13-Hydroxy octadecadienoic acid (13-HODE) inhibits triacylglycerol-rich lipoprotein secretion by CaCo-2 cells

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Abstract **Oxidized lipids present in atherogenic lipoproteins are derived, in part, from the diet. To address the effects of an oxidized lipid on intestinal lipoprotein assembly and secretion, CaCo-2 cells were incubated with 13-HODE or its native fatty acid, linoleic acid, and triacylglycerol-rich lipoprotein synthesis and secretion were investigated. 13-HODE was readily taken up by cells and esterified to lipids. Although both fatty acids were largely esterified to neutral lipids, in comparison to neutral lipids containing linoleic acid, a greater proportion of cellular neutral lipids containing 13-HODE and/or its metabolites was secreted. Compared to linoleic acid, however, 13-HODE caused less triacylglycerol, derived from de novo synthesis, and less triacylglycerol mass to be secreted. Cells incubated with both linoleic acid and 13-HODE together secreted less triacylglycerol mass than did cells incubated with linoleic acid alone. Less newly synthesized apoB and apoB mass were secreted by cells incubated with 13-HODE without altering the abundance of apoB mRNA. The fraction of newly synthesized apoB translocated into the secretory pathway of cells exposed to 13-HODE was significantly less than that observed in cells incubated with linolenic acid, suggesting that 13-HODE interfered with the assembly and secretion of triacylglycerol-rich lipoprotein particles.—**Murthy, S., E. Born, S. Mathur, and F. J. Field. **13-Hydroxy octadecadienoic acid (13-HODE) inhibits triacylglycerol-rich lipoprotein secretion by CaCo-2 cells. *J. Lipid Res.* 1998.** 39: **1254–1262.**

Supplementary key words 13-HODE • triacylglycerol • apoB • CaCo-2 cells

Circulating atherogenic lipoproteins owe their origin, in part, to the small intestine. Intestinally derived triacylglycerol-rich lipoproteins stimulate the secretion of hepatic very low density lipoproteins (VLDL), which upon subsequent catabolism, yield low density lipoproteins (LDL) (1). Moreover, chylomicron remnants are themselves believed to be atherogenic (2).

The finding of oxidized LDL in arterial atherosclerotic lesions and the ability of anti-oxidants to diminish the formation of such lesions, strongly suggest that oxidized lipoproteins play a role in the development of atherosclerosis (3–6). The site of oxidative modification of lipoproteins is unknown, but it is thought to occur partly in the microenvironment of the arterial wall sequestered from circulating antioxidants. Recent observations by Staprans et al. (7–10) suggest that oxidized lipids in the diet could also contribute to oxidized lipids found in circulating lipoproteins and in the arterial wall. In rats, for example, the amount of oxidized lipids found in mesenteric lymph chylomicrons and in plasma VLDL and LDL were directly related to the content of oxidized lipids in the diet (9, 10). Similarly, when diets containing increasing amounts of oxidized lipids were fed to humans, there was a corresponding increase in the amount of oxidized lipids observed in post-prandial chylomicrons (8). Moreover, chylomicron remnants containing these oxidized lipids were taken up by the liver and subsequently secreted in VLDL particles (7). Dietary oxidized lipids, therefore, are taken up and transported by the intestine and contribute to circulating oxidized lipoproteins.

It is not known, however, whether the ingestion of oxidized lipids will alter normal lipoprotein assembly and secretion and/or the composition of triacylglycerol-rich lipoproteins secreted by the intestine. As it is the intestine that takes up dietary oxidized lipids and subsequently transports them in lipoprotein particles, it would be important to understand the effects of an oxidized lipid on intestinal lipid transport. To address this, we compared the effects of 13-HODE and its native fatty acid, linoleic acid, one of the more commonly consumed polyunsaturated fatty acids, on intestinal lipoprotein assembly and secretion in the human intestinal cell line, CaCo-2.

The results suggest that 13-HODE is readily taken up by CaCo-2 cells and esterified to neutral lipids. Compared to lipids containing linoleic acid, a greater proportion of lipids containing 13-HODE and/or its metabolites are secreted into the basolateral medium. Compared to linoleic

Abbreviations: 13-HODE, 13-hydroxy octadecadienoic acid; apoB, apolipoprotein B; apoA-I, apolipoprotein A-I; TLCK, Na-*p*-tosyl-l-lysine chloromethyl ketone; MTP, microsomal triglyceride transfer protein; PMSF, phenylmethylsulfonyl fluoride; VLDL, very low density lipoprotein; LDL, low density lipoprotein; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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acid, however, 13-HODE was less potent in stimulating the synthesis and secretion of triacylglycerols and apoB. The fraction of newly synthesized apoB destined for lipoprotein synthesis and secretion was less in cells incubated with 13-HODE, suggesting that the hydroxy fatty acid interfered with lipoprotein assembly and secretion.

MATERIALS AND METHODS

Materials

13-S-HODE was purchased from Cayman Chemicals (Ann Arbor, MI). Taurocholate, linoleic acid, oleic acid, BSA, Protein G-Sepharose, and GPO Trinder kit were purchased from Sigma Chemicals (St. Louis, MO). 13-[^{3}H]HODE was from DuPont NEN Research Products (Boston, MA). [^{3}H]linoleic acid was purchased from American Radiolabeled Chemicals (St. Louis, MO). The ECL chemiluminescent detection kit and anti-rabbit IgG polyclonal antibody conjugated to horseradish peroxidase (HRP) was from Boehringer Mannheim (Indianapolis, IN). Monoclonal antibody to human apoB, and rabbit anti-human apoB polyclonal antibody conjugated to HRP were bought from Biodesign (Kennebunkport, ME). TMB microwell peroxidase substrate system containing 3,3′,5,5′-tetramethyl benzidine and hydrogen peroxide was purchased from Kirkegaard and Perry (Gaithersburg, MD). Nunc 96-well immunoplates were obtained from PGC Scientific (Gaithersburg, MD). Rabbit anti-human apoB polyclonal antibody was obtained from Calbiochem (San Diego, CA). Recombinant protein A-Sepharose was purchased from Repligen (Cambridge, MA). CellTiter 96 was provided by Promega (Madison, WI). Silica gel G plates were purchased from Fisher Scientific (Batavia, IL). ApoB cDNA was purchased from American Type Culture Collection (Rockville, MD). [α-^{32}P]dCTP (6,000 Ci/mmol) and [^{3}H]glycerol (200 mCi/mmol) were purchased from DuPont NEN Research Products (Boston, MA). *Trans*[^{35}S]methionine (1100 Ci/mmol) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA).

