ASBMB
JOURNAL OF LIPID RESEARCH

Eicosapentaenoic acid reduces hepatic synthesis and secretion of triacylglycerol by decreasing the activity of acyl-coenzyme A:1,2-diacylglycerol acyltransferase

A.C. Rustan, J.Ø. Nossen, E.N. Christiansen,* and C.A. Drevon*

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

Abstract The mechanism for the reduced hepatic production of triacylglycerol in the presence of eicosapentaenoic acid was explored in short-term experiments using cultured parenchymal cells and microsomes from rat liver. Oleic, palmitic, stearic, and linoleic acids were the most potent stimulators of triacyl[^{3}H]glycerol synthesis and secretion by hepatocytes, whereas erucic, α-linolenic, γ-linolenic, arachidonic, docosahexaenoic, and eicosapentaenoic acids (in decreasing order) were less stimulatory. There was a linear correlation ($r = 0.85$, $P < 0.01$) between synthesis and secretion of triacyl[^{3}H]glycerol for the fatty acids examined. The extreme and opposite effects of eicosapentaenoic and oleic acids on triacylglycerol metabolism were studied in more detail. With increasing number of free fatty acid molecules bound per molecule of albumin, the rate of synthesis and secretion of triacyl[^{3}H]glycerol increased, most markedly for oleic acid. Cellular uptake of the two fatty acids was similar, but more free eicosapentaenoic acid accumulated intracellularly. Eicosapentaenoic acid caused higher incorporation of [^{3}H]water into phospholipid and lower incorporation into triacylglycerol and cholesteryl ester as compared to oleic acid. No difference was observed between the fatty acids on incorporation into cellular free fatty acids, monoacylglycerol and diacylglycerol. The amount of some 16- and 18-carbon fatty acids in triacylglycerol was significantly higher in the presence of oleic acid compared with eicosapentaenoic acid. Rat liver microsomes in the presence of added 1,2-dioleoylglycerol incorporated eicosapentaenoic acid and eicosapentaenoyl-CoA into triacylglycerol to a lesser extent than oleic acid and its CoA derivative. Decreased formation of triacylglycerol was also observed when eicosapentaenoyl-CoA was given together with oleoyl-CoA, whereas palmitoyl-CoA, stearoyl-CoA, linoleoyl-CoA, linolenoyl-CoA, and arachidonoyl-CoA had no inhibitory effect. In conclusion, inhibition of acyl-CoA:1,2-diacylglycerol O-acyltransferase (EC 2.3.1.20) by eicosapentaenoic acid may be important for reduced synthesis and secretion of triacylglycerol from the liver.—**Rustan, A.C., J.Ø. Nossen, E.N. Christiansen, and C.A. Drevon.** Eicosapentaenoic acid reduces hepatic synthesis and secretion of triacylglycerol by decreasing the activity of acyl-coenzyme A:1,2-diacylglycerol acyltransferase. *J. Lipid Res.* 1988. **29:**1417–1426.

Supplementary key words triacylglycerol synthesis • VLDL • triacylglycerol secretion • polyunsaturated fatty acids • parenchymal cells • microsomes

Plasma levels of triacylglycerol and very low density lipoprotein (VLDL) are lower in Eskimos than in healthy Danes, although both populations have a quantitatively similar intake of dietary fat (1). This is due to a high amount of saturated fatty acids in the Danish diet, whereas the Eskimos consume much polyunsaturated fatty acids of the n–3 family (2). Results of clinical trials with both hyper- and normolipidemic subjects support the idea that these fatty acids have a lipid-lowering effect (3,4).

Humans consuming diets rich in vegetable polyunsaturated fatty acids (the n–6 family) have plasma triacylglycerol levels between those observed in Eskimos and Danes (5). Dietary studies reveal that n–6 fatty acids also have a triacylglycerol-lowering effect, although they are less potent than marine (n–3) fatty acids (5–8).

The mechanism for lowering plasma triacylglycerol by polyunsaturated fatty acids may be explained by increased removal and/or diminished production of lipoprotein particles (9–13). At present, most evidence supports the notion that n–3 fatty acids decrease hepatic production of VLDL. In human turnover studies, it has been shown that polyunsaturated n–3 fatty acids decrease formation of VLDL-triacylglycerol (12), VLDL-apolipoprotein B (8), and LDL-apolipoprotein

Abbreviations: VLDL, very low density lipoprotein; CoA, coenzyme A (CoASH); LDL, low density lipoprotein; TLC, thin-layer chromatography.

Downloaded from www.jlr.org by guest, on June 18, 2012

B (13). Moreover, purified eicosapentaenoic acid (20:5, n–3) has recently been reported to inhibit secretion of VLDL-triacylglycerol from cultured rat hepatocytes by decreasing cellular triacylglycerol synthesis (14, 15). Reduced VLDL-triacylglycerol secretion was not an effect of this fatty acid on the secretory pathway *per se* (14). Data suggest that eicosapentaenoic acid is a poor substrate for triacylglycerol synthesis, and it reduces the stimulatory effect of oleic acid on synthesis and secretion of triacylglycerol (14). The goal of the present study was to elucidate the mechanism for reduced synthesis of triacylglycerol in the presence of eicosapentaenoic acid during short-term incubations. The effects of eicosapentaenoic (20:5, n–3) and oleic acid (18:1, n–9) on some aspects of cellular lipid metabolism were extensively studied to explore what level of triacylglycerol synthesis is affected by eicosapentaenoic acid. In addition, we have compared the effects of eicosapentaenoic acid and some other fatty acids on synthesis and secretion of triacylglycerol by cultured rat hepatocytes. We present data indicating that eicosapentaenoic acid *per se* inhibits the activity of acyl-CoA:1,2-diacylglycerol acyltransferase (EC 2.3.1.20) and thereby decreases synthesis of triacylglycerol.

MATERIALS AND METHODS

Chemicals

Arginine-free Dulbecco's modified Eagle's medium, newborn calf serum, and gentamicin (10 mg/ml) were obtained from Flow Laboratories, Irvine, Ayrshire, U.K. The medium was supplemented with ornithine (0.5 mM), 4-(hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (10 mM), and $NaHCO_3$ (24 mM) from Sigma Chemical Co., St. Louis, MO. [1,2,3-^{3}H]Glycerol (200 Ci/mol), [^{3}H]water (18 Ci/mol), [U-^{14}C]eicosapentaenoic acid (212 Ci/mol), [U-^{14}C]oleic acid (900 Ci/mol), and [^{14}C]oleoyl-CoA (58.7 Ci/mol) were obtained from New England Nuclear, Dreieich, F.R.G. 1,2-Di-[1-^{14}C]oleoyl-*sn*-glycerophosphocholine (105 Ci/mol) was purchased from Amersham International, Amersham, Bucks., U.K. Collagenase (type 1), bovine insulin, oleic acid, eicosapentaenoic acid (ca. 90% pure; as evaluated by gas–liquid chromatography the main impurities were stearic and oleic acid), the other fatty acids, 1,2-dioleoylglycerol, 1,2-dioleoyl-*sn*-glycerophosphocholine, and bovine serum albumin (essentially fatty acid-free) were from Sigma Chemical Co. Phospholipase C (grade I) from *Bacillus cereus* (EC 3.1.4.3.) was obtained from Boehringer Mannheim, Mannheim, F.R.G. Tissue culture dishes were supplied by Costar, Cambridge, MA. Thin-layer chromatography plates (Silica gel F 1500) were purchased from Schleicher & Schuell, Dassel, F.R.G. All other chemicals and solvents were high purity commercial materials.

