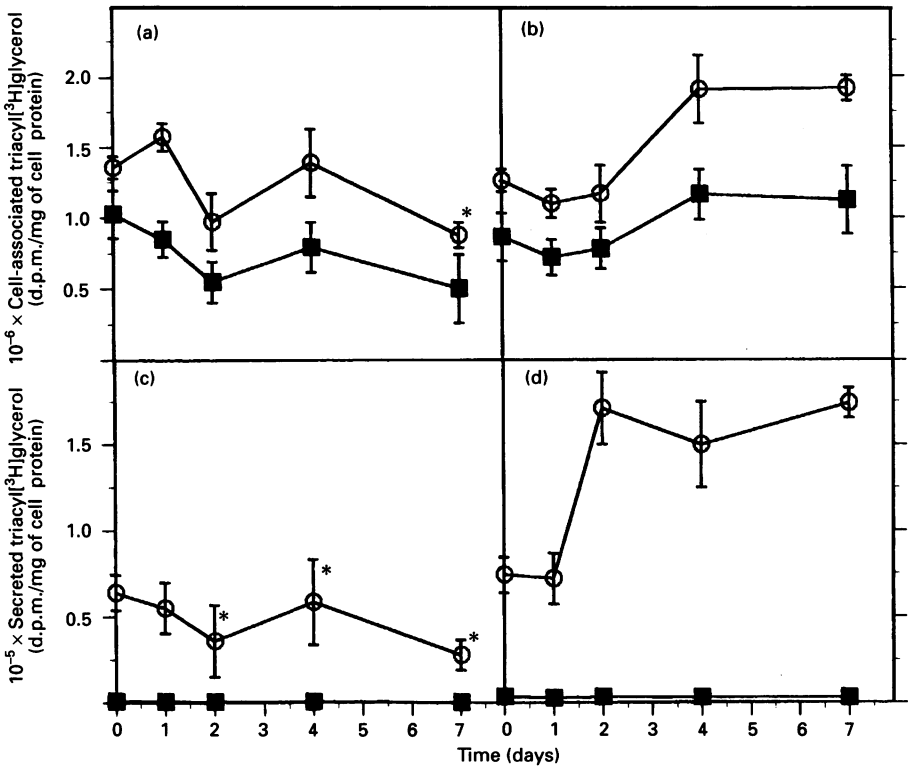

Figure 1 Time course of cell-associated (a,b) and secreted (c,d) triacyl[^{3}H]glycerol after chronic incubation with eicosapentaenoic acid (a,c) and oleic acid (b,d)

CaCo-2 monolayers, cultured on filter membranes for 2 weeks after confluence, were first incubated in DMEM supplemented with 5% FCS for 5 days and then in serum-free DMEM for 2 days. During this 7-day period eicosapentaenoic acid or oleic acid (0.2 mM) was present in the medium for 0, 1, 2, 4 or 7 days. Thereafter, the chronic incubation was accompanied by an acute incubation for 5 h with either eicosapentaenoic acid (■) or oleic acid (○) (0.6 mM) in serum-free DMEM containing [1,2,3-^{3}H]glycerol (13 μCi/ml) to measure cell-associated and secreted labelled triacylglycerol. Data represent means ± S.D. of three cultures given as d.p.m./mg of cell protein. This experiment was repeated with three other cultures, giving similar results. *Significant difference ($P < 0.02$) between oleic acid incubations in cells chronically supplemented with eicosapentaenoic acid or oleic acid.

methanolic HCl (3 M) (Supelco, Supelco Park, Bellefonte, PA, U.S.A.) and 200 μl of 2,2-dimethoxypropane (Supelco) were added, and the vials stored overnight at room temperature. The triacylglycerol was then neutralized with 4.0 ml of $NaHCO_3$ (0.7 M) and extracted with 2 × 2 ml of hexane. Triheptadecanoylglycerol was used as an internal standard.

