Apolipoprotein B mRNA Abundance Is Decreased by Eicosapentaenoic Acid in CaCo-2 Cells

Effect on the Synthesis and Secretion of Apolipoprotein B

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The regulation of apolipoprotein B (apo B) metabolism by eicosapentaenoic acid was investigated in CaCo-2 cells. Cells cultured on semipermeable membranes that separated an upper from a lower well were incubated for 48 hours with albumin alone or 1 mM eicosapentaenoic acid or oleic acid attached to albumin (4:1, mol/mol). Compared with cells incubated with oleic acid, cells incubated with eicosapentaenoic acid synthesized and secreted less [3H]glycerol-labeled triglycerides. Although both fatty acids increased cellular triglyceride mass compared with control cells, less triglycerides accumulated in cells incubated with the n-3 fatty acid. The secretion of triglyceride and apo B mass by cells incubated with eicosapentaenoic acid was less than that observed by cells incubated with oleate. The amount of apo B mass within cells, however, was not altered by either of the fatty acids and was similar to amounts found in control cells. Apo B mRNA abundance was decreased fourfold in cells exposed for 48 hours to eicosapentaenoic acid. In contrast, in cells incubated with oleic acid, apo B mRNA levels were not significantly altered. Pulse-chase experiments were performed to investigate the regulation of apo B synthesis and degradation by the fatty acids. In cells incubated with eicosapentaenoic acid, the synthesis and basolateral secretion of newly synthesized apo B-100 and apo B-48 were significantly less compared with control cells or cells incubated with oleic acid. In contrast, the synthesis and secretion of newly synthesized apo B in cells exposed to oleic acid were similar to control cells. Rates of apo A-1 synthesis were similar in cells incubated with either of the fatty acids. Compared with control cells and cells incubated with eicosapentaenoic acid, the residence time of labeled apo B in cells incubated with oleic acid was prolonged. The percentage of newly synthesized apo B that was degraded was less in cells incubated with oleic acid. In contrast, residence times and the percentages of apo A-1 and apo B-48 degraded were similar in control cells and cells incubated with the fatty acids. Thus, in CaCo-2 cells, compared with the effects of oleic acid, eicosapentaenoic acid impairs triglyceride transport in part by inhibiting apo B synthesis and secretion. The inhibition of apo B synthesis by eicosapentaenoic acid may be related to a decrease in gene transcription or a decrease in mRNA stability, as apo B mRNA levels were significantly decreased in cells incubated with this fatty acid. In contrast, compared with control cells, oleic acid did not increase either the rate of synthesis or the secretion of newly synthesized apo B-100. The monoenoic fatty acid did, however, delay the disappearance of apo B-100 in the cell and decrease the percentage of newly synthesized apo B-100 that was degraded. Because apo B mass secretion was increased by oleic acid, the results suggest that in intestinal cells, there is a preformed pool of apo B that is used for the transport of triglycerides into the lymph. (Arteriosclerosis and Thrombosis 1992;12:691-700)

KEY WORDS • CaCo-2 cells • apolipoprotein B • triglycerides • transcription

The predominant effect of dietary fish oil has been a lowering of very low density lipoprotein triglyceride (see Reference 1 for review). This effect has been ascribed to a decrease in the hepatic production of very low density lipoprotein, although a mechanism for this observation remains unclear. Most investigators have observed a decrease in triglyceride synthesis and secretion in the perfused livers of animals ingesting fish oil or in liver cells exposed for short periods to eicosapentaenoic acid, a major n-3 fatty acid of fish oil.2-4 In cultured rat hepatocytes, however, Lang and Davis5 reported that compared with oleic acid, eicosapentaenoic acid decreased the secretion of newly synthesized triglyceride and triglyceride mass without altering cellular triglyceride synthesis. Evidence was provided to suggest that the n-3 fatty acid impaired the assembly or secretion of very low density lipoprotein particles by inhibiting the secretion of newly synthesized apolipoprotein B (apo B). A decrease in the secretion of newly synthesized apo B by eicosapentaenoic acid was also observed by Wong et al6 in HepG2 cells.

