Differential effects of eicosapentaenoic acid and oleic acid on lipid synthesis and secretion by HepG2 cells

Reynold Homan,[1] James E. Grossman, and Henry J. Pownall[2]

Baylor College of Medicine and The Methodist Hospital, M.S. A-601, Houston, TX 77030

Abstract The effects of eicosapentaenoic acid and oleic acid on lipid synthesis and secretion by HepG2 cells were examined to identify fatty acid specific changes in lipid metabolism that might indicate a basis for the hypolipidemic effect attributed to eicosapentaenoic acid and related n–3 fatty acids. Cellular glycerolipid synthesis, as determined by [3H]glycerol incorporation, increased in a concentration-dependent manner in cells incubated 4 h with either eicosapentaenoic acid or oleic acid at concentrations between 10 and 300 μM. [3H]Glycerol-labeled triglyceride was the principal lipid formed and increased approximately fourfold with the addition of 300 μM oleic acid or eicosapentaenoic acid. Both fatty acids also produced a 20–40% increase in the total cellular triglyceride mass. Although both fatty acids increased triglyceride synthesis to similar extents, eicosapentaenoic acid-treated cells secreted 40% less [3H]glycerol-labeled triglyceride than cells fed oleic acid. Cellular synthesis of [3H]glycerol-labeled phosphatidylethanolamine and phosphatidylcholine was also reduced by 40% and 30%, respectively, in cells given eicosapentaenoic acid versus cells given oleic acid. Similar results were obtained in determinations of radiolabeled oleic acid and eicosapentaenoic acid incorporation. At a fatty acid concentration of 300 μM, incorporation of radiolabeled eicosapentaenoic acid into cellular triglycerides was greater than the incorporation obtained with radiolabeled oleic acid, while the reverse relationship was observed for the formation of phosphatidylcholine from the same fatty acids. ■ Eicosapentaenoic acid is as potent as oleic acid in inducing triglyceride synthesis but eicosapentaenoic acid is a poorer substrate than oleic acid for phospholipid synthesis. The intracellular rise in de novo-synthesized triglyceride in eicosapentaenoic acid-treated cells without corresponding increases in triglyceride secretion suggests that eicosapentaenoic acid is less effective than oleic acid in promoting the transfer of de novo-synthesized triglyceride to nascent very low density lipoproteins. —**Homan, R., J. E. Grossman, and H. J. Pownall.** Differential effects of eicosapentaenoic acid and oleic acid on lipid synthesis and secretion by HepG2 cells. *J. Lipid Res.* 1991. **32:** 231–241.

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Epidemiological studies initially revealed that populations consuming diets rich in fish oils had lower incidences of vascular disease (1, 2). Eicosapentaenoic acid (EPA) and other related n–3 polyunsaturated fatty acids were proposed as the primary agents in fish oil responsible for the reduced risk of coronary heart disease. Subsequent clinical studies with purified preparations of n–3 fatty acids correlated the reversal of plasma lipid levels and thrombosis, both known risk factors for coronary heart disease, with n–3 fatty acid ingestion (recently reviewed in refs. 3 and 4). Marked decreases in the very low density lipoprotein (VLDL) pool of plasma triglyceride are reported for normolipidemic human subjects given n–3 fatty acids (5–7). The hypolipidemic effect is particularly dramatic in hypertriglyceridemic patients fed fish oil where up to fourfold reductions in plasma triglycerides are reported (8, 9). The biochemical basis for these effects remains unclear.

Fasting plasma triglyceride levels are controlled by the relative rates of VLDL secretion and catabolism. Fatty acids stimulate VLDL synthesis and secretion in a manner dependent on the amounts and types of fatty acids available. Triglyceride secretion in perfused rat liver (10) and by rat hepatocytes in culture (11) decreases as the number of double bonds in the fatty acids administered is increased. Thus, the unique lipid lowering effects of the polyunsaturated n–3 fatty acids may derive from decreased hepatic synthesis of VLDL. Evidence obtained from animal model studies indicates that n–3 fatty acids inhibit VLDL synthesis by reducing the synthesis of the component lipids required for VLDL formation. Com-

parisons of oleic acid (OA)- and EPA-dependent lipid metabolism in primary cultured rat hepatocytes (12, 13) and perfused rat liver (14) have shown that EPA inhibits triglyceride synthesis and secretion under conditions where oleic acid stimulates these processes. Observations that n–3 fatty acids enhance fatty acid oxidation and keto-genesis (15, 16) as well as inhibit fatty acid synthesis (17) suggest that n–3 fatty acids reduce the amount of fatty acid substrate available for lipogenesis. Reports of EPA-dependent reductions in the activities of phosphatidic acid phosphatase (14) and acyl-coenzyme A:diacylglycerol acyltransferase (18, 19) in rat hepatocytes suggest that these enzymes, which catalyze successive steps in the final stages of triglyceride synthesis, may be loci of the inhibitory effect. Additionally, it is reported that the synthesis of cholesteryl esters, which also comprise part of the neutral lipid core of lipoproteins, is reduced in the presence of EPA (20, 21).

The present study seeks to obtain biochemical data of similar scope from an experimental cell model of human liver function. The HepG2 cell line, which is derived from a human hepatic carcinoma (22), was used for this purpose. Previous studies have shown that the HepG2 cell line retains many of the normal functions of human liver parenchymal cells, including bioregulatable lipoprotein synthesis and secretion (23, 24). In this report, fatty acid-dependent lipogenesis and lipid secretion in HepG2 cells challenged with OA and EPA is examined to determine the lipid synthetic patterns associated with the administration of these fatty acids.