Statistical analysis

All values are reported as the means ± S.D. of indicated samples and number of experiments. Different treatments were compared by the t test (two-tailed).

RESULTS

Time course of the effect on triacyl[^{3}H]glycerol production of chronic incubation with fatty acids

Cell-associated and secreted triacyl[^{3}H]glycerol during the 7-day incubation with either eicosapentaenoic acid or oleic acid is presented in Figure 1. Seven days of fatty acid treatment was chosen to ensure that significant incorporation of eicosapentaenoic acid was achieved. Cell-associated triacyl[^{3}H]glycerol levels tended to decrease over time with chronic eicosapentaenoic exposure, whereas they were stable or increased over time in cells incubated with oleic acid chronically (Figures 1a and 1b). This was true when either oleic acid or eicosapentaenoic acid was used as the acute substrate. Triacylglycerol secretion in response to oleic acid was lowered when the cells were supplemented with eicosapentaenoic acid for 2 days or more compared with chronic incubation with oleic acid (Figures 1c and 1d). With eicosapentaenoic acid as substrate virtually no triacylglycerol was secreted from cells exposed to either eicosapentaenoic acid or oleic acid. At 2–4 days of chronic oleic acid supplementation, the generation of cell-associated and secreted triacylglycerol had reached a maximum.

Effect of fatty acids on secretion of presynthesized triacyl[^{3}H]glycerol (pulse–chase)

CaCo-2 cells were preincubated for 7 days with either eicosapentaenoic acid or oleic acid and then pulsed for 2 h with [^{3}H]glycerol to generate intracellular triacyl[^{3}H]glycerol. After extensive washing, the cells were reincubated for up to 21 h with one of the two fatty acids and unlabelled glycerol (chase period) (Figure 2). With oleic acid as substrate there was approx 50% reduction in zero-hour cell-associated triacyl[^{3}H]glycerol levels in cells chronically exposed to eicosapentaenoic acid, as compared with chronic incubation with oleic acid (Figures 2a and 2b). Cell-associated triacyl[^{3}H]glycerol was stable during the chase period, indicating that some [^{3}H]glycerol was associated with the cell brush borders or that none was secreted. Secreted triacyl[^{3}H]glycerol was reduced 6-fold after 21 h of incubation with oleic acid as substrate in cells chronically supplemented with