Cell culture

CaCo-2 cells were cultured on T-75 flasks (Corning Glassworks, Corning, NY) in Dulbecco's minimum essential medium (GIBCO, Grand Island, NY) with 4.5 g/L glucose, and supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 4 mm glutamine, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 1% nonessential amino acids. Once the flasks reached 80% confluency, the cells were split and seeded onto polycarbonate micropore membranes (0.4 μm pore size, 6.5 mm diameter) inserted into transwells (Costar, Cambridge, MA) at a density of 0.2×10^5 cells/well. For experiments in which transepithelial electrical resistance, triacylglycerol mass, MTP activity, and lipoprotein secretion were estimated, cells were subcultured in 24.5-mm diameter transwells. Cells were fed every other day and were used 14 days after seeding.

On the day of the experiment, cells were washed twice with M199, and the sodium salts of the fatty acids were solubilized in serum-free Medium 199/1 m HEPES (M199) containing 1 mm taurocholate and then added to the apical chamber. All cells received M199 in the basal chamber. Incubations were carried out at 37°C in an atmosphere of 95% air/5% CO_2.

Cell viability/proliferation

Cell viability and proliferation were assessed by using the Cell Titer 96 assay kit as described previously (11). Compared to the absorbance of the dye released from control cells (1.00), the relative absorbances of the dye released from cells incubated with linoleate and 13-HODE were 1.34 ± 0.08 and 1.00 ± 0.07, respectively.

In addition, cell viability was also assessed by estimating transepithelial electrical resistance. CaCo-2 cells cultured on 24.5-mm diameter transwells were incubated overnight with one of the following treatments: 1 mm taurocholate, 1 mm taurocholate and 250 μm linoleic acid, or 1 mm taurocholate and 250 μm 13-HODE. After the incubation, cells were rinsed with M199 and transepithelial electrical resistance across the monolayers was estimated using chopstick electrodes with silver-plated tips (World Precision Instruments, Inc., Sarasota, FL). The electrodes were equilibrated with M199 before the experiment and rinsed with M199 between readings. Compared to the transepithelial resistance in cells incubated with taurocholate alone (257 ± 7 Ω), the transepithelial resistances in cells incubated with linoleic acid and 13-HODE were 254 ± 7 Ω, and 247 ± 6 Ω, respectively. These values have been corrected for transepithelial electrical resistance across membranes with no cells (197 ± 3 Ω).

Uptake of 13-HODE and linoleic acid

Cells were incubated for 1 h with 1 mm taurocholate containing 250 μm of 13-[^{3}H]HODE (0.1 μCi/well) or 250 μm [^{3}H]linoleic acid (0.1 μCi/well). At the indicated times, cells were washed extensively with M199 containing 83.3 μm BSA to remove any cell-surface adherent fatty acids, scraped from the filters, and sonicated. Cell homogenates were then counted by liquid scintillation counting to determine uptake of the radiolabeled fatty acids.

Incorporation into cellular and media lipids

Cells were incubated for 4 h with 1 mm taurocholate and 250 μm of either 13-[^{3}H]HODE or [^{3}H]linoleic acid (0.2 μCi/well). Cells were scraped from the filters with 0.2 N sodium hydroxide, and after adjusting the pH to 3, lipids from cells and basal media were extracted with chloroform–methanol 2:1 (v/v). A portion of the lipid extract was saponified by methanolic potassium hydroxide and the free fatty acids released were extracted with chloroform–methanol 2:1 (v/v) under acidic conditions (pH 3). Greater than 80% of the incorporated fatty acids was recovered by this procedure. Saponified and unsaponified lipid extracts were then dried under nitrogen, taken up in 0.125 mL chloroform, and applied to silica gel G plates. To resolve the different phospholipid classes, the plates were developed up to 4 cm from the origin with chloroform–methanol–acetic acid–water–acetone 40:25:4:2:4 (v/v). To separate neutral lipids, the plates were then dried and re-eluted with hexanes–diethyl ether–methanol–acetic acid 85:15:1:1: (v/v). The incorporation of the label into the different phospholipids and neutral lipids was estimated by counting the plates on a TLC plate scanner (Vanguard, 2001 TLC Scanner, Digital Diagnostic Corp., Hamden, CT).

Triacylglycerol synthesis and secretion

Cells were incubated for 18 h with 250 μm of either linoleic acid or 13-HODE in 1 mm taurocholate. During the last 4 h of the incubation, 10 μCi of [^{3}H]glycerol was added per well in the apical chambers. Cells were rinsed extensively with M199 and lysed with 0.2 N sodium hydroxide. After adjusting the pH to 3, labeled lipids present in cells and media were extracted with chloroform–methanol 2:1 (v/v). Lipids were dried under nitrogen and applied onto silica gel G TLC plates in 0.125 mL chloroform. The plates were developed in hexanes–diethyl ether–methanol–water 85:15:1:1 (v/v) and newly synthesized triacylglycerols present in cells and media were visualized under iodine vapor, scraped into Budget Solve, and counted by liquid scintillation counting.

Isolation of lipoproteins by ultracentrifugation

Lipoproteins secreted into the basolateral media were isolated by ultracentrifugation as described previously (12). Cells were in-

cubated for 18 h with either linoleic acid or 13-HODE. During the last 4 h of the incubation, 10 μCi of [^{3}H]glycerol was added per well. Basal media containing secreted lipoproteins were pooled from 2 wells, mixed with 20 μL of denatured human plasma and EDTA to yield a final concentration of 1 mm and pH 7.4, and centrifuged for 18 h at 100,000 *g*. One-third volume of the supernatant containing lipoproteins of density < 1.006 g/mL was aspirated from the top and lipids were extracted with chloroform–methanol 2:1 (v/v). Neutral lipids were separated by thin-layer chromatography (TLC) as described above. The bands corresponding to triacylglycerols were scraped into Budget Solve and counted by liquid scintillation counting. To confirm that the majority of esterified fatty acids were being secreted in lipoproteins of d < 1.006 g/mL, an aliquot of the media was taken before centrifugation and the incorporation of labeled glycerol into triacylglycerols was estimated.