The regulation of triglyceride transport by n-3 fatty acids in the intestine has been less well studied. In CaCo-2 cells, we have recently demonstrated that compared with oleic acid, eicosapentaenoic acid signifi-
cantly decreased the synthesis and secretion of newly synthesized triglyceride. In these short-term studies performed over 4 hours, however, the secretion of triglyceride mass by CaCo-2 cells incubated with either of the fatty acids was similar. In human subjects ingesting fish oil on a long-term basis, postprandial lipemia was significantly blunted compared with the postprandial lipemic response in subjects ingesting an oil enriched in n-6 fatty acids for a long time. In contrast, the short-term ingestion of either oil in subjects consuming a normal background diet caused a similar lipemic response. These observations led us to reexamine other mechanisms for the regulation of triglyceride transport by eicosapentaenoic acid in CaCo-2 cells exposed to fatty acids for more prolonged periods.

In the present study, CaCo-2 cells grown on polycarbonate filters were exposed to oleic or eicosapentaenoic acid for periods of up to 48 hours. The results suggest that the n-3 fatty acid impairs the assembly and/or secretion of triglyceride-rich lipoprotein particles by decreasing the transport of not only triglyceride mass but also apo B mass. Moreover, at 48 hours apo B mRNA abundance is significantly decreased, as are the synthesis and secretion of newly synthesized apo B in cells incubated with eicosapentaenoic acid.

**Methods**

**Materials**

- trans-[3H]methionine (1,100 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, Calif.).
- L-[2,3-3H]glycerol (200 mCi/mmol) and [α-32P]deoxycytidine triphosphate (3,000 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.).
- Oleic acid, eicosapentaenoic acid, fatty acid–poor bovine serum albumin, radioimmunoassay-grade bovine serum albumin, and triglyceride analysis kit, salmon sperm, dextran sulfate, and prestained gel electrophoresis standards (molecular weight range, 25–180 kd) were purchased from Sigma Chemical Co. (St. Louis, Mo.).
- Fatty acids were prepared in 95% ethanol and stored under nitrogen at 4°C. Sodium salts of the fatty acids were prepared by adding excess sodium hydroxide to the required aliquots of the stock solutions. The ethanol was then evaporated under nitrogen, and the sodium salt of the fatty acid was dissolved in 0.5 ml hot distilled water and added to a rapidly stirred solution of modified DMEM (GIBCO) supplemented with 20% delipidated fetal calf serum (FCS). The molar ratio of fatty acid to albumin was 4:1.

**Fatty Acid–Albumin Preparation**

Stock solutions of eicosapentaenoic or oleic acid were prepared in 95% ethanol and stored under nitrogen at 4°C. Sodium salts of the fatty acids were prepared by adding excess sodium hydroxide to the required aliquots of the stock solutions. The ethanol was then evaporated under nitrogen, and the sodium salt of the fatty acid was dissolved in 0.5 ml hot distilled water and added to a rapidly stirred solution of modified DMEM (GIBCO) supplemented with 20% delipidated fetal calf serum (FCS). The molar ratio of fatty acid to albumin was 4:1.

**Triglyceride Synthesis**

At the times indicated in the figures, the cells were washed, M199 containing 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4, without serum was placed in the bottom wells, and the DMEM containing 20% delipidated FCS and 1 mM of either eicosapentaenoic or oleic acid attached to albumin was added to the top wells. Control cells received the DMEM containing all components except the fatty acid. Four hours before terminating the experiment, 10 μCi [3H]glycerol was added to the apical chamber. After the 4-hour incubation, the cells were washed and scraped carefully from the filter by a rubber policeman. Lipids in the cells and the basal medium were extracted with chloroform/methanol (2:1, vol/vol), and the chloroform extract was washed with methanol/water (1:1, vol/vol). The chloroform extract was dried under a stream of nitrogen and applied to silica-gel G plates in 125 μl chloroform. The plates were developed in hexanes/diethyl ether/acetic acid/methanol (170:30:2:2, vol/vol/vol/vol), and the lipids were visualized by exposure to iodine vapor. The bands corresponding to triglycerides were scraped from the plate and counted by liquid scintillation.