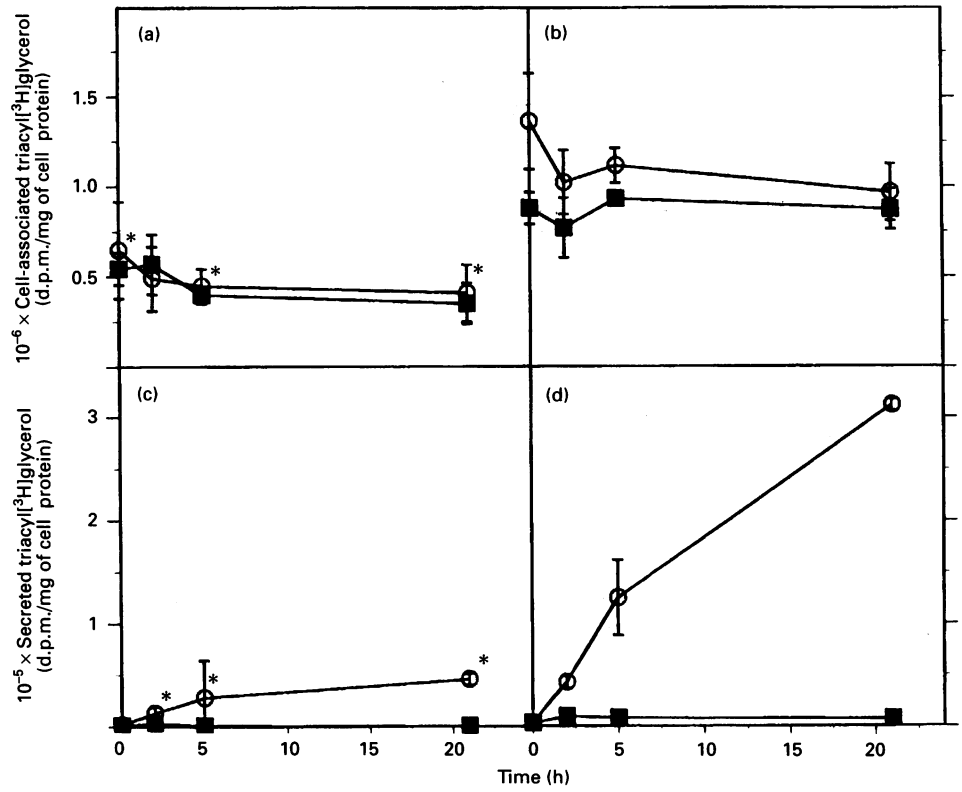

Figure 2 Effect of eicosapentaenoic acid and oleic acid on cell association (a,b) and secretion (c,d) of presynthesized triacyl[^{3}H]glycerol

CaCo-2 monolayers, cultured on filter membranes for 2 weeks after confluence, were incubated first in DMEM supplemented with 5% FCS for 5 days and then in serum-free DMEM for 2 days. During this 7-day period eicosapentaenoic acid (**a,c**) or oleic acid (**b,d**) (0.2 mM) was present in the medium. Thereafter, the cells were incubated in serum-free DMEM containing [1,2,3-^{3}H]glycerol (13 μCi/ml) and eicsoapentaenoic acid (■) or oleic acid (○) (0.6 mM). After 2 h of incubation, the medium was discarded, the cells were washed, and isotope-free medium containing either micellar 0.6 mM eicosapentaenoic acid or 0.6 mM oleic acid was added in addition to glycerol (2.0 mM) and the mixture incubated for up to 21 h. Cell-associated and secreted triacyl[^{3}H]glycerol were determined. Data represent means ± S.D. of six cultures given as d.p.m./mg of cell protein. *Significant difference ($P < 0.02$) between oleic acid incubations in cells chronically supplemented with eicosapentaenoic acid or oleic acid.

Table 1 Incorporation of [1-^{14}C]eicosapentaenoic acid and [1-^{14}C]oleic acid into cell-associated and secreted triacylglycerol

Control CaCo-2 cells were incubated in DMEM supplemented with 20% FCS, cultured for 2 weeks after confluence on filter membranes, incubated for 5 h in serum-free DMEM containing [1-^{14}C]eicosapentaenoic acid or [1-^{14}C]oleic acid (1 μCi/ml; 0.6 mM). Cells incubated chronically with fatty acid were cultured for 1 week after confluence on filter membranes and first incubated in DMEM supplemented with 5% FCS for 5 days and then in serum-free DMEM for 2 days. During this 7-day period, eicosapentaenoic acid or oleic acid (0.2 mM) was present in the medium. Thereafter, the chronically fatty acid-treated cells were incubated acutely for 5 h in serum-free DMEM containing [1-^{14}C]eicosapentaenoic acid or [1-^{14}C]oleic acid (1 μCi/ml; 0.6 mM). Data represent means ± S.D. from six to nine cultures given as d.p.m./mg of cell protein. *Significant difference ($P < 0.02$) between oleic acid incubations in cells chronically supplemented with eicosapentaenoic acid or oleic acid. †Significant difference ($P < 0.05$) between eicosapentaenoic acid incubations in cells chronically supplemented with eicosapentaenoic acid or oleic acid. ‡Significant difference between ($P < 0.01$) eicosapentaenoic acid and oleic acid incubations in cells chronically supplemented with eicosapentaenoic acid or oleic acid.