Estimation of apoB mass

ApoB mass in cells and basal media was determined by sandwich ELISA as previously described (13).

Measurement of apoB synthesis and secretion

After an 18-h incubation with 250 μm of either fatty acid, cells were washed an incubated with methionine-free M199 in the continued presence of the treatments for 1 h. 100 μCi of [^{35}S] methionine was then added to each well in the apical chamber. After 4 h of pulse, cells were harvested in radioimmunoprecipitation assay (RIPA) buffer (10 mm sodium phosphate, pH 7.5, 100 mm sodium chloride, 1% (v/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) sodium deoxycholate, 21 μm leupeptin, 5 mm EDTA, 5 mm EGTA, 1 mm PMSF, 1 mm dithiothreitol, 20 mm methionine, and 1 mm cysteine) and pre-cleared with protein A-Sepharose. Basolateral media and the pre-cleared cell lysates were incubated overnight at 4°C on a shaker with rabbit anti-human apoB antisera (1:250 dilution) and apoA-I antisera (1:1000 dilution). The antigen–antibody complexes were precipitated by incubating with protein A-Sepharose for 2 h at room temperature on a shaker followed by a brief high speed centrifugation. The immune complexes were washed extensively and dissociated from protein A-Sepharose by adding 30 μL 2× Laemmli sample buffer and 10 μL 0.2 m glycine buffer (pH 2). Proteins were separated by SDS-PAGE on 8% porous gels as described by Doucet, Murphy, and Tuana (14). Gels were fixed in 7% acetic acid/5% methanol solution and enhanced in 1 m sodium salicylate solution. After drying, the gels were exposed to X-ray films for 2 h. Bands corresponding to apoB-100, B-48, and A-I were cut from the gels and counted by liquid scintillation counting.

Estimation of apoB mRNA abundance

Total RNA was isolated using TRI Reagent (Sigma Chemical Co., St. Louis, MO) in a modification of the single-step method originally described by Chomczynski and Sacchi (15). Northern blots were run with 20 μg of total RNA and probed for apoB mRNA as previously described (11). Apo B mRNA was quantified by densitometry using Sigma Gel software Jandel Scientific (San Rafeal, CA).

Sodium carbonate-release of labeled apoB

The percent of newly synthesized apoB released into the lumen of microsomes was estimated according to the methodology described by Davis et al. (16). CaCo-2 cells grown on T-75 flasks and on transwells were incubated for 18 h with 1 mm taurocholate containing 250 μm linoleic acid or 250 μm 13-HODE. During the last 4 h of the incubation, cells cultured on transwells were pulsed with radiolabeled methionine. Cells from six transwells and one T-75 flask were pooled and then homogenized in 10 mm Tris buffer, pH 7.4, containing 0.25 m sucrose, 21 μm leupeptin, and 1 mm PMSF. The protease inhibitors, leupeptin and PMSF, were added to all the buffers used in the subsequent steps. After centrifuging for 20 min at 10,000 *g*, microsomes were prepared from the resulting supernatant by centrifuging at 100,000 *g* for 30 min. The microsomal fraction was resuspended in 0.35 mL Tris-sucrose buffer and divided into three aliquots of 0.1 mL each. The first aliquot was diluted to 1 mL with 0.1 × RIPA and taken directly for apoB immunoprecipitation. This fraction was used to estimate apoB present within both the membranes and lumen of the isolated microsomes. The second aliquot was mixed with 0.4 mL water and 0.5 mL of 0.2 m sodium carbonate, pH 11.3. As a control for the sodium carbonate treatment, the third aliquot was diluted to 1 mL with Tris-sucrose buffer alone. After incubating on ice for 30 min, both the sodium carbonate-treated samples and untreated controls were centrifuged for 30 min at 100,000 *g*. The resulting supernatants were adjusted with RIPA to yield a final concentration of 0.1×. The alkaline pH of the supernatants of sodium carbonate-treated samples was neutralized by dialysis for 48 h with four changes of PBS containing 0.1 × RIPA and protease inhibitors. ApoB was immunoprecipitated from all fractions and separated by SDS-PAGE as described above. Bands corresponding to apoB-100 and B-48 were cut from the gel and counted by liquid scintillation counting.

LDL degradation

Human LDL was iodinated with ^{125}I according to the method of McFarlane (17). Ninety-eight percent of the label was precipitated by trichloroacetic acid. ^{125}I-labeled LDL (1 μg, 0.2 μCi) was added per well in the basal chamber and the fatty acids were added to the apical chamber. Glutaraldehyde-fixed cells served as controls for non-cellular-mediated degradation of LDL. To account for nonspecific degradation of LDL, cells were incubated with 50-fold excess of unlabeled LDL in the presence of labeled LDL. After 18 h of incubation, the amount of ^{125}I-labeled LDL that was degraded was estimated according to the method of Goldstein, Basu, and Brown (18).

Chemical analyses

Total protein content in cells was determined by the method of Lowry et al. (19). Triacylglycerol mass in cells was measured using the GPO Trinder kit as described previously (20). Microsomal triglyceride transfer protein (MTP) activity in microsomal preparations was estimated as previously described (21).

Statistical analysis of data was performed by Student's *t* test (22).