**Apolipoprotein B Mass**

At the end of the incubation, the basal medium was diluted with an equal amount of buffer A (phosphate-buffered saline [PBS] containing 1% Triton X-100, 20 mM Tris-HCl, pH 7.4), and the samples were then assayed by the methods of Biochemistry, University of Iowa, Iowa City, Iowa. All other reagents were reagent grade.
mM methionine, 5 mM Na₂EDTA, 0.3 mM aprotinin, 2 μM pepstatin A, 0.1 mM leupeptin, 0.025 mM N-α-p-tosyl-L-lysine chloromethyl ketone, 0.05 mM benzamidine, and 0.05 mM phenylmethylsulfonyl fluoride (PMSF). The cells were rinsed in ice-cold PBS and scraped into 200 μl buffer A. All samples were stored at −80°C until analysis (within 48–72 hours). Apo B mass was estimated by sandwich enzyme-linked immunosorbent assay. Ninety-six-well Nunc immunoplates were precoated with 1 μg monoclonal antibody to apo B in 200 μl 0.05 mM NaHCO₃ buffer, pH 8. This monoclonal antibody binds apo B-100 and apo B-48 as determined by immunoblot. The plates were then covered with Saran Wrap and incubated for 18 hours at 4°C. The bound sites on the wells were blocked by adding to the buffer containing the unbound antibodies was drained from the plate, and the wells were washed three times with PBS containing 0.05% Tween-20 using a Nunc immunowasher (Nunc, Roskilde, Denmark). The unbound sites on the wells were blocked by adding to the wells 200 μl 3% bovine serum albumin (radioimmunoassay grade) in PBS and incubated for 1 hour at room temperature. The wells were then washed and blotted dry. Apo B standard was diluted with buffer B (PBS containing 0.05% Tween-20 and 0.5% bovine serum albumin), and aliquots of 200 μl containing between 0 and 5 ng apo B were added per well in triplicate. Samples of the cell and basolateral media were diluted and added to the wells in triplicate so that the resulting apo B concentrations fell within the standard curve. The plate was wrapped in Saran Wrap and incubated for 18 hours at room temperature. The wells were then washed and blotted dry. Two hundred microliters of buffer B containing 10 ng anti-apo B immunopurified sheep polyclonal antibody conjugated to horseradish peroxidase was added to each well. The plate was again wrapped and incubated for 4 hours at room temperature. The wells were then washed three times with PBS containing 3% bovine serum albumin followed by three washes in PBS and blotted dry. The activity of horse-radish peroxidase was estimated by adding 100 μl 3',5',5'-tetramethylbenzidine and hydrogen peroxide (1:1, vol/vol). After 10 minutes, the reaction was stopped with the addition of 100 μl 1N HCl. The absorbance was determined at 450 nm using a TiterTek Multiscan MCC/340 plate reader (Flow TiterTek, McLean, Va.). The coefficient of variation between assays within a 3-month period was 11% (n=12) and within assays, 6% (n=18).

**Apolipoprotein B and Apolipoprotein A-I Synthesis and Degradation**

CaCo-2 cells were cultured on 6.5-mm Transwell inserts. On day 12, the apical medium was changed to 1 mM oleic or eicosapentaenoic acid complexed to 250 μM albumin in DMEM containing 20% delipidated FCS. Control cells received the same medium but without the fatty acid. The basal chambers were washed and filled with DMEM without serum or fatty acids. After 48 hours, the apical medium was aspirated and the cells were rinsed in methionine-free M199. They were then incubated for 1 hour in methionine-free M199 that contained the fatty acids attached to albumin. Methionine-free M199 was added to the basal well. The addition of 150 μCi [³⁵S]methionine to the apical well was staggered so that all cells were incubated for the same amount of time. After the incubation with the label, the cells were rinsed in ice-cold PBS containing 10 mM methionine and scraped gently from the filter in 0.2 ml buffer A. The cells were sonicated for 10 seconds and centrifuged for 5 minutes at 13,000g in a refrigerated microfuge. Aliquots of the supernatant and the basal medium were taken for estimation of trichloroacetic acid (TCA)–precipitable protein and immunoprecipitation of apo A-I and apo B. A portion of the cell supernatant was taken for protein determination. The aliquot taken for immunoprecipitation was diluted to 0.5 or 1 ml with buffer C (10 mM NaH₂PO₄, pH 7.5, containing 5 mM EDTA, 100 mM NaCl, 1% [vol/vol] Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.5% [wt/vol] sodium deoxycholate, 1 mM PMSF, 20 μM leupeptin, and 1 mM dithiothreitol).