EXPERIMENTAL PROCEDURES

Materials

Oleic acid (99%), eicosapentaenoic acid (90 + %), and bovine serum albumin (Fraction V, essentially fatty acid-free) were purchased from Sigma Chemical Co. (St. Louis, MO). The purity of the fatty acids was verified by gas chromatography (GC). Docosahexaenoic acid (n–3) was the primary contaminant of EPA [1,2,3-^{3}H]Glycerol (200 mCi/mmol) and [5,6,8,9,11,12,14,15,17,18-^{3}H]eicosa-pentaenoic acid (79 Ci/mmol) were obtained from Dupont-New England Nuclear (Boston, MA). [1-^{14}C]Oleic acid (52 mCi/mmol) came from Amersham (Arlington Heights, IL). Dulbecco's modified Eagle's medium (DMEM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and HPLC grade solvents were supplied by Fisher Scientific Co. (Houston, TX). Fetal bovine serum and trypsin solution were ordered from GIBCO (Grand Island, NY). Gentamicin was obtained from Flow Laboratories, Inc. (McLean, VA).

HepG2 cell culture

HepG2 cells (Amercian Type Tissue Culture Collection, Rockville, MD) were grown in DMEM supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml). The cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The media were renewed every 72 h. The cells were routinely replated at 1:4 dilution every 6 to 7 days. Cell experiments were performed 6 days after replating.

Incubation of cells with fatty acid:BSA complexes

Preceding the experimental incubations, the growth media were removed from the cells and the monolayers were incubated for 30 min with DMEM containing 60 μM BSA. Cell incubations were carried out in DMEM (50 μg/ml gentamicin) containing either BSA alone or BSA complexed with OA or EPA at a BSA to fatty acid mole ratio of 1:5 as specified.

To form the BSA:fatty acid complexes, aliquots of 100 mM OA or EPA in ethanol were added to an aqueous solution of 0.1 N KOH containing 1.8 mol-equivalents of KOH per mol of fatty acid. The ethanol was removed under reduced pressure and the fatty acid salts were resuspended in the desired volume of DMEM containing BSA. Brief vortexing and bath sonication under nitrogen dispersed the fatty acids into solution. The ethanolic fatty acid stock solutions for experiments with radiolabeled OA and EPA contained 100 mM ^{14}C-labeled OA (0.12 μCi/μmol) and ^{3}H-labeled EPA (0.5 μCi/μmol), respectively. For experiments examining [^{3}H]glycerol uptake, [1,2,3^{3}H]-glycerol was added directly to the media without carrier. Media for experiments in which only cellular lipid labeling was examined contained 2 μCi ^{3}H per ml. When ^{3}H labeling of secreted lipids was studied, the media contained 4 μCi of ^{3}H per ml.

Lipid extraction and analysis

After incubation, the culture dishes with cells were placed on ice, the media were removed, and the cell monolayers were washed several times with cold (4°C) HEPES-buffered saline (HBS) (150 mM NaCl, 5 mM HEPES, 5 mM EDTA, pH 7.4). Cell monolayers incubated with radiolabeled fatty acid were first washed with HBS containing 0.1% BSA. The cells were scraped from the culture dish in HBS and pelleted at 4°C by ten min of centrifugation at 250 g. The supernate was discarded and cell pellet was dispersed in 1 ml of HBS by bath sonication under nitrogen. An aliquot was removed for protein content assay by the procedure of Lowry et al. (25) using BSA as the standard.

The lipids in the remaining cell suspension were extracted with ethylacetate–acetone 2:1 containing 0.1% butylatedhydroxytoluene (26). The upper solvent phase

was collected and evaporated under a stream of nitrogen. Residual solvent was removed by vacuum. The dried lipid extracts were resuspended in *iso*octane–tetrahydrofuran 99:1. An aliquot was removed for determination of radio-isotope content and the remainder was analyzed by HPLC.

Media lipids were extracted by the same procedure. Media that included [³H]glycerol were first extensively dialyzed against HBS to remove the radiolabeled glycerol. These samples were then lyophilized and rehydrated to the volume necessary for solvent extraction.

Triglyceride and diglyceride were determined by the procedure of Cairns and Peters (27). Phospholipids were assayed according to the method of Rouser, Fleischer, and Yamamoto (28).

High-performance liquid chromatography

Radiolabel distribution among lipid species in the cell extracts was determined by HPLC. Fifty to 100 μl of sample was injected into a Hewlett-Packard 1090M high-performance liquid chromatograph (Avondale, PA) equipped with a silica column (Spherisorb, 3 μm, 100 mm × 4.6 mm, Phase Separations, Inc., Norwalk, CT). The lipids were eluted by a ternary solvent elution scheme, essentially as described by Christie (29). The column was kept at 35°C. Radiolabel in the column effluent was detected with an LKB-Wallac 1208 Betacord radioactivity monitor (LKB, Bromma, Sweden). The Betacord liquid scintillator pump and mixer combined column effluent with 1.5 volumes of Scintiverse-E (Fisher Scientific) upstream of the detector flow cell (1 ml volume). Scintillator quenching by the solvent gradient was calculated and used to correct peak areas as previously described (30).

A Spectra-Physics 800B liquid chromatograph (Spectra-Physics, Inc., Santa Clara, CA) equipped with a Waters radial compression module containing a Radial Pak 10-μm silica cartridge (5 mm i.d.) (Millipore-Waters Division, Amherst, MA) was used to isolate the individual lipid components in samples containing 1 to 2 mg of total cell lipids (30).

Fatty acid analysis

The fatty acid compositions of purified cellular lipids were determined by capillary GC. The fatty acids were transesterified with BF₃–methanol and methanolic base. The methyl esters were separated on a 30-meter Supel-cowax-10 capillary column (Supelco, Bellefonte, PA). The column temperature was increased from 180 to 245°C at 1.3°C/min. Methyl ester standards (Nu-Check Prep Inc., Elysian, MN) were routinely chromatographed to calibrate the column and detector. Heptadecanoic acid was used as an internal standard.