RESULTS

Uptake and incorporation into lipids

Initial rates of uptake of a fatty acid, by an intestinal cell, will affect its intracellular esterification and eventual secretion as part of a lipoprotein particle. Rates of uptake into CaCo-2 cells of linoleic acid and 13-HODE were, therefore, addressed. CaCo-2 cells were incubated for up to 1 h with a taurocholate solution containing 250 μm of labeled 13-HODE or linoleic acid and the amount of cell-associated fatty acid was estimated (**Fig. 1**). Compared to the uptake of linoleic acid, CaCo-2 cells accumulated significantly more 13-HODE. By the end of 1 h, 18% of the added 13-HODE was cell-associated, compared to 12% for linoleic acid. To address the incorporation of these fatty acids into cellular lipids and their secretion into the basolateral medium, cells were incubated for 4 h with the re-

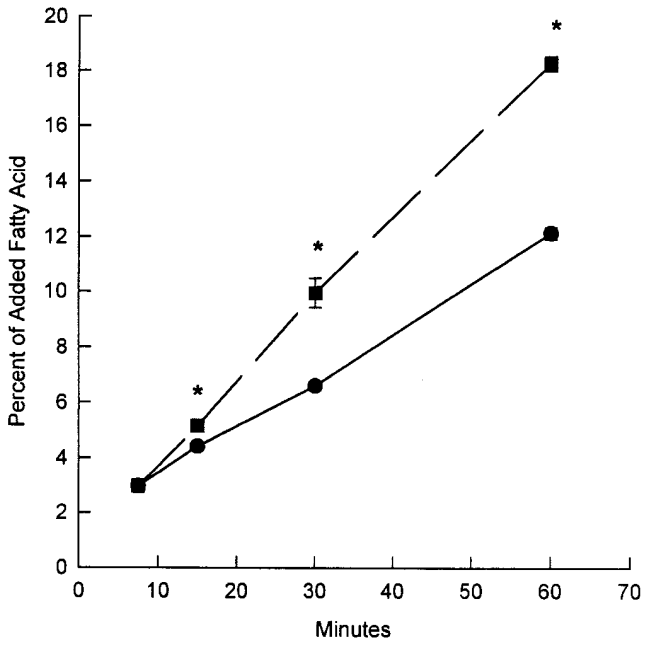

Fig. 1. Initial rates of uptake of 13-HODE and linoleic acid. CaCo-2 cells were incubated with 250 μm of 13-[^{3}H]HODE (0.1 μCi/well) (■) or [^{3}H]linoleic acid (0.1 μCi/well) (●). After 7.5, 15, 30, and 60 min incubation, cells were washed extensively with M199 containing 83.3 μm BSA to remove any cell-surface adherent fatty acids. Cells were scraped from the filters, sonicated, and counted by liquid scintillation counting. The results are expressed as a percent of added fatty acids that were taken up by cells. The data represent mean ± SEM of 6 dishes. *$P < 0.05$ vs. linoleic acid.

spective labeled fatty acid. After incubation, total lipids were extracted from cells and basolateral media and individual lipids were isolated by TLC (**Table 1**). Most of the 13-HODE (73%) and linoleic acid (78%) that was taken up by cells was esterified into lipids, with both fatty acids being largely incorporated into neutral lipids. Compared to linoleic acid, however, less 13-HODE was incorporated into phospholipids, with the decrease being secondary to a lower incorporation into phosphatidylethanolamine. The remainder of linoleic acid was largely retained by the cell as the free fatty acid. In contrast, unesterified 13-HODE was not retained by the cell but secreted into the basolateral medium as polar metabolites. Compared to lipids containing linoleic acid, a greater proportion of intracellular lipids containing 13-HODE and/or its metabolites were secreted into the basolateral medium (38% vs. 4%). Negligible amounts of phospholipids containing either fatty acid and/or their metabolites were secreted. Saponification of lipids extracted from cells and media incubated with 13-HODE revealed that about 85% of the hydroxy fatty acid that was taken up was further metabolized to more polar products, of which 68% was esterified and either retained within the cell or secreted basolaterally (data not shown). The identity of these polar products is currently being determined. The data suggest that although a substantial amount of 13-HODE is oxidized by CaCo-2 cells, a significant amount is esterified to cellular lipids and secreted.

Synthesis and secretion of triacylglycerols

Although the results from the previous experiment indicate that 13-HODE was readily esterified to lipids and efficiently secreted from the cell, they do not, address the ability of the fatty acid to promote de novo triacylglycerol synthesis and/or its secretion. Triacylglycerol synthesis in CaCo-2 cells occurs predominantly via de novo synthesis from glycerol-3-phosphate (23). The effect of 13-HODE on the synthesis and secretion of triacylglycerols was therefore addressed by pulsing cells with labeled glycerol after an 18-h incubation with a 1 mm taurocholate solution containing 250 μm 13-HODE or linoleic acid. The incorporation of the labeled glycerol into cellular and media triacylglycerols was then estimated. **Figure 2** shows the results of this experiment. Compared to the de novo synthesis of triacylglycerols in cells incubated with linoleic acid, cells incubated with 13-HODE synthesized less triacylglycerols, although the difference between the two fatty acids was quite modest. However, the basolateral secretion of labeled triacylglycerols by cells incubated with 13-HODE was dramatically less than that observed by cells incubated with linoleic acid. At 4 h, in cells incubated

TABLE 1. Incorporation of 13-HODE and linoleic acid into cellular and media lipids

	Cells		Media	
	Linoleic Acid	13-HODE	Linoleic Acid	13-HODE
	%		%	
Phosphatidylcholine	5.23 ± 0.15	5.23 ± 0.34		
Phosphatidylserine/phosphatidylinositol	2.61 ± 0.31	2.25 ± 0.52		
Phosphatidylethanolamine	12.94 ± 0.97	4.57 ± 0.03[a]		
Total phospholipids	20.78 ± 1.43	12.05 ± 0.34[a]	trace	trace
Neutral esterified lipids	54.79 ± 0.40	44.74 ± 0.24[a]	2.04 ± 0.06	16.52 ± 0.96[a]
Total esterified lipids	75.57 ± 1.03	56.80 ± 0.70[a]	2.04 ± 0.06	16.52 ± 0.86[a]
Unesterified fatty acids	19.6 ± 1.07	—	2.79 ± 0.02	26.69 ± 0.16[a]

CaCo-2 cells were incubated for 4 h with 250 μm 13-[^{3}H]HODE (0.2 μCi/well) or [^{3}H]linoleic acid (0.2 μCi/well). Lipids from cells and basal media were extracted and separated by TLC as described in Methods. Results are expressed as percentage of the total uptake of linoleic acid and 13-HODE which was 9449 ± 299 and 9906 ± 203 pmol/well, respectively; n = 3.