To estimate degradation, cells were pulsed for 20 minutes with 200 μCi [³⁵S]methionine. After the cells were rinsed in M199 containing 10 mM methionine, they were incubated in M199 with the excess methionine. The inserts were transferred to a new 24-well plate containing 0.6 ml M199 and 10 mM methionine. At the specified time, the cells were harvested in 0.2 ml buffer A. The cell homogenate and the basal medium were analyzed as described above for estimation of synthesis.

**Immunoprecipitation of Apolipoprotein B and Apolipoprotein A-I**

Aliquots from cell and basolateral media containing 5×10⁴ TCA-precipitable counts were preclarified of nonspecific counts by incubation with 0.2 ml IgG sorb (10% wt/vol) on a tube rocker for 1 hour at 4°C. This was followed by centrifugation for 5 minutes in a refrigerated microfuge. To the supernatant was added rabbit immunopurified polyclonal antibody directed against human apo B and rabbit sera containing antibodies to human apo A-I. The amount of antibody used was in excess, as estimated by repeating the immunoprecipitation on the supernatant from the first immunoprecipitation. The samples were incubated for 18 hours at 4°C in a rocker. The antigen–antibody complexes were precipitated with 50 μl protein A bound to agarose (10%, wt/vol) (binding capacity, 4 mg human IgG/ml). The samples were rocked for 1 hour and centrifuged for 30 seconds at 13,000g. The pellet containing the antigen–antibody complex bound to protein A was washed six times with 1 ml buffer C and once with 1 ml PBS. The washed pellet was taken up in 40 μl 1× Laemmli sample buffer, and the proteins were separated by electrophoresis on 5% stacking and 8% separating porous gels as described by Doucet et al. After electrophoresis, the gels were treated with fixative solution (40% methanol, 7% acetic acid) for 30 minutes, washed three times with distilled water, and soaked for 30 minutes at room temperature in 1 M sodium salicylate. The gels were dried at 80°C for 2 hours, and the radioactive bands were detected by exposing the gels to Kodak X-Omat AR film for 48–72 hours. The radioactivity from gel portions corresponding to apo B-100, apo B-48, and apo A-I bands was determined by liquid scintillation.
Plots showing accumulation and secretion of newly synthesized triglycerides. Seven-day-postconfluent CaCo-2 cells were incubated for 48 hours with 1 mM of either eicosapentaenoic or oleic acid attached to albumin as described in "Methods." Control cells were incubated with albumin alone. During the last 4 hours of incubation, the incorporation of [3H]glycerol into cellular triglycerides and triglycerides secreted into the basolateral medium was estimated. Results are expressed as mean±SEM of radiolabel incorporated into triglycerides per milligram of cell protein. ○, Control; ●, oleic acid; V, eicosapentaenoic acid. n=6, *p<0.001 vs. control and eicosapentaenoic acid.