Data analysis

Statistical significance of differences between different treatments was calculated by the Student's t test. Values of $P < 0.05$ (double-tailed) were considered significant.

RESULTS

Effect of fatty acid on cellular lipid content

HepG2 cells were incubated for 4 h in the absence or presence of 300 μM OA or EPA to determine the dependence of cellular lipid composition on short-term exposure to these fatty acids. The concentration of fatty acid was chosen because it was within the range of free fatty acid concentrations found in normal human plasma (31) and was less than the millimolar levels of fatty acid reported to be cytotoxic to cells in culture (23). TG was the single most abundant lipid in HepG2 cells and was found to be 217 nmol/mg in cells incubated in fatty acid-free media (**Table 1**). Cellular TG rose to 264 nmol/mg protein and 306 nmol/mg protein with the addition of 300 μM OA and 300 μM EPA, respectively ($P < 0.05$ vs. albumin). The difference in TG content between OA- and

TABLE 1. Effect of oleic acid and eicosapentaenoic acid on HepG2 cell lipid composition

Albumin Complex	Lipid							
	TG	DG	CE	Chol	PE	PI	PS	PC
	nmol/mg cell protein							
Fatty acid-free	217 ± 11	16 ± 4	63 ± 15	49 ± 2	32 ± 2	10 ± 2	11 ± 2	75 ± 7
Oleic acid	264 ± 23[a]	18 ± 4	47 ± 21	41 ± 1	31 ± 1	12 ± 1	10 ± 1	76 ± 3
Eicosapentaenoic acid	306 ± 10[b]	20 ± 3	51 ± 17	48 ± 3	33 ± 2	13 ± 0	9 ± 1	81 ± 4

HepG2 cells were incubated for 4 h in media containing fatty acid-free BSA (60 μM) or BSA complexed with 300 μM oleic acid or eicosapentaenoic acid. Cell lipids were extracted with ethyl acetate–acetone 2:1 and the component lipid species in the extracts were isolated by HPLC. The lipid content in each fraction was determined by the appropriate colorimetric assay, as described in Experimental Procedures. The results are reported as the mean ± SEM of three separate experiments.

[a]$P < 0.05$ relative to "Fatty acid-free".
[b]$P < 0.001$ relative to "Fatty acid-free".

EPA-treated cells was marginally significant ($P < 0.08$). No significant changes in the cellular content of any other lipid classes were detected.

Fatty acid composition of cellular lipids

The fatty acid compositions of the lipid classes in HepG2 cells were significantly modified by the 4-h exposure to EPA and OA (**Table 2**). The OA content of TG increased by 40% in cells given OA, and the OA content of PC rose by more than 50%. In contrast, no significant changes in the OA content of DG and PE were detected. With the exception of an increase in the palmitic acid content of cellular TG, OA addition did not significantly change the amounts of other fatty acid species esterified to TG.

The analysis of cellular lipid fatty acid composition revealed that nearly 80% of the EPA incorporated into the lipids of HepG2 cells exposed to EPA was esterified as TG, while approximately 12% was incorporated into PC. EPA was also detected in DG and PE at mol percents similar to the values determined for TG and PC, respectively. Docosapentaenoic acid (22:5(n–3)) and docosahexaenoic acid (22:6(n–3)) were also detected in TG, DG, and PE isolated from cells fed EPA. While some of the docosahexaenoic acid may have been introduced as impurities in the stock EPA (see Experimental Procedures), the presence of docosapentaenoic acid in cellular lipids suggests chain elongation of EPA occurred as well. The presence of docosahexaenoic acid in the PE isolated from EPA-treated cells was not unique, since PE was enriched in polyunsaturated fatty acids, including docosahexaenoic acid, under all incubation conditions. Docosapentaenoic acid and docosahexaenoic acid were also detected in the PC from EPA-treated cells, but the amounts were less than 1 mol%. EPA addition also resulted in significant increases in the amounts of palmitic acid (16:0) and vaccenic acid (18:1(n–7)) esterified to TG.

TABLE 2. Fatty acid composition of lipids isolated from HepG2 cells incubated with oleic acid and eicosapentaenoic acid