Preparation of cultured hepatocytes

Hepatocytes were prepared from male albino rats (200–300 g, Wistar) fed an ordinary pellet diet and water ad libitum. In short, the rats were anesthetized with barbiturates, and the hepatocytes were isolated by collagenase perfusion, washed, and plated in Dulbecco's modified Eagle's medium containing newborn calf serum (20%), insulin, and gentamicin (16).

Experiments with cultured hepatocytes

After 20 hr incubation, the cells were changed to serum-free Dulbecco's modified Eagle's medium containing glucose (12.5 mM), fatty acids bound to albumin, and the compounds indicated in legends to figures and tables. The protein content of each culture dish was determined using bovine serum albumin as standard (17).

Triacylglycerol measurement; isotope studies

It has been shown that more than 95% of the triacyl[^{3}H]glycerol secreted into the medium by cultured rat hepatocytes is recovered in the VLDL fraction (d<1.006 g/ml) (18). Triacyl[^{3}H]glycerol in the medium was therefore used as an estimate for VLDL-triacylglycerol secretion. Moreover, when [^{3}H]glycerol and oleic acid (1 mM) are added to the culture medium, hepatocytes secrete triacyl[^{3}H]glycerol in a linear manner for at least 2 hr (18). Medium and cellular triacyl[^{3}H]glycerol were measured after extraction and separation as described elsewhere (18).

Measurement of intracellular labeled lipids

Incorporation of [^{3}H]water and [^{14}C] fatty acid into cell-associated lipids was determined by liquid scintillation after extraction (19) and separation by thin-layer chromatography (TLC).

Fatty acid pattern of cells and media

Triheptadecanoin (20 μg) was added to cells and media as internal standard before extraction (19) and isolation of triacylglycerol. The triacylglycerol spots on TLC plates were visualized by fluorescein, scraped into vials, redissolved in 2 ml benzene, and methylated by overnight incubation in 2 ml methanolic HCl (3 N) and 200 μl 2,2-dimethoxypropane. The mixtures were neutralized by 4 ml $NaHCO_3$ (0.7 M) and extracted twice with 2 ml n-hexane. After evaporation of the solvent, the lipids were redissolved in 2.0 ml n-hexane, and an aliquot was injected into the gas–liquid chromatograph (Carlo Erba Strumentazione, Fractovap Series 2150), which was equipped with an apolar cap-

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH

illary column (CP Sil 19 CB, length 50 m, diameter 0.2 mm); the carrier gas was helium (flowrate: 0.5 ml/min). The oven temperature was programmed to rise from 200°C to 260°C at 0.5 °C/min. Retention times and peak areas were automatically computed by a Hewlett-Packard 3390 A integrator. Identification of the individual methyl esters was performed by frequent comparison with authentic standard mixtures analyzed under the same conditions.

Uptake of fatty acids by hepatocytes

Cellular uptake of radioactive eicosapentaenoic and oleic acids was determined after trichloroacetic acid precipitation as outlined elsewhere (14).

Assay of acyl-CoA:1,2-diacylglycerol acyltransferase

Rat liver microsomes were prepared as described elsewhere (20) and stored at −70°C. Three different methods were used to measure esterification of diacylglycerol.

Method 1, using labeled fatty acid, was based on an acyl-CoA generating system (21, 22). The incubations were performed at 37°C in 200 μl of potassium phosphate buffer (0.1 M, pH 7.4) containing 1,2-dioleoylglycerol (150 μM) dissolved in ethanol (10%, v/v, final concentration), dithiothreitol (2 mM), ATP (2 mM), $MgCl_2$ (4 mM), CoA (0.2 mM), microsomes, and [^{14}C]fatty acid (0.5 μCi/ml, 100 μM) bound to defatted bovine serum albumin (90 μM).

Method 2, using preformed labeled acyl-CoA (23), was performed as described elsewhere (24). Enzyme activity was assayed at 23°C in 500 μl of Tris-buffer (175 mM, pH 7.8) containing 1,2-dioleoylglycerol (125 μM) dissolved in ethanol (10%, v/v, final concentration), bovine serum albumin (15 μM), $MgCl_2$ (8 mM), microsomes, and [^{14}C]acyl-CoA.

Method 3, using labeled diacylglycerol, was performed as outlined in Method 2. 1,2-Di[1-^{14}C]oleoylglycerol was prepared from 1,2-di[1-^{14}C]oleoyl-*sn*-glycerophosphocholine by treatment with purified phospholipase C as described elsewhere (25). More than 90% of the labeled phosphatidylcholine was converted to 1,2-dioleoylglycerol by this method. The dioleoylglycerol was isolated and purified by thin-layer chromatography (n-hexane–diethyl ether–acetic acid 80:20:1, (v/v/v), redissolved in absolute ethanol and stored at −70°C. Greater than 98% of the ^{14}C-labeled material migrated similar to 1,2-dioleoylglycerol. Acyl-CoA:1,2-diacylglycerol acyltransferase activity measured by this method closely agreed with method 2, and was linear with protein up to 20 μg and with time up to 15 min when oleoyl-CoA was used as substrate (data not shown). The incubations were terminated by adding 20 volumes of chloroform–methanol 2:1 (v/v) (19). Serum (20 μl) was added to supply triacylglycerol as unlabeled carrier. After extraction, the residual lipids were redissolved in n-hexane and applied to TLC plates. Radioactivity in triacylglycerol was measured by liquid scintillation spectrometry.

Presentation of results

Results are presented as mean ± SD of at least triplicate samples representative for three or more separate experiments. Radioactivity incorporated into triacylglycerol (and other lipid classes) was divided by the specific radioactivity of the precursor in the incubation medium. The counting efficiencies for ^{3}H and ^{14}C were 54 and 80%, respectively, and quenching was similar in all experiments.

Statistics

The data were analyzed by the Mann-Whitney non-parametric test (one-tailed) (MINITAB statistical program, Minitab, Inc., State College, PA). Linear least squares analysis was determined using MINITAB.

RESULTS

Effects of different fatty acids on synthesis and secretion of triacyl[^{3}H]glycerol

We first examined the effect of different fatty acids on the incorporation of [^{3}H]glycerol into synthesized and secreted triacylglycerol. Oleic (18:1, n–9), palmitic (16:0), and linoleic (18:2, n–6) acids had a stimulatory effect (300–600%) on synthesis and secretion of triacyl[^{3}H]glycerol as compared to fatty acid-free albumin (**Fig. 1**). On the other hand, eicosapentaenoic acid (20:5, n–3) promoted only a very small and insignificant increase in triacylglycerol synthesis and secretion as compared to albumin. Stearic (18:0), α-linolenic (18:3, n–3), γ-linolenic (18:3, n–6), arachidonic (20:4, n–6), erucic (22:1, n–9), and docosahexaenoic (22:6, n–3) acids increased synthesis as well as secretion of triacylglycerol by 200–300%. A significant linear relationship ($r = 0.85$, $P < 0.01$) was found between synthesis and secretion of triacylglycerol for the fatty acids examined (Fig. 1, insert). Since oleic and eicosapentaenoic acids had the most extreme effects on synthesis and secretion of triacylglycerol, we focused our further studies on these two fatty acids.