[a] $P < 0.05$ versus linoleic acid.

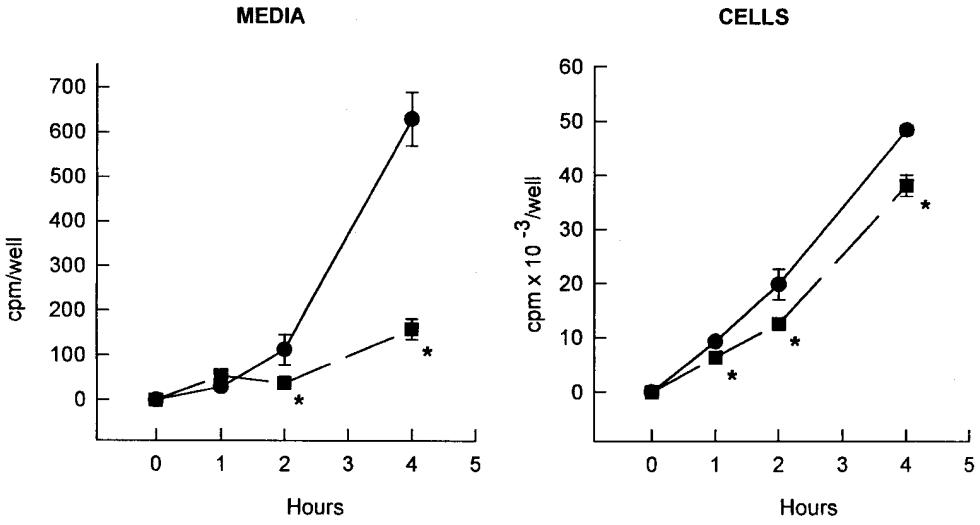

Fig. 2. Effect of 13-HODE on the synthesis and secretion of newly synthesized triacylglycerols. CaCo-2 cells were incubated for 18 h with 250 μm linoleic acid (●) or 250 μm 13-HODE (■). During the last 4 h of the incubation, 10 μCi of [^{3}H]glycerol was added per well. Lipids present in cells and media were extracted and separated by TLC as described in Methods. Triacylglycerol bands were visualized under iodine vapor and scraped into Budget Solve and counted. The data are shown as the mean ± SEM of the incorporation of [^{3}H]glycerol into media and cellular triacylglycerols (n = 6). *$P < 0.05$ vs. linoleic acid.

with 13-HODE, the percent of newly synthesized triacylglycerols derived from de novo synthesis that was secreted was 3-fold less than what was observed in cells incubated with linoleic acid (0.4 vs. 1.3%, $P < 0.01$, respectively). To confirm that the labeled triacylglycerols secreted into the basolateral medium represented the secretion of triacylglycerol-rich lipoproteins, lipoproteins of density ≤ 1.006 g/mL were isolated from the basolateral medium. The percent of labeled triacylglycerols recovered at this density was similar between cells incubated with either fatty acid and approximated 76% of the total secreted. Thus, 13-HODE interfered with the secretion of newly synthesized triacylglycerols recovered in a triacylglycerol-rich lipoprotein particle.

To address whether the decrease in secretion of triacylglycerols synthesized de novo by cells incubated with 13-HODE also reflected a decrease in the secretion of triacylglycerol mass, cells were incubated overnight with increasing concentrations of linoleic acid or 13-HODE solubilized in 1 mm taurocholate. The amount of triacylglycerol mass secreted into the basolateral medium was then estimated (**Fig. 3**). At 125 μm and 250 μm concentrations, the amount of triacylglycerol mass found in the basolateral medium of cells incubated with 13-HODE was less compared to the amount observed with linoleic acid. Moreover, when 13-HODE was mixed with linoleic acid in equimolar concentrations and added to cells, less triacylglycerols were secreted compared to the amount secreted by cells incubated with linoleic acid alone. Only at 250 μm was there less accumulation of triacylglycerols in cells incubated with 13-HODE compared to that found with linoleic acid. These results, taken together, suggest that the uptake of the hydroxy fatty acid altered the transport into the basolateral medium of triacylglycerols.

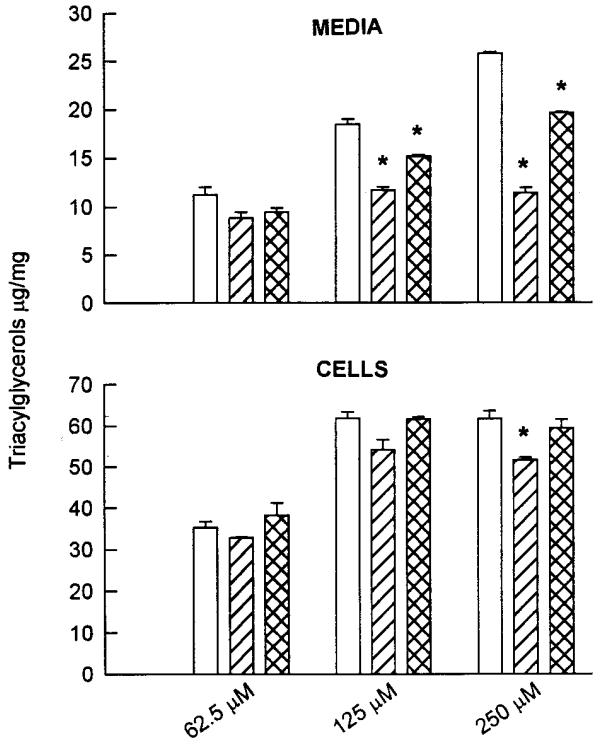

Fig. 3. Effect of 13-HODE on the secretion of triacylglycerol mass. CaCo-2 cells were incubated for 18 h with 0–250 μm of linoleic acid (open bars), 13-HODE (hatched bars), or equivalent concentrations of each of linoleic acid and 13-HODE (cross-hatched bars). Lipids in cells and media were extracted and triacylglycerol mass was analyzed using the GPO Trinder kit as described in Methods. The accumulation of triacylglycerol mass (μg/mg protein) in media and cells is shown as mean ± SEM of 6 wells per treatment. *$P < 0.05$ vs. linoleic acid.