Lipid	14:0	16:0	16:1 (n-7)	18:0	18:1 (n-9)	18:1 (n-7)	18:2 (n-6)	20:3 (n-6)	20:4 (n-6)	20:5 (n-3)	22:5 (n-3)	22:6 (n-3)
						nmol fatty acid/mg cell protein (*mol %*)						
Albumin												
TG	48.7	225.1	79.0	21.1	148.7	114.2						
	(7.5)	(34.6)	(12.1)	(3.2)	(22.8)	(17.5)						
DG	3.8	11.3	3.6	1.9	6.5	4.4	0.8					
	(11.3)	(34.9)	(11.2)	(5.9)	(20.0)	(13.6)	(2.6)					
PE	8.0	13.9	12.3	6.4	12.1	7.5	1.7	2.3	3.2			1.5
	(11.7)	(20.4)	(18.0)	(9.4)	(17.7)	(11.0)	(2.4)	(3.2)	(4.7)			(2.2)
PC	8.0	56.0	30.5	4.1	27.2	17.7						
	(5.5)	(38.4)	(20.9)	(2.8)	(18.6)	(12.1)						
18:1(n–9)												
TG	66.7	255.1[a]	96.5	22.7	210.6[a]	119.3						
	(8.5)	(32.6)	(12.3)	(2.9)	(26.9)	(15.2)						
DG	2.3	11.7	3.6	2.2	9.2	5.4	0.5					
	(6.6)	(33.1)	(10.1)	(6.2)	(26.1)	(15.3)	(1.4)					
PE	0.4	9.6	7.9	7.2	17.4	8.8	2.1	2.2	4.1			1.4
	(1.0)	(15.7)	(12.8)	(11.7)	(28.4)	(14.4)	(3.5)	(3.5)	(6.7)			(2.3)
PC	6.1	54.1	24.3	3.4	42.7[a]	18.5	1.5					
	(4.0)	(35.7)	(16.0)	(2.3)	(28.1)	(12.2)	(1.0)					
20:5(n–3)												
TG	66.5	295.9[a]	108.6	24.5	160.3	149.3[a]				82.3[a]	18.6[a]	9.2[a]
	(7.3)	(32.4)	(11.9)	(2.7)	(17.5)	(16.3)				(9.0)	(2.0)	(1.0)
DG	2.4	13.3	3.7	2.3	7.3	5.8	0.4			4.0[a]	0.8[a]	0.4[a]
	(6.0)	(33.2)	(9.2)	(5.9)	(18.1)	(14.4)	(1.1)			(10.0)	(2.2)	(1.0)
PE	2.4	8.7	9.7	8.7	14.4	9.7	1.0	2.6	3.5	4.8[a]	0.8[a]	1.5
	(3.6)	(13.0)	(14.4)	(13.0)	(21.4)	(14.5)	(1.6)	(3.9)	(5.3)	(7.1)	(1.3)	(2.3)
PC	8.2	62.7	24.9	3.5	24.6	21.0	1.5			12.3[a]		
	(5.0)	(38.5)	(15.3)	(2.1)	(15.1)	(12.9)	(1.0)			(7.5)		

HepG2 cells were incubated for 4 h in Dulbecco's modified Eagle's medium containing 60 µM bovine serum albumin alone or complexed with 300 µM oleic acid (18:1(n–9)) or 300 µM eicosapentaenoic acid (20:5(n–3)). The cell lipids were then extracted and the individual lipid species were isolated by HPLC (see Experimental Procedures). Fatty acid methyl esters, derived from the purified lipids, were analyzed by gas chromatography. Data represent the mean of three separate experiments. The mole percent distribution of fatty acids is given in parentheses. Value less than 1 mol% are not shown.
[a]Indicates $P < 0.05$ relative to albumin.

Time course of radiolabeled fatty acid incorporation into cellular lipids

The kinetics of [14C]-labeled OA and [3H]-labeled EPA transport into HepG2 cells and cellular transformation of the labeled fatty acids to acylated lipid species was investigated to further distinguish differences in the utilization of these fatty acids for lipid synthesis. The data in **Fig. 1** demonstrate that both fatty acids entered the cells at similar rates and in a biphasic manner. After an initial rise in the first hour of incubation, the rate of label uptake declined but then increased again after 2 h. Both fatty acid labels were rapidly converted to acylated lipids, as demonstrated by the low levels of unesterified fatty acid label in the cells which never exceeded 5% of the total

label present at any time. The majority of fatty acid label was incorporated into TG, DG, PE, and PC. Both fatty acid labels were also converted to CE, PI, and PS in the cells; but, together with unesterified fatty acid label, these latter lipids accounted for less than 10% of the total label taken up by the cells.