Apolipoprotein B mRNA Estimation
After 48 hours of incubation with the respective fatty acids, RNA was extracted with an acid guanidinium isothiocyanate/phenol/chloroform mixture as described by Chomczynski and Sacchi.15 Total RNA (0.10–1.0 µg) was applied to nylon membrane filters (Nytran, Midwest Scientific, Valley Park, Md.) via slot-blot apparatus. The filters were dried at 80°C in a vacuum oven. Prehybridization was performed at 42°C for 2 hours in buffer containing 20 ml formamide, 2 ml Denhardt's solution, 10 ml 20X saline–sodium phosphate–EDTA buffer (SSPE), 0.2 ml 20% SDS, 0.4 ml salmon sperm (10 mg/ml), and 7.4 ml diethyl pyrocarbonate water. After labeling the respective cDNA probes by random priming,16 duplicate filters were hybridized with either 0.1 µg 32P–apo B cDNA or 32P–α-actin cDNA. The filters were washed twice for 15 minutes at room temperature in 6× SSPE and 0.1% SDS, once at 42°C for 30 minutes in 1× SSPE and 0.1% SDS, and once at 65°C for 45 minutes in 1× SSPE and 0.1% SDS. The filters were exposed to x-ray film with intensifying screens for 24–48 hours. mRNA abundances for apo B and actin were quantified by laser densitometry of the autoradiograms. The densities of apo B mRNA were normalized to the densities of α-actin mRNA.

Cell Viability
The viability of cells grown on permeable membranes after 48 hours of incubation with the fatty acids was evaluated by measuring the release of cellular lactate dehydrogenase (LDH).7 In the basal medium, the percent release of cellular LDH was 0.96±0.06%, 1.07±0.11%, and 0.71±0.10% for eicosapentaenoic acid, oleic acid, and control cells, respectively. The percentage of cellular LDH activity detected in the apical media was higher, 2.56±0.07%, 2.16±0.18%, and 2.46±0.09%, respectively. No differences between any of the treatments were observed.

Chemical Analysis
Protein was measured according to the method of Lowry et al.17 Triglyceride mass was measured as previously described.7

Statistical Analysis
One-way analysis of variance was used to determine differences between the means of the three treatments—oleic acid, eicosapentaenoic acid, and control. Pairwise comparisons of the means were performed using Tukey's t test.

Results
Synthesis and Basolateral Secretion of Labeled Triglyceride
CaCo-2 cells were cultured on polycarbonate filters separating an upper and a lower well. Experiments were performed on days 12–14 after plating, approximately 7–9 days after confluence was reached. In the first experiment, CaCo-2 cells were incubated for 48 hours in DMEM containing 20% delipidated FCS with 1 mM oleic or eicosapentaenoic acid attached to albumin (4:1, mol/mol) or albumin alone. The rate of triglyceride synthesis and its secretion into the basolateral medium was determined by the addition of labeled glycerol to the apical medium during the last 4 hours of the incubation. As shown in Figure 1 (left panel), the basolateral secretion of labeled triglyceride was significantly less in cells incubated with eicosapentaenoic acid compared with that observed in cells incubated with oleic acid. Moreover, the n-3 fatty acid did not increase the secretion of newly synthesized triglyceride above
that observed for control cells. Compared with cells incubated with oleic acid, the rate of triglyceride synthesis was also significantly decreased by eicosapentaenoic acid (right panel).

**Secretion of Triglyceride Mass**

To investigate whether the marked inhibition of triglyceride synthesis by the n-3 fatty acid would lead to a decrease in secretion of triglyceride mass, the triglyceride mass within cells and that secreted into the basolateral medium were determined after 12-, 24-, and 48-hour incubations with the respective fatty acid. The results are shown in Figure 2. Compared with the mass of triglyceride secreted by cells incubated with oleic acid, the secretion of triglyceride mass by cells incubated with the n-3 fatty acid was significantly diminished (left panel). Both fatty acids stimulated triglyceride secretion above that observed in control cells incubated with albumin alone. Triglycerides did accumulate in CaCo-2 cells incubated with either of the fatty acids; however, significantly less triglycerides accumulated in cells incubated with eicosapentaenoic acid (right panel).

**Secretion of Apolipoprotein B Mass**

If the amount of labeled triglyceride or triglyceride mass secreted into the basolateral medium was normalized to the amount present within the cell, the fractional secretion of triglyceride by cells incubated with eicosapentaenoic acid compared with cells incubated with oleic acid was significantly less (2% versus 6% and 12% versus 20% for labeled triglyceride and triglyceride mass, respectively, at 48 hours).