ApoB secretion

To investigate whether the decrease in triacylglycerol secretion caused by 13-HODE could be related to a decrease in the number of lipoprotein particles secreted, cells were incubated with increasing concentrations of the respective fatty acid, and the amount of apoB mass secreted into the basolateral medium was estimated (**Fig. 4**). Compared to the amount of apoB secreted by cells incubated with linoleic acid, cells incubated with 100 or 250 μm 13-HODE secreted significantly less apoB into the basolateral medium. Changes in the accumulation of apoB in the basolateral media were not a reflection of a change in the reuptake of secreted apoB as less than 0.1% of apoB-labeled LDL added to the basolateral medium of cells incubated with either fatty acid was taken up and degraded. The amount of apoB found within cells was essentially unchanged and was similar in cells treated with either linoleic acid or 13-HODE. Thus, 13-HODE alters not only the amount of triacylglycerols in triacylglycerol-rich lipoproteins but also the number of lipoprotein particles secreted.

To address whether the changes observed in apoB secretion were associated with a lower rate of synthesis of apoB in cells incubated with 13-HODE, the effect of linoleic acid and 13-HODE on the synthesis and secretion of newly synthesized apoB was estimated. Cells were incubated for 18 h with 250 μm linoleic acid or 13-HODE. During the last 4 h of the incubation, cells were pulsed with labeled methionine and the amount of label incorporated into apoB-100, B-48, and A-I was estimated after immunoprecipitation and separation of the proteins by SDS-PAGE. Bands corresponding to apoB-100, B-48 and A-I were cut from the gel and counted. The results are shown in **Fig. 5**. Compared to cells incubated with linoleic acid, cells incubated with 13-HODE secreted less newly synthesized apoB-100 and apoB-48. In contrast, the secretion of apoA-I by cells incubated with either linoleic acid or 13-HODE was similar. In cells incubated with 13-HODE, the amount of newly synthesized apoB-100 was significantly less than the amount observed in cells incubated with linoleic acid. The amount of label incorporated into apoB-48 and total TCA-precipitable proteins, however, was similar. In data not shown, apoB mRNA levels were similar in cells incubated with either fatty acid, suggesting that the effect of 13-HODE on apoB-100 synthesis was independent of changes in apoB gene expression.

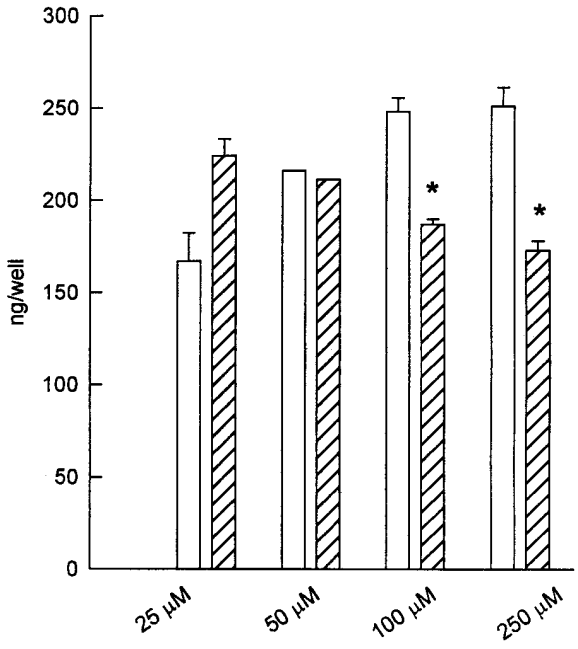

Fig. 4. Effect of 13-HODE on the secretion of apoB mass. CaCo-2 cells were incubated for 18 h with 250 μm linoleic acid (open bars) or 250 μm 13-HODE (hatched bars). ApoB mass in basal media was analyzed by sandwich ELISA. Mean ± SEM of 6 dishes are shown in the figure. *$P < 0.05$ vs. linoleic acid.

ApoB present within the lumen of the endoplasmic reticulum (ER) has been shown to represent the amount of apolipoprotein destined for secretion (16). To address whether 13-HODE decreased the secretion of triacylglycerols and apoB by altering the translocation of apoB into the lumen of the ER, the proportion of newly synthesized apoB present within the lumen of microsomes prepared from cells incubated with 13-HODE or linoleic acid was determined. As shown in **Table 2**, the percentage of newly synthesized apoB-100 estimated to be within the lumen of microsomes prepared from cells incubated with 13-HODE was less than that observed in microsomes prepared from cells incubated with linoleic acid. In cells incubated with either fatty acid, however, the percent of microsomal apoB-48 that was found within the lumen was similar. Thus, 13-HODE interferes with the assembly and secretion of triacylglycerol-rich lipoprotein particles by altering the proportion of apoB-100 that is destined for secretion.

To address whether the decrease in the amount of newly synthesized apoB destined for transport in cells incubated with 13-HODE was associated with a decrease in MTP activity, MTP activity in microsomes prepared from cells incubated overnight with either fatty acid was assessed. Similar levels of MTP activity were detected in cells incubated with either linoleic acid or 13-HODE (0.48 ± 0.02 pmol transferred / mg protein per min vs. 0.46 ± 0.02 pmol transferred / mg protein per min).