Although the rates of fatty acid transport into cells were nearly identical, the time-dependent formation of acylated lipid products was distinct for each fatty acid label. During the first 2 h of incubation, the conversions of [14C]OA and [3H]EPA to acylated lipids were nearly indistinguishable. But after 2 h, the relative cellular distributions of the fatty acid labels diverged. At 4 h [3H]EPA incorporation into TG exceeded that of [14C]OA and, at
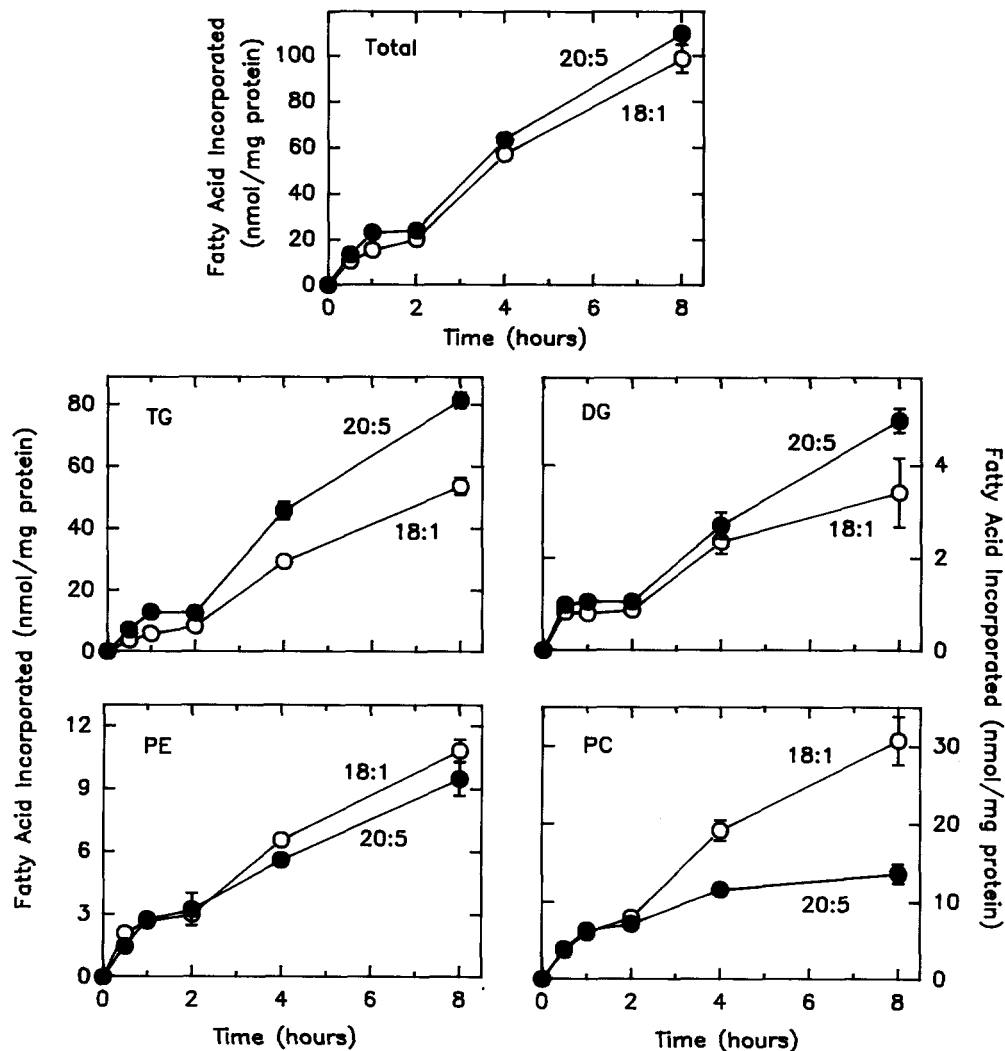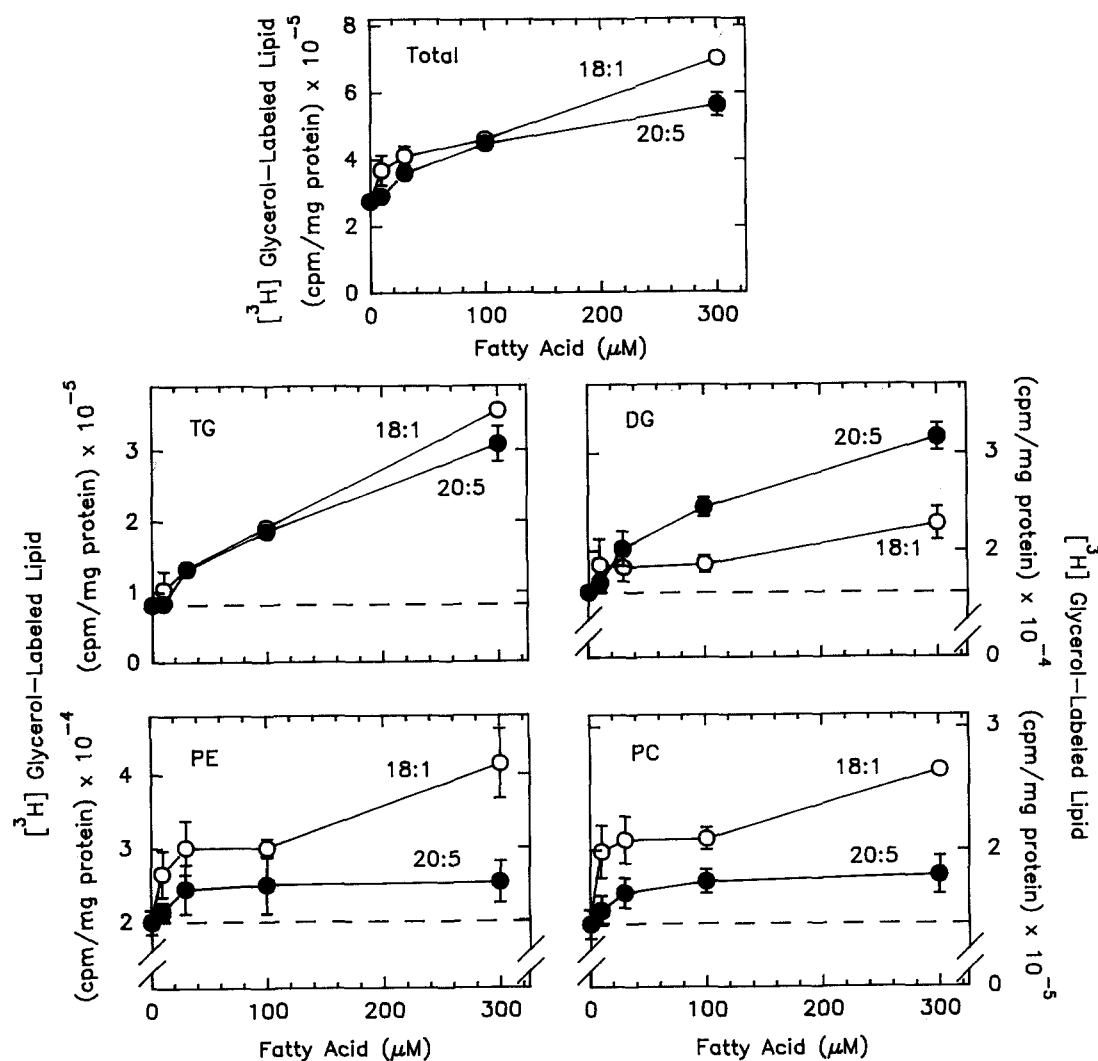

Fig. 1. Time course of [14C]oleic acid and [3H]eicosapentaenoic acid incorporation into cellular lipids. HepG2 cells were incubated in serum-free media containing 60 μM fatty acid-free bovine serum albumin complexed with 300 μM of either [1-14C]oleic acid (18:1) or [5,6,8,9,11,12,14,15,17,18-3H]eicosapentaenoic acid (20:5). At the times indicated, cells were harvested, the lipids were extracted, and the amounts of fatty acid label incorporated into TG, DG, PE, and PC were quantified by HPLC, as described in Experimental Procedures. The data are plotted as the mean ± SD for three separate incubations. Error bars smaller than the symbol size are not shown.