To determine whether eicosapentaenoic acid caused a decrease in the number of triglyceride-rich lipoprotein particles being secreted, apo B mass was estimated in the basolateral medium of cells incubated with eicosapentaenoic acid compared with controls, this difference did not reach statistical significance. In contrast, apo B mass within cells did not change during the 48-hour incubation with the fatty acids and remained similar to the amounts observed in control cells, i.e., approximately 0.80 μg/mg protein (data not shown).

**Apolipoprotein B mRNA Abundance**

Apo B mRNA abundance was estimated by slot-blot and Northern hybridizations from RNA extracted from cells incubated for 48 hours with the fatty acids. The blots were hybridized with a labeled cDNA probe for apo B and actin. The slot blot is shown in Figure 4. Compared with apo B mRNA levels in control cells (relative density of 1.00) or cells incubated with oleic acid, the fractional occupancy of the blots was significantly less (2% versus 6% and 12% versus 20% for labeled triglyceride and triglyceride mass, respectively, at 48 hours).

To determine whether eicosapentaenoic acid caused a decrease in the number of triglyceride-rich lipoprotein particles being secreted, apo B mass was estimated in the basolateral medium of cells incubated with eicosapentaenoic acid compared with controls, this difference did not reach statistical significance. In contrast, apo B mass within cells did not change during the 48-hour incubation with the fatty acids and remained similar to the amounts observed in control cells, i.e., approximately 0.80 μg/mg protein (data not shown).
Acid, apo B mRNA abundance was significantly decreased in cells incubated with the n-3 fatty acid (density of 0.26). Compared with control cells, apo B mRNA levels were similar in cells incubated with oleic acid (density of 1.20). The slot blot that was hybridized with labeled actin substantiated that the amounts of RNA applied to the filters were similar. In data not shown, the Northern provided similar results, demonstrating a significant decrease in the 14-kb mRNA for apo B in cells incubated with eicosapentaenoic acid compared with control cells or cells incubated with oleic acid.

Apolipoprotein B Synthesis

To investigate whether the changes observed in apo B mRNA levels were associated with alterations in the synthesis of apo B, CaCo-2 cells incubated for 48 hours with the respective fatty acid were pulsed with 150 μCi [35S]methionine, and the incorporation of labeled methionine into immunoprecipitable apo B-100, apo B-48, and apo A-I was estimated. An autoradiogram of the polyacrylamide gel from this experiment is shown in the left panel of Figure 5, and the amount of radioactivity in the apoprotein bands cut from this gel is shown in the right panel of Figure 5. The incorporation of labeled methionine into cellular and medium apo B was significantly decreased in cells incubated with eicosapentaenoic acid compared with that observed in cells incubated with oleic acid. In contrast, oleic acid did not alter the incorporation of label into either cellular or medium apo B compared with controls. Moreover, the ratio of label observed in apo B-100 and apo B-48 was not changed by any of the treatments, suggesting that apo B mRNA editing was likely unaltered by the fatty acids. Compared with control cells, there was a modest decrease in the rate of synthesis and secretion of apo A-I in cells incubated with either of the fatty acids, particularly at the later time points (2 and 3 hours). Overall rates of apo A-I synthesis (cell plus medium), however, were similar in cells incubated with eicosapentaenoic or oleic acid. Neither of the fatty acids significantly affected the incorporation of labeled methionine into TCA-precipitable protein within cells or that found in the basolateral medium.