Mass quantitation and fatty acid pattern of cellular and secreted triacylglycerol

Oleic acid was incorporated into cellular and secreted triacylglycerol to a larger extent than eicosapentaenoic acid as determined by gas–liquid chromatography (**Table 1**). Compared to oleic acid, eicosapentaenoic acid decreased cell-associated and secreted triacylglycerol by approximately 45%. Eicosapentaenoic acid

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH

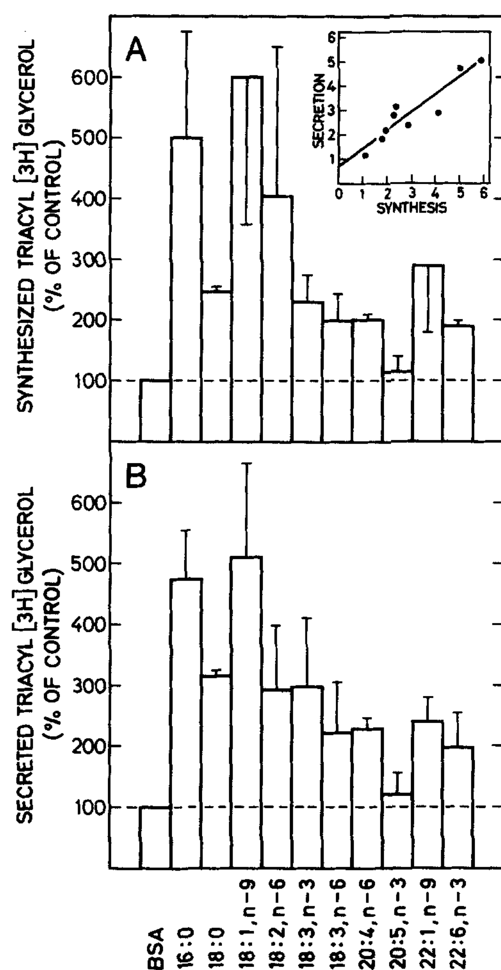

Fig. 1 Effect of different fatty acids on synthesis (A) and secretion (B) of triacyl[^{3}H]glycerol. Hepatocytes were incubated in Dulbecco's modified Eagle's medium containing glucose (12.5 mM), [^{3}H]glycerol (5 μCi/ml, 25 μM), bovine serum albumin (0.24 mM), and one of the indicated fatty acids (0.6 mM). The ratio of fatty acids to albumin was 2.5:1. After 2 hr incubation, medium and cells were harvested and analyzed for triacyl[^{3}H]glycerol. Results are presented as mean ± SD of 6 to 18 individually handled dishes, where control values (BSA, bovine serum albumin) are in the range 27–43 and 2.6–4.8 pmol/mg cell protein for synthesized and secreted triacyl[^{3}H]glycerol, respectively. All values, except for eicosapentaenoic acid, are different from control values at $P<0.006$. Insert: Linear correlation between synthesis and secretion of triacylglycerol. Regression equation: Secretion = 73.7 + 0.721; Synthesis, $r=0.85$, $P<0.01$. Values are given as % × 1/100.

increased cellular content of triacylglycerol by 60%, whereas secretion was slightly reduced (10%), when compared to a fatty acid-free control (albumin, 0.24 mM). On the other hand, oleic acid increased synthesis and secretion of triacylglycerol to 282 and 165% of control, respectively (data not shown).

The amount of palmitic (16:0), stearic (18:0), oleic (18:1, n–9), *cis*-vaccenic (18:1, n–7), and linoleic (18:2, n–6) acids in cell-associated triacylglycerol was significantly lower in the presence of eicosapentaenoic acid than in the presence of oleic acid (Table 1). The fatty acid pattern of secreted triacylglycerol reflected the pattern of cellular triacylglycerol. Eicosapentaenoic acid promoted a significant reduction for palmitic (16:0), palmitoleic (16:1, n–7), stearic (18:0), and oleic (18:1, n–9) acids in secreted triacylglycerol as compared to oleic acid (Table 1).

Effect of the ratio between fatty acids and albumin

At 0.6 mM fatty acid bound to 1.2 mM albumin (ratio = 0.5:1), oleic acid promoted higher synthesis (160%) and secretion (40%) of triacylglycerol than did eicosapentaenoic acid (**Fig. 2**). As compared to 1.2 mM albumin alone, oleic acid increased synthesis of triacylglycerol by 130%, whereas eicosapentaenoic acid had no significant effect. When the concentration of albumin was reduced while fatty acid concentration was kept constant (0.6 mM), oleic acid promoted a much higher incorporation of [^{3}H]glycerol into cellular and secreted triacylglycerol than eicosapentaenoic acid. At a binding ratio of fatty acid to albumin of 7.5:1, oleic acid increased synthesis and secretion of triacylglycerol 18- and 10-fold, respectively, whereas eicosapentaenoic acid increased synthesis 2-fold and showed only a slight increase in secreted triacylglycerol.

Uptake of radiolabeled eicosapentaenoic and oleic acid

Eicosapentaenoic and oleic acids (0.6 mM) were taken up by the hepatocytes to the same extent (**Fig. 3**). Cellular uptake of [^{14}C]oleic acid (0.6 mM) was not affected by the presence of unlabeled eicosapentaenoic acid (0.6 mM) (Fig. 3 B).

Distribution of radiolabeled eicosapentaenoic and oleic acids in cell-associated and medium lipids

Labeled fatty acids were used to examine intracellular metabolism. The incorporation of [^{14}C]eicosapentaenoic acid into cellular phospholipid was increased by 90%, whereas incorporation into cholesteryl ester was 50% lower relative to [^{14}C]oleic acid (**Table 2**). The incorporation of eicosapentaenoic acid and oleic acids into diacylglycerol and triacylglycerol was similar. Cell-associated free eicosapentaenoic acid was 110% higher than oleic acid (Table 2). There was no significant difference between eicosapentaenoic and oleic acids in medium phospholipid, triacylglycerol, and acid-soluble radioactivity (Table 2). Most of the radioactivity added to the hepatocytes was found in the medium free fatty acids (66–71%); results for eicosapentaenoic and oleic acid were similar. [^{14}C]Eicosapentaenoic acid was incorporated into triacylglycerol to the same extent as [^{14}C]oleic acid. This is inconsistent with data obtained by mass measurement (Table 1) and incorporation of [^{3}H]glycerol

Downloaded from www.jlr.org by guest, on June 18, 2012
ASBMB
JOURNAL OF LIPID RESEARCH