8 h, the amounts of [³H]EPA esterified to DG and TG exceeded those obtained for [¹⁴C]OA in the same lipids ($P < 0.003$ when fatty acid incorporations into TG at 8 h of incubation are compared). Conversely, the amount of [¹⁴C]OA esterified to PC was greater than the amount of [³H]EPA incorporated into the same lipid at 4 and 8 h of incubation ($P < 0.003$). The increases in [³H]EPA-labeled TG over [¹⁴C]OA-labeled TG at 4 h and 8 h are approximately equivalent to the combined increases in [¹⁴C]OA-labeled phospholipid over [³H]EPA-labeled phospholipid at these time points.

Effect of fatty acids on de novo glycerolipid synthesis

[³H]Glycerol incorporation was used to monitor de novo glycerolipid synthesis in HepG2 monolayers chal-

lenged with a range of OA and EPA concentrations. HepG2 cells were incubated in media containing [³H]glycerol and 0 to 300 μM of either OA or EPA complexed to BSA (20 mol%). As the concentration of either fatty acid was increased from 10 to 300 μM, the cellular content of [³H]glycerol-labeled lipids rose correspondingly (**Fig. 2**). Total [³H]glycerolipid formation was greatest with OA. Three hundred μM EPA produced a twofold increase in cellular [³H]glycerolipid relative to cells grown in fatty acid-free albumin, while addition of the equivalent amount of OA resulted in nearly a threefold increase in cellular [³H]glycerol-labeled lipid. Fatty acid-free BSA alone at concentrations of 0 to 60 μM had no effect on [³H]glycerolipid formation (data not shown). HPLC analysis of the extracted cellular lipids revealed that [³H]glyc-

Fig. 2. Effect of oleic acid (18:1) and eicosapentaenoic acid (20:5) concentration on the incorporation of [³H]glycerol into cellular glycerolipids. HepG2 cells were incubated for 4 h in serum-free media containing [³H]glycerol and either oleic acid (18:1) or eicosapentaenoic acid (20:5) at the concentrations shown. The fatty acids were complexed with 0.2 mol-equivalents of fatty acid-free bovine serum albumin. After incubation, the cells were harvested, the cell lipids were extracted, and the amounts of [³H]-label incorporated into TG, DG, PE, and PC were determined by HPLC, as described in Experimental Procedures. The data are plotted as the mean ± SD for three separate incubations. No bars are shown for data with SDs smaller than the symbol size. The dashed lines indicate the level of radiolabel incorporation obtained for fatty acid-free incubations.

erol was incorporated into TG, DG, PE, PI, PS, and PC. In all cases, PC and TG accounted for greater than 70% of the radiolabel that was incorporated. The bulk of the remaining label was in the form of PE and DG. [³H]-Labeled PI and PS were also detected, but they accounted for less than 5% of the total [³H]-label incorporated.

Incorporation of [³H]glycerol into TG was similar for both oleate and EPA at fatty acid concentrations between 10 and 100 μM (Fig. 2). At 300 μM fatty acid, OA increased ³H-labeled TG by 440% over the albumin control, while the equivalent amount of EPA resulted in a 380% gain. More significant differences between OA- and EPA-dependent conversion of radiolabeled glycerol to DG, PE, and PC were also observed. For example, 300 μM EPA caused a 200% increase in cellular [³H]DG over fatty acid-free controls, while the equivalent amount of OA produced only an 80% gain (P < 0.005, OA vs. EPA). The reverse relationship was observed for [³H]glycerol-labeled PE and PC synthesis. At all fatty acid concentrations tested, [³H]glycerol incorporation into PC and PE was greater with OA than with EPA. At 300 μM fatty acid, [³H]glycerol incorporation into PC increased 20% over basal levels in the presence of EPA, but [³H]glycerol incorporation increased 180% with OA under identical conditions (P < 0.002, OA vs. EPA). Similarly, [³H]glycerol incorporation into PE rose 25% in cells treated with 300 μM EPA, whereas [³H]glycerol-labeled PE increased 200% in cells given OA (P < 0.02, OA vs. EPA).

Analysis of secreted lipids

The effects of fatty acid addition on TG and PC secretion were determined by incubating HepG2 cells for 4 h under the same conditions as those described in Fig. 2. After the incubation, the TG and PC secreted into the media by the cells were isolated for determination of mass and radiolabel content as described in Methods. The results plotted in **Fig. 3** show that oleic acid addition resulted in a fourfold increase in the [³H]glycerol content of secreted TG, compared to fatty acid-free incubations. EPA-treated cells secreted twofold more [³H]glycerol-labeled TG, compared to BSA controls. These changes in the radiolabel content of secreted TG were not reflected in the masses of TG secreted. A slight increase in the mass of TG secreted by OA-treated cells was observed. In all cases, the mass of TG secreted was approximately 1% of the total cellular TG mass. Fatty acid addition did not cause a significant change in the mass of PC secreted. OA addition did result in a 30% increase in the [³H]glycerol content of secreted PC.