Apolipoprotein B Degradation

Degradation of labeled apo B was estimated by pulse-chase experiments after 48 hours of incubation with the respective fatty acids. Cells were pulsed for 20 minutes with 200 μCi [35S]methionine and then chased with excess unlabeled methionine for 3 hours. Apo B and apo A-I were immunoprecipitated from the cells and the basolateral medium. Figure 6 shows the autoradiogram (left panel) and radioactivity data (right panel) from this experiment. Maximal incorporation of the radiolabel into apo B and apo A-I occurred 15 minutes into the chase; therefore, this time point was taken as 100%. As shown on the autoradiogram, it can again be appreciated that in cells incubated with eicosapentaenoic acid, there was a significant decrease in the secretion of labeled apo B-100 and apo B-48 compared with that observed in control cells or cells incubated with oleic acid. In contrast, labeled apo A-I secretion was not significantly altered by the fatty acids. The amount of labeled apo B-100 remaining within the cell during the chase (residence time) was increased in cells incubated with oleic acid, whereas the residence time of apo B in control cells and cells incubated with eicosapentaenoic acid was similar. The percentage of labeled apo B-100 that was not recovered was 39%, 15%, and 33% for control cells, cells incubated with oleic acid, and cells incubated with eicosapentaenoic acid, respectively. These percentages estimate the amount of apo B-100 degraded for each treatment. Residence times and the percentages of apo A-I and apo B-48 degraded were similar among the treatment groups.

Discussion

It is clear from the results of this study that compared with oleic acid, the n-3 fatty acid, eicosapentaenoic acid, inhibits the transport of triglyceride mass by CaCo-2 cells. This can be partially explained by a rather marked inhibition of triglyceride synthesis and a decrease in the accumulation of cellular triglycerides in cells incubated with this fatty acid. In a previous report of CaCo-2 cells, after 4 hours of incubation eicosapentaenoic acid did not inhibit the secretion of triglyceride mass despite having caused a significant decrease in triglyceride synthesis. This would suggest that there is a lag period between the time of inhibition of triglyceride synthesis and the time required to deplete a critical cellular pool of triglyceride resulting in a decrease in triglyceride mass secretion. Inhibition of triglyceride synthesis by...
**Figure 5.** [35S]methionine incorporation into immunoprecipitable apolipoprotein B-100, B-48, and A-I. The incubation was performed as described in the legend to Figure 1 and “Methods.” After 1-hour incubation in methionine-free medium containing albumin alone or the respective fatty acid, the cells were pulsed with 150 μCi [35S]methionine. Addition of the label was staggered so that cells were incubated for the same amount of time. At the end of 3 hours, the incorporation of labeled methionine into apolipoprotein B-100, B-48, and A-I cells and that secreted into the basolateral medium was estimated by immunoprecipitation and separation of the apoproteins by polyacrylamide gel electrophoresis as described in “Methods.” This figure represents an autoradiogram of that gel (left panel) and the radioactivity in the bands corresponding to apolipoprotein B-100, B-48, and apo A-I (right panel). ○, Control; ●, oleic acid; ▲, eicosapentaenoic acid (EPA).
Degradation of apolipoprotein B. The incubation was performed as described in the legend to Figure 1 and “Methods.” After 1-hour incubation in methionine-free albumin alone or the respective fatty acid, the cells were pulsed with 200 μCi [35S]methionine for 20 minutes and then chased with 10 mM unlabeled methionine as described in “Methods.” At the specified time the cells and basolateral media were harvested, and apolipoproteins B and A-I were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis as described. The left panel of Figure 6 represents an autoradiogram of that gel. From two individual experiments, the right panel of Figure 6 demonstrates the radioactivity found in the bands corresponding to apolipoprotein B-100, B-48, and A-I (expressed as a percentage of the radioactivity present within the cell 15 minutes into the chase). Values for maximal radioactivity incorporated into the apoproteins after 15 minutes of chase were as follows: for apo B-100—control, 970; oleate, 917; and eicosapentaenoic acid, 655; for apo B-48—control, 520; oleate, 667; and eicosapentaenoic acid, 477; for apo A-I—control, 355; oleate, 917; and eicosapentaenoic acid, 2810. □, Control; ●, oleic acid; ▼, eicosapentaenoic acid (EPA).
eicosapentaenoic acid is but one mechanism to explain the inhibition of triglyceride secretion by CaCo-2 cells. This is not the whole explanation, however. Of the amount of cellular triglyceride mass or the amount of newly synthesized triglyceride present within cells, the fraction that was secreted by cells incubated with eicosapentaenoic acid remained significantly less compared with the fraction secreted by cells incubated with oleic acid. The n-3 fatty acid, therefore, most likely impairs the assembly and/or secretion of lipoprotein particles by CaCo-2 cells. This was substantiated by the observation that compared with cells incubated with oleic acid, the secretion of apo B mass was significantly less in cells exposed to eicosapentaenoic acid, suggesting that fewer lipoprotein particles were being secreted. Although the suggestion that eicosapentaenoic acid impairs lipoprotein assembly/secretion has been made previously,3 this is the first study that demonstrates a decrease in the secretion of both triglyceride and apo B mass by eicosapentaenoic acid in cultured cells. In cultured rat hepatocytes, Lang and Davis5 observed a decrease in the secretion of triglyceride mass in cells incubated with eicosapentaenoic acid compared with oleate. In contrast to our results, however, similar amounts of lipoproteins were accumulated within the cells in response to the two fatty acids. The secretion of labeled apo B by hepatocytes exposed to eicosapentaenoic acid was also decreased when compared with the amount secreted by cells incubated with oleic acid. Wong et al6 made very similar observations in HepG2 cells and concluded that n-3 fatty acids interfered with the normal secretion of lipoproteins. The present study supports those conclusions in CaCo-2 cells.