TABLE 1. Fatty acid pattern of cellular and secreted triacylglycerol

Fatty Acid	Cellular Triacylglycerol		Secreted Triacylglycerol	
	Eicosapentaenoic Acid	Oleic Acid	Eicosapentaenoic Acid	Oleic Acid
	nmol fatty acid/mg cell protein			
16:0	31.4±4.8	46.5±3.1*	2.52±0.38	4.90±0.38*
16:1 (n−7)	11.7±1.3	14.1±3.1	0.59±0.21	1.07±0.21*
18:0	8.3±1.0	12.1±0.7*	5.66±1.20	9.00±0.93*
18:1 (n−9)	32.4±5.9	162.7±4.1*	1.59±0.41	5.76±0.38*
18:1 (n−7)	9.3±1.7	16.2±1.7*	0.93±0.24	1.07±0.10
18:2 (n−6)	35.9±6.6	46.8±2.4*	0.48±0.24	1.21±0.75
20:3 (n−6)	1.0±0.3	1.0±0.3	nd	nd
20:4 (n−6)	8.3±1.3	8.3±0.7	nd	nd
20:5 (n−3)	35.9±5.5	2.8±0.3*	0.24±0.17	nd
22:6 (n−3)	9.3±2.4	10.6±0.7	nd	nd
Total	183.5	321.1	11.77	22.92

Hepatocytes were incubated in Dulbecco's modified Eagle's medium containing glucose (12.5 mM), bovine serum albumin (0.24 mM), and eicosapentaenoic (0.6 mM) or oleic (0.6 mM) acid. The fatty acid–albumin ratio was 2.5:1. After incubation for 2 hr, the medium was discarded and the cells were reincubated in identical medium for 4 hr. The fatty acid pattern of triacylglycerol was determined by gas–liquid chromatography. Data represent mean ± SD from six individual culture dishes. Asterisks (*) show differences from eicosapentaenoic acid at $P<0.006$; nd, not detectable.

(Figs. 1 and 2), and may be explained by different pool sizes of these fatty acids in the hepatocytes. We found that there was about a 10-fold higher content of oleic acid compared to eicosapentaenoic acid in cell-associated triacylglycerol of hepatocytes incubated with 0.24 mM albumin only (data not shown). This means that added [^{14}C]oleic acid is diluted more (lower specific activity) than added [^{14}C]eicosapentaenoic acid.

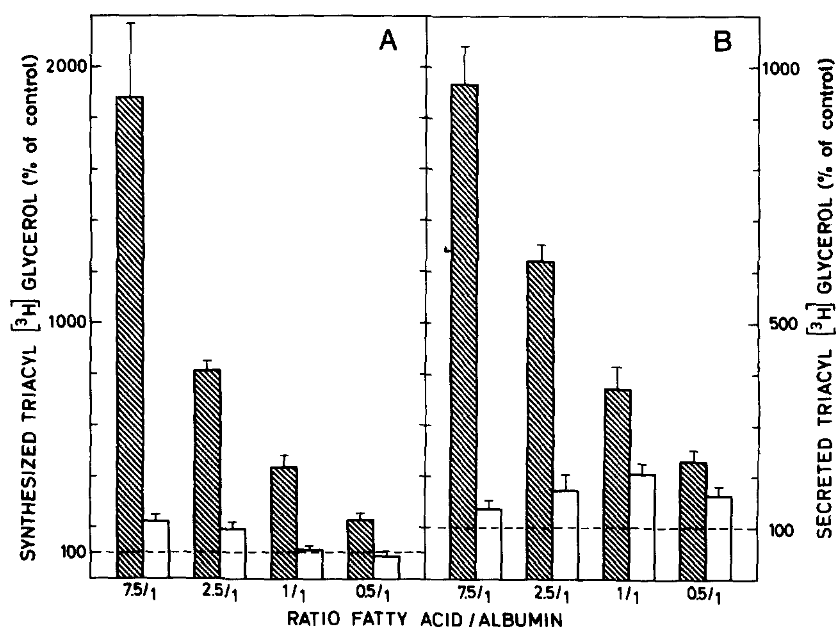

Fig. 2. Effect of increasing ratio of free fatty acid to albumin on synthesis (A) and secretion (B) of triacyl[^{3}H]glycerol. Hepatocytes were incubated in Dulbecco's modified Eagle's medium containing glucose (12.5 mM), [^{3}H]glycerol (5 μCi/ml, 25 μM), eicosapentaenoic or oleic acid (0.6 mM) and various concentrations of bovine serum albumin (range 0.08–1.2 mM) for 2 hr. Open bars represent eicosapentaenoic acid, hatched bars represent oleic acid. Data are presented as mean ± SD of six individually handled dishes. All values for eicosapentaenoic and oleic acids are different at $P<0.006$. Control values (pmol/mg cell protein) are:

Bovine serum albumin concentration mM	Triacyl[^{3}H]glycerol Synthesized	Triacyl[^{3}H]glycerol Secreted	Ratio Fatty Acid:Albumin
0.08	30±2.5	7.3±0.2	7.5:1
0.24	26±2.0	5.3±0.5	2.5:1
0.60	29±1.6	3.9±0.8	1:1
1.20	31±3.7	2.6±0.4	0.5:1

Downloaded from www.jlr.org by guest, on June 18, 2012
JOURNAL OF LIPID RESEARCH
ASBMB

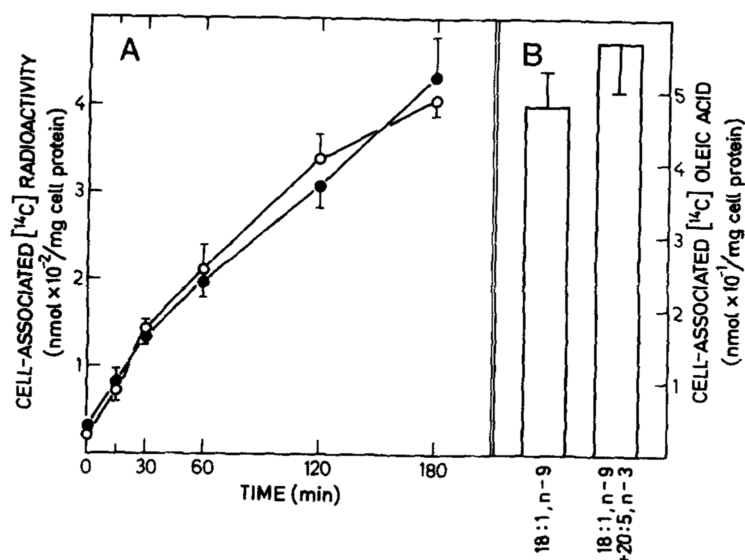

Fig. 3. Cellular uptake of eicosapentaenoic and oleic acid. Hepatocytes were incubated in Dulbecco's modified Eagle's medium containing glucose (12.5 mM), bovine serum albumin (0.08 mM), and either: A) [14C]eicosapentaenoic (0.05 μCi/ml, 0.6 mM) (○) or [14C]oleic acid (0.05 μCi/ml, 0.6 mM) (●), or: B) [14C]oleic acid (0.05 μCi/ml, 0.6 mM) with or without unlabeled eicosapentaenoic acid (0.6 mM) for 2 hr. Cellular content of acid-insoluble radioactivity (A) and [14C]oleic acid (B) was measured. The ratio between fatty acid and albumin was kept constant at 7.5:1.