DISCUSSION

Significant amounts of oxidized lipids are found in deep fat-fried and processed foods (24–28). As high as 15% of polyunsaturated fatty acids used in oils for the commercial preparation of French fries is oxidized (28). This is equivalent to 3.5–7 mmol oxidized fatty acids per serving. It is not unreasonable to assume, therefore, that the intestine will absorb micromolar amounts of oxidized derivatives of polyunsaturated lipids in a meal of fried food. In vivo studies conducted in animals have clearly demonstrated that dietary oxidized lipids are absorbed by the intestine and released into the circulation within triacylglycerol-rich lipoproteins (7–10). Consistent with these results, the present study performed in CaCo-2 cells shows that the hydroxy fatty acid, 13-HODE, is readily taken up

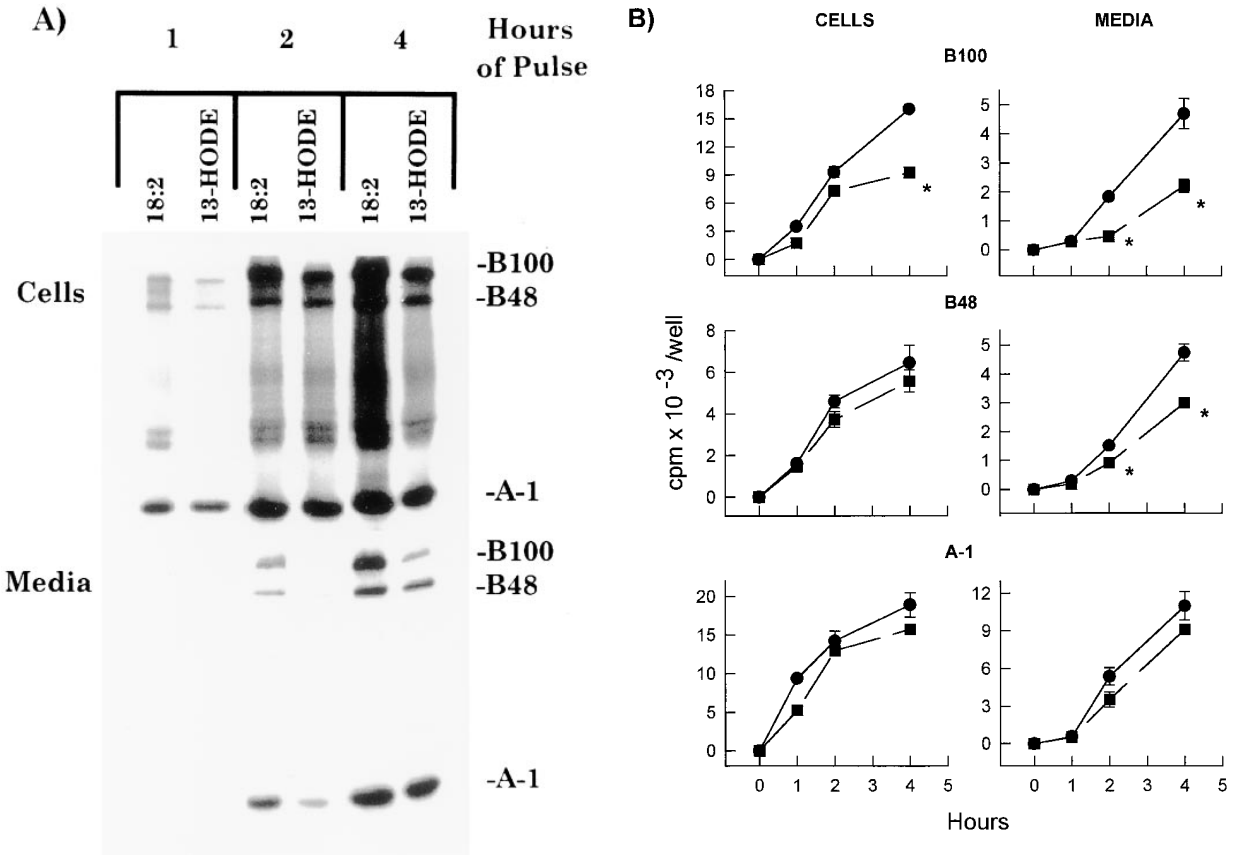

Fig. 5. Effect of 13-HODE on the synthesis and secretion of newly synthesized apoB. CaCo-2 cells were incubated for 18 h with 250 μm of 13-HODE (■) or 250 μm linoleic acid (18:2) (●). After incubation for 1 h in methionine-free M199, cells were pulsed for 4 h with 100 μCi of [^{35}S]methionine in the continued presence of methionine-free M199 containing the respective treatments. ApoB present in cells and basal media was immunoprecipitated and separated by SDS-PAGE. Gels were dried and exposed to X-ray film for 8 h. Bands corresponding to apoB-100, B-48 and A-I were cut from the gel and counted by liquid scintillation counting. A: A representative gel showing the migration of apoB-100, B-48 and A-I. B: Results from one of triplicate experiments are shown as mean ± SEM of radioactivity (cpm/well) in cells and basal media (n = 3/treatment). *$P < 0.05$ vs. linoleic acid.

by CaCo-2 cells, esterified to cellular lipids, and secreted into the basolateral medium. Moreover, 13-HODE also interferes with the assembly, secretion, and composition of triacylglycerol-rich lipoproteins secreted by intestinal cells.

Compared to linoleic acid, cells incubated with 13-HODE secreted significantly less triacylglycerol mass. As 13-HODE was taken up by cells at a rate greater than that observed with linoleic acid, a decrease in the availability, for lipid synthesis, of cellular 13-HODE and/or its metabolites cannot explain the observed decrease in the secretion of triacylglycerols. Similar to endothelial and smooth muscle cells (29), CaCo-2 cells have been shown not only to take up and esterify long chain hydroxy fatty acids but to oxidize them as well (30, 31). In fact, most of an hydroxylated derivative of arachidonic acid, 15-HETE, which is also taken up by CaCo-2 cells, is metabolized via oxidation with little being directly esterified to cell lipids (31). Similarly, in macrophages, another HETE, 12-HETE, is largely β-oxidized to polar products and released into the medium, leaving little for incorporation into lipids (32). In the present study, however, although 13-HODE was metabolized to more polar products, a significant amount of the hydroxy fatty acid taken up by cells (73%) was esterified to neutral lipids. In addition, compared to lipids containing linoleate, a significantly greater proportion of lipids containing 13-HODE and/or its metabolites was secreted basolaterally. When the de novo synthesis of triacylglycerols and its secretion were examined, however, 13-HODE, compared to linoleic acid, was found to significantly lower the synthesis and secretion of newly synthesized triacylglycerols. Moreover, the fraction of triacylglycerols derived from de novo synthesis within the cell that was secreted was considerably less in cells incubated with 13-HODE than in cells incubated with linoleic acid. This is further substantiated by the observation that compared to cells incubated with linoleic acid, cells incubated with 13-HODE secreted significantly less triacylglycerol mass without altering (or only modestly altering) cellular triacylglycerol mass. The small decrease in cellular triacylglycerol mass observed in cells incubated with 250 μm of 13-HODE cannot account for the 40% decrease in the secretion of triacylglycerols compared to linoleic acid. Moreover, when linoleic acid and 13-HODE were added together to cells, the secretion of triacylglycerol mass remained depressed despite triacylglycerol mass within cells being similar to that observed in cells incubated with linoleic acid alone.