The lipids secreted by cells given exogenous fatty acid were greatly enriched in the particular fatty acid species included in the media (**Table 3**). For example, OA comprised almost 50% of the fatty acid content of TG secreted by HepG2 cells fed OA. In contrast, stearic acid,
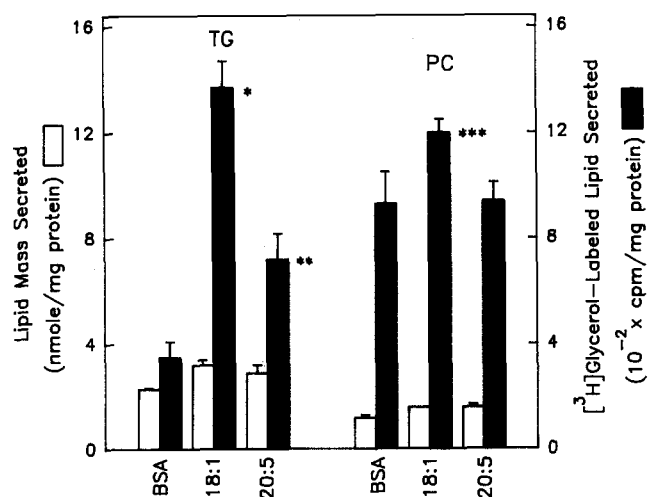
Fig. 3. Effect of oleic acid (18:1) and eicosapentaenoic acid (20:5) on lipid secretion by HepG2 cells. HepG2 cells were incubated for 4 h in media containing [³H]glycerol (4 μCi/ml) and supplemented with fatty acid-free BSA (60 μM) alone or BSA complexed with 300 μM oleic acid or eicosapentaenoic acid. The triglyceride (TG) and phosphatidylcholine (PC) secreted by the cells were extracted from the media and purified by HPLC as described in Table 1. The radiolabel content and lipid mass in each fraction were determined as described in Experimental Procedures. The results are plotted as the mean ± SD of three incubations. *P < 0.002 for [³H]TG (18:1) vs. [³H]TG (BSA); **P < 0.04 for [³H]TG (20:5) vs. [³H]TG (BSA), [³H]TG (20:5) vs. [³H]TG (18:1); ***P < 0.04 for [³H]PC (18:1) vs. [³H]PC (20:5).

not OA, was the principal fatty acid component of TG secreted by cells incubated in fatty acid-free media. Similarly, EPA constituted nearly 50% of the fatty acids in TG secreted by cells incubated with EPA whereas no EPA was detected in the TG secreted in fatty acid-free incubations. Exogenously added OA was also extensively incorporated into secreted PC. EPA was also incorporated into the PC secreted by EPA-treated cells but the extent of incorporation was less than the enrichment obtained with OA addition (Table 3).

DISCUSSION

The results of this study demonstrate significant differences between OA- and EPA-induced lipogenesis and lipid secretion by HepG2 cells. Examination of total cellular lipid composition and the incorporation of radiolabeled fatty acids or radiolabeled glycerol reveals that, under conditions of acute fatty acid addition, EPA is as effective as OA in promoting cellular TG synthesis in HepG2 cells. The fatty acid composition of TG in EPA-treated cells and incorporation of radiolabeled EPA into cellular TG clearly indicate that EPA is an efficient substrate for TG synthesis. Despite the increase in cellular TG synthesis with EPA, secretion of de novo-synthesized TG by EPA-treated cells, as measured by [³H]glycerol in-

TABLE 3. Effect of fatty acid supplementation on fatty acid composition of lipids secreted by HepG2 cells

Fatty Acid	Albumin		Oleic Acid		Eicosapentaenoic Acid	
	TG	PC	TG	PC	TG	PC
	(nmol fatty acid/mg cell protein) $\times$ 10^{-2} (mol %)					
14:0	10 (12%)	13 (43%)	14 (7%)	43 (34%)	13 (5%)	61 (37%)
16:0	22 (27%)	10 (33%)	35 (16%)	34 (27%)	37 (15%)	43 (27%)
18:0	33 (40%)	4 (12%)	32 (15%)	6 (5%)	41 (16%)	8 (5%)
18:1 (n–6 + 7)	11 (14%)	3 (12%)	130 (60%)	42 (34%)	44 (17%)	45 (27%)
20:4 (n–6)	5 (6%)		4 (2%)		5 (2%)	
20:5 (n–3)					101 (41%)	7 (7%)
22:6 (n–3)					9 (3%)	

HepG2 cells were incubated for 4 h in media containing fatty acid-free BSA (60 μM) alone or complexed with either 300 μM oleic acid or 300 μM eicosapentaenoic acid. The triglyceride (TG) and phosphatidylcholine (PC) in the lipoproteins (d < 1.063 g/ml) secreted by the cells were isolated and transesterified to fatty acid methylesters for GC analysis, as described in Experimental Procedures.

corporation (Fig. 3), was only half of the amount of [³H]glycerol-labeled lipid secreted by cells given OA. This last result is similar to earlier reports of EPA-dependent inhibition of TG secretion in rat hepatocytes (12–14). The studies with rat hepatocytes, however, determined that under conditions of acute EPA administration incorporation of [³H]glycerol into both secreted TG and cellular TG was reduced. The reduction in cellular TG synthesis obtained in those studies was then hypothesized to be the source of the decline in secretion. The increased cellular TG synthesis and yet reduced TG secretion by cells exposed to EPA in this study do not indicate that TG synthesis is the limiting factor for TG secretion by EPA-treated HepG2 cells.

The divergence between the results of this report and the earlier studies with rat hepatocytes may, in part, be accounted for by the differences in cell types and culture conditions employed. These differences may also be a result of the higher concentrations of fatty acid, in the range of 1 mM or greater, used in the previous studies. In spite of these variations, some qualitative similarities do exist between the results obtained in this study and previous work with HepG2 cells. For example, HepG2 cells given 1 mM OA or 1 mM EPA are reported to respond with fivefold or fourfold increases, respectively, in the mass of cellular TG (32). In the same study, the secretion of [³H]glycerol-labeled TG increased 20-fold with OA and only twofold with EPA. In an even more recent study (33), HepG2 cells incubated with either 1 mM OA

or 1 mM EPA exhibited elevated rates of [³H]glycerol-labeled TG synthesis and secretion; however, the increases in radiolabeled TG secretion from EPA-treated cells were approximately 75% of those obtained with OA treatment.