Incorporation of [3H]water into cellular lipids

[3H]Water is incorporated into lipids through hydride transfer reduction. Thus, potential problems encountered with labeling precursor pools should be avoided, and [3H]water incorporation closely reflects actual rates of synthesis (26). Incorporation of [3H]water into cell-associated phospholipid was significantly higher (30%) in the presence of eicosapentaenoic acid as compared to oleic acid, whereas incorporation into cellular triacylglycerol and cholesteryl ester was markedly lower (40–60%) (**Table 3**). The lower incorporation of [3H]water into cellular triacylglycerol in the presence of eicosapentaenoic acid agreed with the data obtained by mass quantitiation (Table 1). Eicosapentaenoic acid had no effect on incorporation of [3H]water into cellular free fatty acid, monoacylglycerol, and diacylglycerol as compared to oleic acid (Table 3). These data suggest that eicosapentaenoic acid decreases triacylglycerol synthesis primarily by affecting the last step in formation of triacylglycerol. This possibility was further evaluated by studying acyl-CoA:1,2-diacylglycerol acyltransferase activity in isolated rat liver microsomes.

Activity of acyl-CoA:1,2-diacylglycerol acyltransferase

Microsomes to which were added 1,2-dioleoylglycerol incorporated 40–100% more [14C]oleic acid than

TABLE 2. Distribution of [14C]eicosapentaenoic acid and [14C]oleic acid in cells and medium

Cellular Lipids	Cellular Radioactivity: Eicosapentaenoic Acid	Cellular Radioactivity: Oleic Acid	Medium Radioactivity: Eicosapentaenoic Acid	Medium Radioactivity: Oleic Acid
	cpm/mg cell protein			
Free fatty acid	6,128 ± 1,014	2,896 ± 309*	112,455 ± 9,114	118,041 ± 12,348
Diacylglycerol	647 ± 74	691 ± 29	nd	nd
Phospholipid	10,613 ± 1,441	5,468 ± 779*	648 ± 279	353 ± 88
Triacylglycerol	30,282 ± 1,617	28,371 ± 3,381	1,176 ± 88	1,205 ± 59
Cholesteryl ester	279 ± 15	544 ± 74 *	nd	nd
TCA-soluble radioactivity	nd	nd	7,659 ± 280	7,556 ± 147
Recovered radioactivity			169,887 (96%)	165,125 (94%)

Hepatocytes were incubated for 2 hr in Dulbecco's modified Eagle's medium containing glucose (12.5 mM), bovine serum albumin (0.08 mM), and [14C]eicosapentaenoic acid or [14C]oleic acid (0.05 μCi/ml, 0.6 mM). The fatty acid–albumin ratio was 7.5:1. Asterisks (*) show differences from eicosapentaenoic acid at $P<0.03$; nd, not detectable.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

TABLE 3. Incorporation of [^{3}H]water into cellular lipids

Cellular Lipids	Eicosapentaenoic Acid	Oleic Acid
	nmol/mg cell protein	
Free fatty acid	14.2 ± 1.6	13.6 ± 0.5
Monoacylglycerol	8.7 ± 1.1	9.8 ± 1.6
Diacylglycerol	34.3 ± 3.3	38.6 ± 3.8
Phospholipid	62.7 ± 3.3	47.9 ± 2.2*
Triacylglycerol	100.8 ± 9.8	215.8 ± 7.1*
Cholesteryl ester	4.4 ± 0.5	8.2 ± 1.1*

Hepatocytes were incubated for 10 hr in Dulbecco's modified Eagle's medium containing glucose (12.5 mM), [^{3}H]water (2 mCi/ml), bovine serum albumin (0.08 mM), and eicosapentaenoic (0.6 mM) or oleic acid (0.6 mM). The fatty acid–albumin ratio was 7.5:1. Asterisks (*) show differences from eicosapentaenoic acid at $P<0.03$.

[^{14}C]eicosapentaenoic acid into triacylglycerol in the presence of ATP, CoA, Mg^{2+}, and dithiothreitol (DTT) (**Fig. 4**). In the absence of cofactors the enzyme activity was very low (Fig. 4C).

The content of labeled triacylglycerol in microsomes increased threefold in the presence of [^{14}C]oleoyl-CoA after 15 min of incubation or at the highest concentration of microsomes tested, compared to incubation with [^{14}C]eicosapentaenoic-CoA (**Figs. 5A and 5B**). When increasing the concentration of acyl-CoA, oleoyl-CoA increased triacylglycerol formation far more than eicosapentaenoyl-CoA did (**Fig. 6**). Triacylglycerol formation achieved saturation at 20–40 μM acyl-CoA. At these concentrations triacylglycerol formation was two- to threefold higher with oleoyl-CoA than with eicosapentaenoic-CoA (Fig. 6). Furthermore, unlabeled eicosapentaenoic-CoA decreased incorporation of [^{14}C]oleoyl-CoA into triacylglycerol (**Fig. 7**). By using 20 μM labeled oleoyl-CoA in the presence of increasing concentrations of unlabeled eicosapentaenoyl-CoA, there was noted a significant reduction of triacylglycerol synthesis already at 2 μM added eicosapentaenoyl-CoA. With 20 μM eicosapentaenoyl-CoA added to the incubation mixture, the enzyme activity was decreased to approximately 30% of the activity observed in the presence of oleoyl-CoA only. Similar observations were noted when using [^{14}C]1,2-dioleoylglycerol as labeled substrate (Fig. 7). At the highest concentration of eicosapentaenoyl-CoA examined (20 μM), microsomal triacylglycerol formation was reduced by 60%. The other acyl-CoA derivatives tested (palmitoyl-CoA, stearoyl-CoA, linoleoyl-CoA, linolenoyl-CoA, arachidonoyl-CoA) did not inhibit triacylglycerol formation in the presence of oleoyl-CoA (**Table 4**).

DISCUSSION

This study demonstrates that during short-term incubation with cultured rat hepatocytes, eicosapentaenoic acid promotes lower synthesis and secretion of triacylglycerol than many other fatty acids (Fig. 1). Oleic acid increased synthesis and secretion of triaclglycerol more than any other fatty acid examined (15, 27, 28). A linear correlation between synthesis and secretion was found, which suggests that triacylglycerol secretion is dependent on triacylglycerol synthesis (availability) within the hepatocyte (28).

It has previously been shown that eicosapentaenoic acid inhibits VLDL-triacylglycerol secretion by reducing triacylglycerol synthesis within the hepatocytes (14, 15). The fatty acid pattern of synthesized and secreted triacylglycerol showed that eicosapentaenoic acid was incorporated into triacylglycerol to a smaller degree

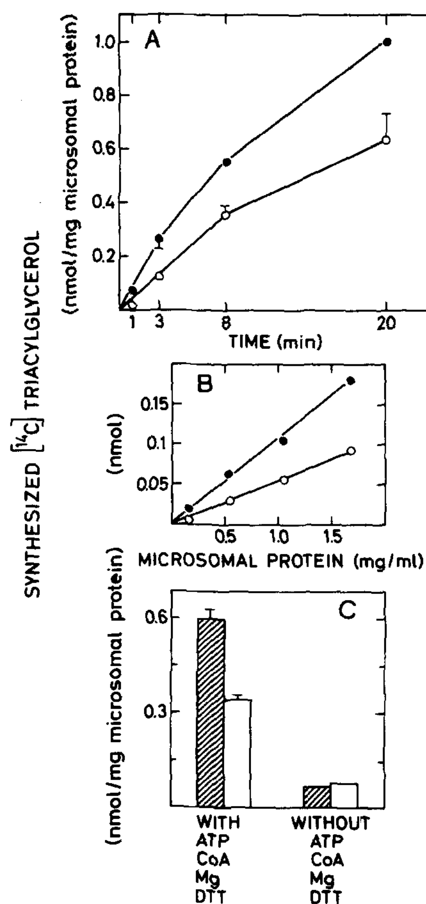

Fig. 4. Microsomal incorporation of [^{14}C]eicosapentaenoic and [^{14}C]oleic acid into triacylglycerol. Assays were performed using method 1. Microsomes were preincubated for 5 min at 37°C in potassium phosphate buffer (0.1 M) containing 1,2-dioleoylglycerol (150 μM), dithiothreitol (DTT)(2 mM), ATP (2 mM), $MgCl_2$ (4 mM) and CoA (0.2 mM) before [^{14}C]eicosapentaenoic (○) or [^{14}C]oleic acid (●) (0.5 μCi/ml, 100 μM) bound to bovine serum albumin (90 μM) was added. Panel A: Incubated with microsomal protein (1 mg/ml) for the indicated time periods. Panel B: Incubated with the indicated amounts of microsomal protein for 8 min. Panel C: Incubated with microsomal protein (1 mg/ml) with or without the cofactors for 8 min. Open bars represent eicosapentaenoic acid and hatched bars represent oleic acid.