	10^{-4} × Cell-associated triacylglycerol (d.p.m./mg of cell protein)			10^{-4} × Secreted triacylglycerol (d.p.m./mg of cell protein)		
Fatty acid substrate	Control	Chronic eicosapentaenoic acid	Chronic oleic acid	Control	Chronic eicosapentaenoic acid	Chronic oleic acid
Eicosapentaenoic acid	73.28 ± 3.0	46.03 ± 8.4	46.13 ± 9.9	7.81 ± 0.5	0.5 ± 0.2†‡	0.94 ± 0.3‡
Oleic acid	74.73 ± 2.5	50.08 ± 6.5	48.18 ± 5.4	8.58 ± 0.7	7.9 ± 1.7*	14.08 ± 1.9

eicosapentaenoic acid, indicating that the secretion was inhibited (Figures 2a and 2b).

Studies with [1-^{14}C]fatty acids

CaCo-2 cells, chronically exposed to fatty acids, were incubated with either [1-^{14}C]eicosapentaenoic acid or [1-^{14}C]oleic acid for 5 h (Table 1). There was a similar appearance of cell-associated triacylglycerol labelled with eicosapentaenoic acid and oleic acid after chronic incubation with both fatty acids. Secretion of labelled triacylglycerol was reduced by approx. 50% with oleic acid as substrate by chronic supplementation with eicosa-

Table 2 Incorporation of ^3H_2O into cell-associated and secreted triacylglycerol

CaCo-2 monolayers, cultured on filter membranes for 2 weeks after confluence, were first incubated in DMEM supplemented with 5% FCS for 5 days and then in serum-free DMEM for 2 days. During this 7-day period eicosapentaenoic acid or oleic acid (0.2 mM) was present in the medium. Thereafter, the cells were incubated for 5 h in serum-free DMEM containing ^3H_2O (9 mCi/ml), Hepes (20 mM) and eicosapentaenoic acid or oleic acid (0.6 mM). Data represent means ± S.D. from six cultures given as d.p.m./mg of cell protein. *Significant difference ($P < 0.003$) between oleic acid incubations in cells chronically supplemented with eicosapentaenoic acid or oleic acid. †Significant difference ($P < 0.03$) between eicosapentaenoic acid incubations in cells chronically supplemented with eicosapentaenoic acid or oleic acid.

	10^{-2} × Cell-associated triacylglycerol (d.p.m./mg of cell protein)		10^{-2} × Secreted triacylglycerol (d.p.m./mg of cell protein)	
Fatty acid substrate	Chronic eicosapentaenoic acid	Chronic oleic acid	Chronic eicosapentaenoic acid	Chronic oleic acid
Eicosapentaenoic acid	39.39 ± 10	32.16 ± 8.5	2.01 ± 0.6†	3.40 ± 0.5
Oleic acid	35.54 ± 7.3	31.28 ± 4.2	2.96 ± 0.3*	5.63 ± 1.2

Table 3 Effect of eicosapentaenoic acid (EPA) and oleic acid on mass of triacylglycerol and apolipoprotein B

CaCo-2 monolayers, cultured on filter membranes for 2 weeks after confluence, were incubated in DMEM supplemented with 5% FCS for 1, 2 or 3 days. Fresh eicosapentaenoic acid or oleic acid (0.6 mM) was added to the apical media every day. Basolateral media were not changed during the fatty acid incubation period. Cells and media were harvested, and lipoproteins from the basolateral media were isolated by ultracentrifugation at $d < 1.006$ g/ml. Mass determination of cell-associated and secreted triacylglycerol was performed by g.l.c. Analysis of cell-associated and secreted apolipoprotein B was performed by radioimmunoassay. Data represent means ± S.D. of six cultures given as μg/mg of cell protein. *Significant difference ($P < 0.03$) between cells incubated with EPA and oleic acid.