The primary effect of fatty acid addition on TG secretion in this study was a several fold increase in the [³H]glycerol content of secreted TG, whereas the mass of TG secreted into the media increased only fractionally, at best. These results suggest that, under the experimental conditions used in this study, the majority of TG secreted into the media was derived from endogenous lipid stores. This effect is similar to that observed in rat hepatocytes which are reported to obtain a significant portion of VLDL TG from intracellular TG stores (34). The fatty acid-dependent boost in ³H-labeled TG secretion suggests that exogenous fatty acid promotes the incorporation of de novo-synthesized TG into lipoproteins. This is substantiated by the finding that the TG secreted by cells exposed to fatty acid was specifically enriched in the particular fatty acid species that was added to the medium. Such fatty acid enrichment of secreted TG is analogous to the enrichment detected in VLDL TG secreted by rat livers perfused with OA and EPA (14).

The reduction in [³H]glycerol-labeled TG secreted from cells fed EPA would suggest that less TG was secreted, but direct TG mass measurements did not indicate this. Instead, the principle effect of EPA in these studies was to reduce the incorporation of de novo-synthesized TG into lipoproteins. This result is analogous

to data obtained in rat liver (35), which showed that transfer of newly synthesized TG to nascent VLDL is reduced by fasting without a corresponding decrease in the transport of TG to intracellular lipid stores, thereby indicating that incorporation of TG into VLDL is specifically regulated. Similarly, in perfused livers from African green monkeys that were maintained on fish oil-rich diets for 2.5 years, no differences between incorporation of radiolabeled OA and radiolabeled EPA into hepatic TG were detected, whereas secretion of TG containing labeled EPA was significantly less than secretion of TG containing the OA label (36). Thus, the hypolipidemic properties of EPA may stem from inhibition of the factors that enhance the transfer of de novo-synthesized TG into nascent VLDL.

The near equivalency between EPA and OA in promoting cellular TG synthesis contrasted with the enhanced incorporation of OA into phospholipids, compared to EPA-treated cells. These results indicate a selective utilization of fatty acids by HepG2 cells which may reflect the substrate specificities of cellular lipogenic enzymes. DG is the common metabolic precursor for TG, PC, and PE (37). DG is utilized by acyl-coenzyme A:1,2-diacylglycerolacyltransferase, which produces TG; by CDP-choline:1,2-diacylglyceroltransferase, which converts DG to PC; and by CDP-ethanolamine:1,2-diacylglyceroltransferase, which forms PE. The results for conversion of [³H]glycerol into cellular glycerolipids reflect the relative activities of these enzymes. The greater amounts of [³H]glycerol-labeled PC and PE in OA-treated cells, as compared to EPA-treated cells, and the elevated [³H]glycerol-labeled DG in EPA-treated cells suggest that conversion of DG to PE and PC is reduced in the presence of EPA. The reduced incorporation of radiolabeled EPA into cellular PC, compared to the results for OA, suggests that DG, containing EPA, is a poorer substrate for PC synthesis. In contrast to the results for PC, radiolabeled EPA and radiolabeled OA incorporation into PE were similar. This difference between EPA-dependent glycerol incorporation and direct EPA incorporation into PE may reflect the activity of deacylation/reacylation processes in the cell, which are known to be responsible for the preferential incorporation of polyunsaturated fatty acids into PE (38). This is supported by the determinations of fatty acid compositions, which showed enrichment of PE with polyunsaturated fatty acids. It should also be noted that the accumulation of ³H-labeled DG in EPA-treated cells without any accumulation of ³H-labeled phosphatidic acid, the precursor of DG, or alternate products of phosphatidic acid, such as phosphatidylinositol, indicate that phosphatidic acid phosphatase activity was not inhibited by EPA, as previously suggested for perfused rat livers (14). The results of this study also indicate phospholipid synthesis is balanced by phospholipid breakdown. The short-term in-

cubations with fatty acid resulted in only a net increase in the cellular content of TG. Although [³H]glycerol incorporation and radiolabeled fatty acid incorporation into the phospholipids were comparable to those for TG, particularly in the case of PC in OA-treated cells, no significant increases in cellular phospholipid mass were found.

The effects of EPA on PC synthesis are intriguing since there are several examples of the direct dependence of lipoprotein secretion on PC synthesis. Yao and Vance (39) recently demonstrated the obligatory role of PC synthesis in VLDL assembly and secretion by rat hepatocytes. They found that TG secretion was reduced in hepatocytes kept in choline- and methionine-deficient media. They were able to restore TG secretion by adding back choline or methionine (a precursor for the conversion of PE to PC (37)). The significance of PC synthesis in the secretion of VLDL is also indicated by earlier dietary studies that showed that rats fed choline-deficient diets exhibited reduced plasma TG levels accompanied by accumulation of TG in the liver (40). The requirement for choline lipid synthesis is not unique to the liver. Chylomicron secretion by the intestine is also directly dependent on active PC synthesis (41, 42).

The differences in the acylated lipid products formed form OA and EPA addition result from cellular selectivity in the utilization of fatty acids for acylated lipid synthesis. This selectivity may have profound effects on hepatic lipid secretion. Intracellular formation of the lipoprotein particles depends on the continuous supply of lipids for lipoprotein assembly. Interruptions in the lipid supply by substrate-dependent changes in acylated lipid synthesis could impair overall lipid secretion. The data obtained in this and other studies thus far demonstrate n-3 fatty acid-dependent changes in the metabolism of TG and PC, both of which are essential lipoprotein components. A better understanding of the hypolipidemic properties of n-3 fatty acids will, in large part, depend on a more detailed knowledge of the metabolic mechanisms underlying lipoprotein assembly and secretion. ∎

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