TABLE 2. Effect of 13-HODE on newly synthesized apoB released into the lumen of microsomes

	ApoB-100		ApoB-48	
	Linoleic Acid	13-HODE	Linoleic Acid	13-HODE
	cpm/preparation			
Microsomes (M) (total = sediment + supernatant)	6462 ± 239	5708 ± 123	2259 ± 72	2084 ± 130
Supernatants				
Without sodium carbonate (A)	1083 ± 83	743 ± 56	599 ± 65	542 ± 34
With sodium carbonate (B)	3359 ± 116	1891 ± 82[a]	1172 ± 61	1056 ± 71
Specific release of apoB due to sodium carbonate treatment				
Lumen (B − A)	2276 ± 70	1148 ± 105[a]	573 ± 34	515 ± 60
Percent in lumen (100 × (B − A)/M	36 ± 1%	20 ± 2%[a]	26 ± 2%	25 ± 3%

CaCo-2 cells were grown on T-75 flasks and transwells were incubated for 18 h with 250 μm 13-HODE or 250 μm linoleic acid. During the last 4 h of the incubation, cells grown on transwells were pulsed with radiolabeled methionine. Microsomes were prepared from cells pooled from six transwells and one flask. To monitor recoveries of apoB, one-third of the microsomal preparation was taken for immunoprecipitation of apoB. The remainder was divided into two equal portions and treated with or without sodium carbonate. The samples were then centrifuged and the supernatants containing the released intraluminal contents were immunoprecipitated with anti-apoB antisera. ApoB-100 and apoB-48 were separated by SDS-PAGE and the corresponding bands were cut from the gel and counted. Results represent mean ± SEM of 8 samples per treatment pooled from 3 experiments.

[a] $P < 0.05$ versus linoleic acid.

These results taken together suggest that the hydroxy fatty acid, independent of its availability for lipid synthesis and secretion, interferes with the secretion of triacylglycerols.

The secretion of apoB is believed to be regulated largely by post-translational events (33–35, review). Despite causing marked alterations in the amount of apoB secreted by cells in culture, apoB message levels usually remain unchanged (36, 37). Similarly, apoB message levels were similar in cells incubated with either 13-HODE or linoleic acid despite less apoB being synthesized and less newly synthesized apoB and apoB mass secreted by cells incubated with the hydroxy fatty acid. The availability of lipids within the lumen of ER and the rate of degradation of apoB regulate the amount of apoB secreted (16, 35, 37–40). Newly synthesized apoB is either incorporated into lipoproteins and secreted or, by default, degraded within the cell. During translation of apoB, the presence of lipids within the lumen of the ER appears to dictate the amount of apoB that is translocated into the lumen and destined for secretion. In the absence of lipids, or under conditions of inadequate lipid supply, newly synthesized apoB remains associated with the ER membrane and is ultimately degraded. Compared to microsomes prepared from cells incubated with linoleic acid, significantly less of newly synthesized apoB was found in the lumen of microsomes prepared from cells incubated with 13-HODE, suggesting that less apoB was translocated, and thus, destined for secretion. It is not likely that an inadequate supply of lipid could completely account for the differences in apoB secretion observed in cells incubated with either of the two fatty acids. As argued above, 13-HODE impaired the secretion of triacylglycerol and apoB even in the presence of adequate lipid substrates.

One possible explanation for these findings is that 13-HODE could have altered the activity of microsomal triglyceride transfer protein (MTP), an enzyme that catalyzes the co-translational lipidation of nascent apoB within the lumen of the ER (35). This was dismissed as, compared to MTP activity in cells incubated with linoleic acid, MTP activity in cells incubated with 13-HODE was similar. This finding was not unexpected in view of our recent observations in CaCo-2 cells demonstrating that MTP is not regulated by an influx of fatty acids and is likely not the rate-limiting step in fatty acid-mediated regulation of lipoprotein secretion (21). Because a significant proportion of lipids containing 13-HODE and/or its metabolites are secreted into the basolateral medium, it is possible that during lipoprotein assembly, the presence of hydroxyl groups in the hydroxy fatty acid-containing triacylglycerols may sterically hinder these molecules from associating with additional lipids and/or with newly synthesized apoB. If true, compared to cells incubated with linoleic acid, less triacylglycerol molecules would be packaged per lipoprotein particle in cells incubated with 13-HODE. Our findings are consistent with this hypothesis. The basolateral media from cells incubated with 13-HODE contained 69 ng of triacylglycerol mass per ng of apoB. In contrast, cells incubated with linoleic acid secreted 108 ng of triacylglycerol per ng of apoB. To address whether triacylglycerols containing hydroxy fatty acids are poor substrates for MTP would require the estimation of MTP-catalyzed transfer of hydroxy fatty acid-containing labeled triacylglycerols (41). Cost and unavailability of the labeled triacylglycerols prevented these experiments from being performed.

The results of the present study demonstrate that compared to linoleic acid, 13-HODE was readily taken up and esterified to cellular lipids. It was not, however, as effective as linoleic acid in promoting the synthesis and secretion of triacylglycerol-rich lipoproteins. Compared to linoleic acid, fewer lipoprotein particles containing less triacylglycerol molecules per particle were secreted in response to the hydroxy fatty acid. Furthermore, significantly more 13-HODE than linoleic acid was secreted in neutral lipid esters, suggesting that the composition of lipoprotein parti-

cles was altered by the oxidized fatty acid. As suggested by results of previous studies that have demonstrated the enhancement of foam cell and fatty streak formation after the ingestion of a diet enriched in oxidized lipids (42, 43), intestinally derived lipoproteins containing hydroxy fatty acids are likely to be more atherogenic than lipoproteins containing linoleic acid.

Manuscript received 13 October 1997 and in revised form 12 February 1998.

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