	Cell-associated (μg/mg of cell protein)				Secreted (μg/mg of cell protein)			
	Triacylglycerol		Apolipoprotein B		Triacylglycerol		Apolipoprotein B	
Time (days)	Eicosapentaenoic acid	Oleic acid	Eicosapentaenoic acid	Oleic acid	Eicosapentaenoic acid	Oleic acid	Eicosapentaenoic acid	Oleic acid
1	113 ± 20	97 ± 2	0.6 ± 0.1	0.6 ± 0.1	1.12 ± 0.12	1.22 ± 0.14	0.09 ± 0.02*	0.13 ± 0.01
2	234 ± 43	339 ± 68	0.8 ± 0.1	0.7 ± 0.1	1.2 ± 0.01*	2.35 ± 0.55	0.13 ± 0.01*	0.20 ± 0.01
3	318 ± 33	288 ± 56	1.0 ± 0.3	0.6 ± 0.2	1.24 ± 0.3*	2.21 ± 0.63	0.14 ± 0.01*	0.24 ± 0.02

Table 4 Fraction of synthesized triacylglycerol secreted

The values are based on data given in Figure 1 and Tables 1 and 2. The cells were supplemented with either eicosapentaenoic acid or oleic acid for 7 days, and thereafter incubated for 5 h with oleic acid as substrate. The values for the triacylglycerol mass experiment are based on data given in Table 3; the cells were incubated with eicosapentaenoic acid or oleic acid for 3 days. More details are given in the Figure and Table legends. *Significant difference ($P < 0.03$) between cells incubated with eicosapentaenoic acid or oleic acid.

	Secreted triacylglycerol (% of synthesized)	
Experiment	Chronic eicosapentaenoic acid	Chronic oleic acid
[^{3}H]Glycerol (Figure 1)	3.1 ± 0.4*	8.3 ± 0.7
[^{14}C]Fatty acids (Table 1)	11.1 ± 2.4*	20.5 ± 1.7
^3H_2O (Table 2)	8.0 ± 2.1*	15.2 ± 2.1
Triacylglycerol mass (Table 3)	0.39 ± 0.05*	0.83 ± 0.09

pentaenoic acid. Very little triacylglycerol labelled with [1-^{14}C]eicosapentaenoic acid was secreted from cells maintained chronically on either fatty acid. The acute suppression of triacylglycerol secretion by eicosapentaenoic acid is inconsistent with earlier studies, in which we found no major differences between the effects of eicosapentaenoic acid and oleic acid on the rate of lipoprotein secretion from CaCo-2 cells [13,14]. This could be due to the different preincubation procedures used. In this study the cells were incubated for 2 weeks after confluence, but in contrast with the previous experiments, during the last 7 days micellar fatty acids were included in the media (Table 1).

Studies with ^3H_2O

The problems associated with introducing labelled fatty acids into pools of potentially different sizes was minimized by incubating the CaCo-2 cells with tritiated water in the presence of the two fatty acids, as ^{3}H enters the lipid pathways at early steps because of rapid equilibration of ^3H_2O with the cellular water phase. Cells incubated with either eicosapentaenoic acid or oleic acid for 5 h after 7 days of preconditioning with either fatty acid showed a similar incorporation of ^{3}H into cellular triacylglycerol. However, in cells chronically exposed to eicosapentaenoic acid, the acute addition of oleic acid or eicosapentaenoic acid led to a significantly lower secretion of labelled triacylglycerol than was observed in cells incubated chronically with oleic acid (Table 2).

Mass of triacylglycerol and apolipoprotein B

Synthesized triacylglycerol was measured after 1, 2 or 3 days of incubation with either eicosapentaenoic acid or oleic acid (0.6 mM) present in the apical media. The apical media were changed daily, whereas the basolateral media were not changed during the fatty acid incubation period. Thus chylomicrons accumulated in the basolateral media. The rate of triacylglycerol

Table 5 Fatty acid distribution in cellular triacylglycerol

CaCo-2 monolayers, cultured on filter membranes for 2 weeks after confluence, were first incubated in DMEM supplemented with 5% FCS for 5 days and then in serum-free DMEM for 2 days. The cells were supplemented with eicosapentaenoic acid or oleic acid (0.2 mM) every day for 7 days. One portion of the cells was immediately extracted (basal levels). Other portions were extracted after subsequent incubation for 5 h in serum-free DMEM with eicosapentaenoic acid (EPA) or oleic acid (OA) (0.6 mM) (fatty acid values). The fatty acid composition of triacylglycerol was determined by g.l.c. Data represent means ± S.D of three cultures given as percentage of total fatty acids. n.d., not detected.