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 18, 2012

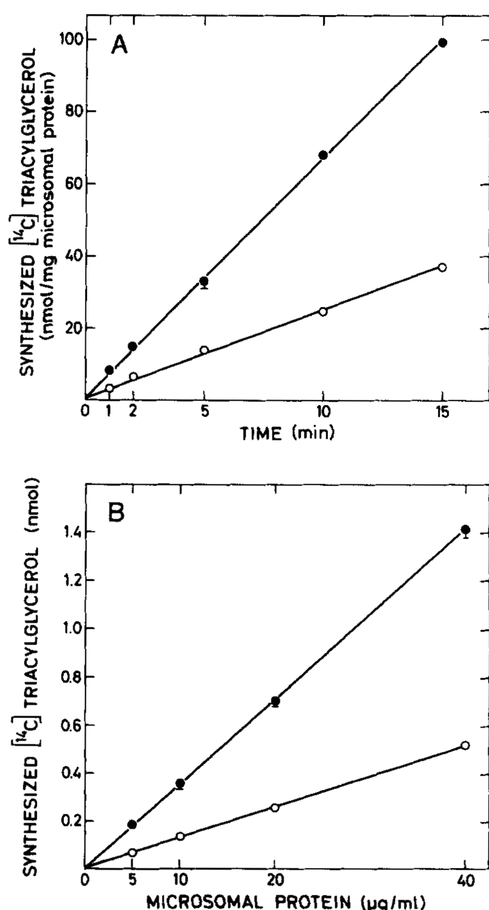

Fig. 5. Activity of acyl-CoA:diacylglycerol acyltransferase in microsomes incubated with [^{14}C]eicosapentaenoyl-CoA (○) and [^{14}C]oleoyl-CoA (●). Assays were performed using method 2. Panel A: After preincubation for 5 min in Tris-buffer (175 mM) containing bovine serum albumin (15 µM), $MgCl_2$ (8 mM) and [^{14}C]acyl-CoA (0.056 µCi/ml, 30 µM), microsomes (10 µg/ml) and 1,2-dioleoylglycerol (125 µM) were added and the mixtures were reincubated for different time periods. Panel B: After 5 min preincubation, different amounts of microsomal protein were added to Tris-buffer containing the components indicated under A, and reincubated for 10 min.

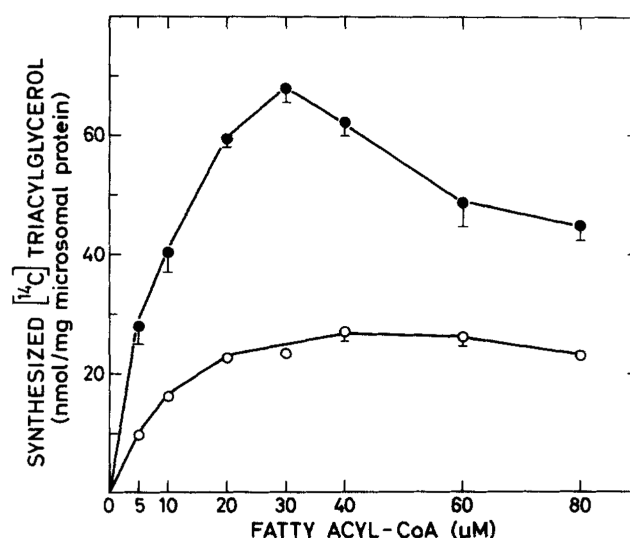

Fig. 6. Effect of [^{14}C]eicosapentaenoyl-CoA (○) and [^{14}C]oleoyl-CoA (●) concentration on acyl-CoA:diacylglycerol acyltransferase activity. Assays were performed using method 2. The incubation mixture contained Tris-buffer (175 mM), bovine serum albumin (15 µM), $MgCl_2$ (8 mM), and different amounts of [^{14}C]acyl-CoA. After 5 min preincubation, microsomes (10 µg/ml) and 1,2-dioleoylglycerol (125 µM) were added and the incubation was continued for 10 min.

The observed effects of eicosapentaenoic and oleic acid on triacylglycerol synthesis and secretion depended on the ratio between the fatty acids and albumin in the incubation mixture (Fig. 2). At a molar ratio of

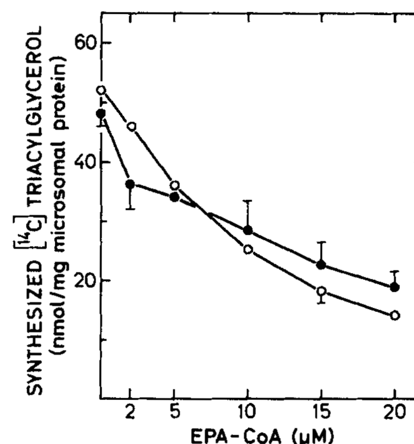

Fig. 7. Effect of eicosapentaenoyl-CoA on acyl-CoA:diacylglycerol acyltransferase activity in the presence of oleoyl-CoA. Assays were performed using either method 2 (○) or method 3 (●). Method 2: the incubation mixture contained Tris-buffer (175 mM), bovine serum albumin (15 µM), $MgCl_2$ (8 mM), [^{14}C]oleoyl-CoA (0.1 µCi/ml, 20 µM) and different concentrations of unlabeled eicosapentaenoyl-CoA. After 5 min preincubation, microsomes (10 µg/ml) and 1,2-dioleoylglycerol (125 µM) were added and the incubation was continued for 10 min. Method 3: the incubation mixture contained Tris-buffer (175 mM), bovine serum albumin (15 µM), $MgCl_2$ (8 mM), oleoyl-CoA (20 µM), and different concentrations of eicosapentaenoyl-CoA. After 5 min preincubation, microsomes (10 µg/ml) and [^{14}C]1,2-dioleoylglycerol (0.11 µCi/ml, 125 µM) were added and incubation was continued for 10 min.

than oleic acid (Table 1). Furthermore, eicosapentaenoic acid caused lower incorporation of some fatty acids with 16 and 18 carbon atoms into triacylglycerol as compared to oleic acid. These observations indicate that eicosapentaenoic acid itself is not a good promoter of triacylglycerol synthesis, and indeed inhibits incorporation of other fatty acids into triacylglycerol. It has previously been shown that eicosapentaenoic acid decreases the stimulatory effect of oleic acid on synthesis and secretion of triacylglycerol (14). These observations may explain the reduced production of triacylglycerol in the presence of eicosapentaenoic acid. Moreover, compared to oleic acid, a higher amount of unesterified eicosapentaenoic acid accumulated intracellularly, indicating that this fatty acid is not as rapidly metabolized as oleic acid (Table 2).