	Fatty acid composition of cellular triacylglycerol (% of total fatty acids)					
	Chronic eicosapentaenoic acid			Chronic oleic acid		
Fatty acid	Basal level	+ Eicosapentaenoic acid	+ Oleic acid	Basal level	+ Eicosapentaenoic acid	+ Oleic acid
16:0	9.3 ± 0.4	8.4 ± 1.1	7.5 ± 0.2	10 ± 0.6	8.8 ± 0.9	7.4 ± 0.4
18:0	4.7 ± 0.1	3.9 ± 0.4	3.5 ± 0.1	6.2 ± 0.3	4.4 ± 0.2	5.9 ± 1
18:1 (*n*−9)	21.2 ± 0.8	9.1 ± 0.5	32.2 ± 2	70.1 ± 0.6	45.2 ± 0.4	77.2 ± 0.6
18:2 (*n*−6)	n.d.	n.d.	nd.d	1.2 ± 0.6	0.4 ± 0.3	1.2 ± 0.9
20:1 (*n*−9)	n.d.	n.d.	n.d.	2.5 ± 0.1	0.9 ± 0.7	2.4 ± 0.4
20:4 (*n*−6)	0.5 ± 0.4	0.5 ± 0.4	0.4 ± 0.4	n.d.	n.d.	n.d.
20:5 (*n*−3)	35.1 ± 0.8	48.8 ± 4.8	29.4 ± 1.2			
	n.d.	24.6 ± 1.1	n.d.			
22:1 (*n*−9)	3.8 ± 0.5	4.6 ± 1.1	3.6 ± 0.3	3.1 ± 0.6	3.8 ± 0.5	1.8 ± 1.5
22:5 (*n*−3)	15.9 ± 0.8	14 ± 0.5	14.1 ± 0.3	n.d.	3.1 ± 0.3	n.d.
22:6 (*n*−3)	0.6 ± 0.3	1.7 ± 0.1	0.9 ± 0.3	n.d.	n.d.	n.d.

secretion was not reduced in cells supplemented with eicosapentaenoic acid for 1 day, but was decreased after 2 days of supplementation. The reduced rate of triacylglycerol secretion from cells supplemented with eicosapentaenoic acid for 2 and 3 days (Table 3) was similar to that found with the radiolabelled substrates.

The effects of eicosapentaenoic acid incubation on cell-associated and secreted apolipoprotein B (Table 3) were similar to those observed for triacylglycerol, except that even on day 1, there was a small but significant decrease in apolipoprotein B with eicosapentaenoic acid. Cell-associated apolipoprotein B levels were stable over the time of the experiment similar for both fatty acids.

Fraction of total synthesized triacylglycerol (secreted + cell-associated) secreted

The percentage of total synthesized triacylglycerol secreted is summarized in Table 4. The results are based on Figure 1 and Tables 1, 2 and 3, and demonstrate that secreted triacylglycerol was significantly decreased by chronic supplementation with eicosapentaenoic acid compared with supplementation with oleic acid.

Fatty acid composition of cellular triacylglycerol

The fatty acid compositions of CaCo-2 cells incubated with either eicosapentaenoic acid or oleic acid are given in Table 5. There was an accumulation of 20:5 (*n*−3), 22:5 (*n*−5) and 22:6 (*n*−3) and a reduction of 18:1 (*n*−9) in cells exposed to eicosapentaenoic acid for 1 week. The 20:5 (*n*−3) and 22:6 (*n*−3) levels increased even more at the expense of 18:1 (*n*−9) during the additional 5 h incubation with 0.6 mM eicosapentaenoic acid.

DISCUSSION

Consumption of diets enriched in marine *n*−3 polyunsaturated fatty acids reduces postprandial lipaemia [9,10,26]. In the present work we have investigated a potential mechanism for this effect, namely reduced rate of chylomicron secretion. Cells were incubated with fatty acids for up to 1 week to mimic chronic treatment with fish oil as reported in humans [9]. We found that the principal effect of eicosapentaenoic acid was to decrease the rate of secretion of triacylglycerol from CaCo-2 cells, whereas total synthesis was unaffected. This was observed in both isotope and mass studies (Table 4), and was supported by the decrease in apolipoprotein B secretion (Table 3). Murthy et al. [27] showed that eicosapentaenoic acid impairs triacylglycerol transport in part by inhibiting apolipoprotein B synthesis and secretion. Our results support this conclusion.

In the present paper we found that the rate of triacylglycerol secretion from cells chronically supplemented with eicosapentaenoic acid was reduced. In support of this, Kowalski et al. [28] reported that intestinal biopsies from malnourished patients re-fed with a diet enriched in *n*−3 fatty acids for 15 days produced less triacylglycerol than tissue taken from patients re-fed with a standard diet. Thus the decrease in postprandial lipaemia in humans produced by dietary *n*−3 fatty acids may be explained partly by a reduction in the rate of triacylglycerol secretion in enterocytes.

The combination of chronic and acute incubation with eicosapentaenoic acid blocked triacylglycerol secretion. This is consistent with findings in humans [9,10], where the lowest postprandial lipoprotein levels were achieved with a combination of chronic and acute *n*−3 fatty acid feeding. It is unlikely that eicosapentaenoic acid caused reduced triacylglycerol secretion by lowering fatty acid absorption, because the amount of synthesized labelled triacylglycerol was similar in cells chronically treated with either eicosapentaenoic acid or oleic acid (Table 1) [29]. In addition, the amounts of intracellular labelled free fatty acids were similar (1.63×10^5 versus 1.65×10^5 d.p.m./mg of cell protein) in cells chronically exposed to eicosapentaenoic acid or oleic acid. The most likely explanation for the present data is a decreased rate of triacylglycerol secretion. This is seen most clearly in Table 4. It is unlikely that this low rate of secretion was due to enhanced oxidation as the fraction of oxidized fatty acid is very small (5–7%), and probably does not alter lipoprotein production [13,30].

Cellular triacylglycerols contained high amounts of eicosa-

pentaenoic acid and docosapentaenoic acid (22:5, $n-3$) after 1 week of incubation with eicosapentaenoic acid. There was also an additional accumulation of eicosapentaenoic acid and a decrease in oleic acid after a 5 h incubation with eicosapentaenoic acid (Table 5), indicating that the cells were still completely metabolically active after 1 week. Thus, after chronic supplementation with eicosapentaenoic acid, CaCo-2 cells apparently have the capacity to elongate eicosapentaenoic acid to docosapentaenoic acid, but produce only small amounts of docosahexaenoic acid, consistent with observations made *in vivo* [31].

CaCo-2 cells incorporate significant amounts of exogenous fatty acids into their membrane phospholipids [32]. In the present study the incorporation of eicosapentaenoic acid into cellular phospholipids was 24% after 1 week of fatty acid supplementation, causing reduction of the arachidonic acid concentration by 50%. Eicosapentaenoic acid may therefore influence the functional properties of intestinal cell membranes or alter metabolism by decreasing the synthesis of eicosanoids from arachidonic acid [33,34].

In summary, we demonstrate a reduction in the rate of triacylglycerol from CaCo-2 cells chronically incubated with eicosapentaenoic acid using different labelled precursors, mass measurements and apolipoprotein B analysis. This may suggest that the reduction in postprandial triglyceridaemia in humans on dietary $n-3$ fatty acids may be explained partly be a decrease in the rate of triacylglycerol secretion in enterocytes. However, the present findings in isolated cells must await confirmation from studies *in vivo*.

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