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH

TABLE 4. Activity of acyl-CoA:diacylglycerol acyltransferase in microsomes incubated with oleoyl-CoA in the presence of different acyl-CoA derivatives

Acyl-CoA	Synthesized Triacylglycerol
	nmol/mg microsomal protein
Control	45.1 ± 4.5
Palmitoyl-CoA (16:0)	43.8 ± 1.2
Stearoyl-CoA (18:0)	40.6 ± 1.3
Oleoyl-CoA (18:1, n–9)	48.0 ± 4.1
Linoleoyl-CoA (18:2, n–6)	39.0 ± 2.3
Linolenoyl-CoA (18:3, n–3)	44.2 ± 6.4
Arachidonoyl-CoA (20:4, n–6)	39.5 ± 4.9
Eicosapentaenoyl-CoA (20:5, n–3)	20.5 ± 3.0*

Assays were performed using method 3. The incubation mixture contained Tris-buffer (175 mM), bovine serum albumin (15 μM), $MgCl_2$ (8 mM), oleoyl-CoA (20 μM) without (control) or with different acyl-CoA derivatives (20 μM). After 5 min preincubation, microsomes (10 μg/ml) and 1,2-di-[^{14}C]oleoylglycerol (0.11 μCi/ml, 125 μM) were added and incubation continued for 10 min. The asterisk (*) shows difference from control at $P<0.03$.

fatty acid to albumin in the physiological range of 0.5 to 2, when fatty acids are bound to high affinity binding sites on albumin (29, 30), there was a significant difference between the effects of eicosapentaenoic and oleic acids on triacylglycerol metabolism. When the flux of fatty acids into the cells was increased by a higher ratio of fatty acid to albumin and medium viscosity was reduced (31), oleic acid promoted a marked increase in synthesis and secretion of triacylglycerol, whereas eicosapentaenoic acid had little stimulatory effect. Although the difference between eicosapentaenoic and oleic acids on synthesis and secretion of triacylglycerol was smaller at a low binding ratio, the less stimulatory effect still persisted.

Since cellular uptake of oleic and eicosapentaenoic acid was similar, this could not explain the difference in synthesis of triacylglycerol (Fig. 3). No competition between these fatty acids during uptake was found. Furthermore, incorporation of [^{3}H]water into free fatty acids, monoacylglycerol, and diacylglycerol was similar for eicosapentaenoic and oleic acid, suggesting that synthesis and activation (acyl-CoA synthetase) of fatty acids was not impaired by eicosapentaenoic acid (Table 3). In addition, incorporation of tritiated water into cellular phospholipid, which also depends on acyl-CoA, was increased in the presence of eicosapentaenoic acid (Table 3). Increased phospholipid synthesis in the presence of eicosapentaenoic acid has previously been observed (15), and it may be due to reduced triacylglycerol synthesis and/or increased lysophospholipid acylation.

The observations discussed so far may suggest that eicosapentaenoic acid primarily affects the final step of the triacylglycerol synthetic pathway. This reaction involves the conversion of 1,2-diacylglycerol into triacylglycerol catalyzed by acyl-CoA:1,2-diacylglycerol acyltransferase. Incubation with isolated microsomes revealed that eicosapentaenoic acid is a poorer substrate for acyl-CoA:1,2-diacylglycerol acyltransferase than oleic acid. In the presence of microsomes, oleic acid was incorporated into triacylglycerol to a larger extent than eicosapentaenoic acid, both when the fatty acids were added as free fatty acids or as acyl-CoA (Figs. 4, 5, and 6). Eicosapentaenoyl-CoA also decreased microsomal triacylglycerol formation when given together with oleoyl-CoA (Fig. 7). This observation is in agreement with data from hepatocytes which show decreased triacylglycerol synthesis when eicosapentaenoic acid is given together with oleic acid (14), and may explain the reduced incorporation of some other fatty acids into triacylglycerol in the presence of eicosapentaenoic acid (Table 1). The other acyl-CoA derivatives examined had little or no effect on microsomal triacylglycerol formation when given in combination with oleoyl-CoA (Table 4). Although the fatty acid specificity of acyl-CoA synthetase is demonstrated in glomeruli (32) and liver microsomes (33) from rats, one might assume that production of acyl-CoAs is not the rate-limiting step in triacylglycerol synthesis, since the capacity of acyl-CoA synthetase is estimated to be 5- to 10-fold above the capacity of triacylglycerol production in our microsomes.

The results of this study suggest that eicosapentaenoic acid decreases hepatic triacylglycerol formation primarily by inhibiting the activity of microsomal acyl-CoA:1,2-diacylglycerol acyltransferase. Eicosapentaenoic acid itself is a poor substrate for this enzyme, and it decreases incorporation of other fatty acids (e.g., oleic acid) into triacylglycerol by inhibiting this reaction. Lower hepatic synthesis of triacylglycerol due to decreased activity of acyl-CoA:1,2-diacylglycerol acyltransferase (34) may be one important reason for the reduced VLDL secretion and lower plasma triacylglycerol levels observed when diets rich in marine polyunsaturated fatty acids are consumed.

We have recently shown that eicosapentaenoic acid also decreases cholesterol esterification in cultured rat hepatocytes and microsomes by reducing the activity of acyl-CoA:cholesterol acyltransferase. This subsequently causes reduced secretion of VLDL cholesteryl ester (35). Thus, it is possible that eicosapentaenoic acid decreases VLDL-cholesterol and VLDL-triacylglycerol via a similar mechanism by inhibiting enzymes responsible for synthesis of VLDL core lipids.

Special thanks are extended to Professor Harald Osmundsen for supplying CoA-derivatives of eicosapentaenoic acid. This investigation was supported by grants from Nansenfondet, Langfeldts fond, Nordisk Insulinfond, Familien Blix' fond, and the Norwegian Council on Cardiovascular Disease and the Norwegian Council of Science and the Humanities.

Manuscript received 28 October 1987 and in revised form 28 April 1988.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH

REFERENCES

1. Bang, H. O., J. Dyerberg, and A. B. Nielsen. 1971. Plasma lipid and lipoprotein pattern in Greenlandic west coast Eskimos. *Lancet.* **i:** 1143–1146.
2. Bang, H. O., J. Dyerberg, and N. Jørne. 1976. The composition of food consumed by Greenland Eskimos. *Acta Med. Scand.* **200:** 69–73.
3. von Lossonczy, T. O., A. Ruiter, H. C. Bronsgeest-Schoute, C. M. van Gent, and R. J. J. Hermus. 1978. The effect of a fish diet on serum lipids in healthy human subjects. *Am. J. Clin. Nutr.* **31:** 1340–1346.
4. Phillipson, B. E., D. W. Rothrock, W. E. Connor, W. S. Harris, and D. R. Illingworth. 1985. Reduction of plasma lipids, lipoproteins, and apoproteins by dietary fish oils in patients with hypertriglyceridemia. *N. Engl. J. Med.* **312:** 1210–1216.
5. Nestel, P. J., K. R. Carrol, and N. Havenstein. 1970. Plasma triglyceride response to carbohydrates, fats and caloric intake. *Metabolism.* **19:** 1–18.
6. Vega, G. L., E. Groszek, R. Wolf, and S. M. Grundy. 1982. Influence of polyunsaturated fats on composition of plasma lipoproteins and apolipoproteins. *J. Lipid Res.* **23:** 811–822.
7. Harris, W. S., W. E. Connor, and M. P. McMurry. 1983. The comparative reduction of the plasma lipids and lipoproteins by dietary polyunsaturated fats: salmon oil versus vegetable oils. *Metabolism.* **32:** 179–184.
8. Nestel, P. J., W. E. Connor, M. R. Reardon, S. Connor, S. Wong, and R. Boston. 1984. Suppression by diets rich in fish oil of very low density lipoprotein production in man. *J. Clin. Invest.* **74:** 82–89.
9. Spritz, N., and M. A. Mishkel. 1969. Effects of dietary fats on plasma lipids and lipoproteins: an hypothesis for the lipid-lowering effect of unsaturated fatty acids. *J. Clin. Invest.* **48:** 78–86.
10. Pawar, S., and H. C. Tidwell. 1968. Effect of ingestion of unsaturated fat on lipolytic activity of rat tissues. *J. Lipid Res.* **9:** 334–336.
11. Shepherd, J., C. J. Packard, S. M. Grundy, D. Yeshurun, A. M. Gotto, Jr., and O. D. Taunton. 1980. Effects of saturated and polyunsaturated fat diets on the chemical composition and metabolism of low density lipoproteins in man. *J. Lipid Res.* **21:** 91–99.
12. Harris, W. S., W. E. Connor, D. R. Illingworth, and D. M. Foster. 1984. The mechanism of the hypotriglyceridemic effect of dietary omega-3 fatty acids in man. *Clin. Res.* **32:** 560A (abstract).
13. Illingworth, D. R., W. S. Harris, and W. E. Connor. 1984. Inhibition of low density lipoprotein synthesis by dietary omega-3 fatty acids in humans. *Arteriosclerosis.* **4:** 270–275.
14. Nossen, J. Ø., A. C. Rustan, S. H. Gloppestad, S. Målbakken, and C. A. Drevon. 1986. Eicosapentaenoic acid inhibits synthesis and secretion of triacylglycerols by cultured rat hepatocytes. *Biochim. Biophys. Acta.* **879:** 56–65.
15. Wong, S., M. Reardon, and P. Nestel. 1985. Reduced triglyceride formation from long-chain polyenoic fatty acids in rat hepatocytes. *Metabolism.* **34:** 900–905.
16. Rustan, A. C., J. Ø. Nossen, T. Berg, and C. A. Drevon. 1985. The effects of monensin on secretion of very low density lipoprotein and metabolism of asialofetuin by cultured rat hepatocytes. *Biochem. J.* **227:** 529–536.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
18. Nossen, J. Ø., A. C. Rustan, T. Barnard, and C. A. Drevon. 1984. Inhibition by chloroquine of the secretion of very low density lipoproteins by cultured rat hepatocytes. *Biochim. Biophys. Acta.* **803:** 11–20.
19. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497-509.
20. Hashimoto, S., C. A. Drevon, D. B. Weinstein, J. S. Bernett, S. Dayton, and D. Steinberg. 1983. Activity of acyl-CoA:cholesterol acyltransferase and 3-hydroxy-3-methylglutaryl-CoA reductase in subfractions of hepatic microsomes enriched with cholesterol. *Biochim. Biophys. Acta.* **754:** 126–133.
21. Drevon, C. A., and K. Norum. 1975. Cholesterol esterification and lipids in plasma and liver from newborn and young guinea pigs raised on milk and non-milk diet. *Nutr. Meta.* **18:** 137–151.
22. Balasubramaniam, S., K. A. Mitropoulos, and S. Venkatesan. 1978. Rat-liver acyl-CoA:cholesterol acyltransferase. *Eur. J. Biochem.* **90:** 377–383.
23. Kawaguchi, A., T. Yoshimura, and S. Okuda. 1981. A new method for the preparation of acyl-CoA thioesters. *J. Biochem. (Tokyo)* **89:** 337–339.
24. Coleman, R., and R. M. Bell. 1976. Triacylglycerol synthesis in isolated fat cells: studies on the microsomal diacylglycerol acyltransferase activity using ethanol-dispersed diacylglycerols. *J. Biol. Chem.* **251:** 4537–4543.
25. Mavis, R. D., R. M. Bell, and P. R. Vagelos. 1972. Effect of phospholipase C hydrolysis of membrane phospholipids on membrane enzymes. *J. Biol. Chem.* **247:** 2835–2841.
26. Davis, R. A., M. Malone McNeal, and R. L. Moses. 1982. Intrahepatic assembly of very low density lipoprotein: competition by cholesterol ester for the hydrophobic core. *J. Biol. Chem.* **257:** 2634–2640.
27. Kohout, M., B. Kohoutova, and M. Heimberg. 1971. The regulation of hepatic triglyceride metabolism by free fatty acids. *J. Biol. Chem.* **246:** 5067–5074.
28. Davis, R. A., and J. R. Boogaerts. 1982. Intrahepatic assembly of very low density lipoproteins: effect of fatty acids on triacylglycerol and apolipoprotein synthesis. *J. Biol. Chem.* **257:** 10908–10913.
29. Spector, A. A. 1975. Fatty acid binding to plasma albumin. *J. Lipid Res.* **16:** 165–179.
30. Kragh-Hansen, U. 1981. Molecular aspects of ligand binding to serum albumin. *Pharmacol. Rev.* **33:** 17–53.
31. Davis, R. A., S. C. Engelhorn, D. B. Weinstein, and D. Steinberg. 1980. Very low density lipoprotein secretion by cultured rat hepatocytes. Inhibition by albumin and other macromolecules. *J. Biol. Chem.* **255:** 2039–2045.
32. Morisaki, N., T. Kanzaki, Y. Saito, and S. Yoshida. 1986. Fatty acid specificity of acyl-CoA synthetase in rat glomeruli. *Biochim. Biophys. Acta.* **875:** 311–315.
33. Normann, P. T., M. S. Thomassen, E. N. Christiansen, and T. Flatmark. 1981. Acyl-CoA synthetase activity of rat liver microsomes. Substrate specificity with special reference to very-long-chain and isomeric fatty acids. *Biochim. Biophys. Acta.* **664:** 416–427.
34. Strum-Odin, R., B. Adkins-Finke, W. L. Blake, S. D. Phinney, and S. D. Clarke. 1987. Modification of fatty acid composition of membrane phospholipid in hepatocyte monolayer with n–3, n–6 and n–9 fatty acids and its relationship to triacylglycerol production. *Biochim. Biophys. Acta.* **921:** 378–391.
35. Rustan, A. C., J. Ø. Nossen, H. Osmundsen, and C. A. Drevon. 1988. Eicosapentaenoic acid inhibits cholesterol esterification in cultured parenchymal cells and isolated microsomes from rat liver. *J. Biol. Chem.* **263:** 8126–8132.

Downloaded from www.jlr.org by guest, on June 18, 2012