Neither oleic nor eicosapentaenoic acid altered the levels of apo B mass within CaCo-2 cells despite having stimulated triglyceride transport. Moberly et al16 made a similar observation in HepG2 and CaCo-2 cells incubated with eicosapentaenoic acid compared with oleate. Compared with control cells, oleate caused a significant increase in the secretion of apo B mass by both cell types without changing the amount of cellular apo B. It could be postulated that hepatocytes and intestinal cells rigidly maintain an intracellular pool of preformed apo B that can be called on during times of acute fatty acid influx. This makes good sense. The intestinal absorptive cell must respond rapidly to a large influx of fatty acids after the ingestion of a fatty meal. For normal absorption to occur, the cell must reestablish the fatty acids, assemble the lipoprotein and package it, and secrete it into the lymph. To synthesize a protein the size of apo B each time an intestinal cell is exposed to a large influx of fatty acids seems impractical and inefficient. This postulate is supported by data from the present study as well as others. Compared with CaCo-2 cells incubated with albumin alone, the secretion of apo B mass was significantly increased by oleic acid. This supports a previous observation by Dashi et al.19 The incorporation of labeled methionine into cellular or medium apo B, however, was not increased by oleate. Lang and Davis5 and Wong et al6 found similar results in cultured hepatocytes. Thus, in response to an influx of oleic acid, an increase in the rate of apo B synthesis is not required by CaCo-2 cells or liver cells to transport triglycerides or apo B. There is a report, however, that disagrees with these observations. Although intracellular synthesis of apo B was not determined, Moberly et al16 observed an increase in the secretion of newly synthesized apo B in HepG2 and CaCo-2 cells incubated with oleic acid. However, only a 24-hour time point was reported, and labeled apo B was not immunoprecipitated from the medium before gel electrophoresis. Other than these differences in experimental design, the reasons for this disparity among the three studies are unclear.

This is the first study to demonstrate an inhibition of apo B synthesis and a decrease in mRNA abundance for apo B by eicosapentaenoic acid in cultured cells. A 48-hour incubation period was chosen for this study because shorter periods of incubation were thought to be too short to detect changes in mRNA abundance.6 Pullinger et al17 estimated the half-life of apo B mRNA in HepG2 cells to be 16 hours. Wong et al6 failed to detect changes in apo B mRNA abundance by eicosapentaenoic acid after 3 hours of incubation with HepG2 cells, a duration probably much too short to observe changes in apo B mRNA levels. Lang and Davis5 in their study did not measure apo B message levels. These investigators failed to detect differences in [35S]methionine-labeled apo B in hepatocytes incubated for 18 hours with the long-chain fatty acids and either oleic acid or eicosapentaenoic acid. It is likely, however, that after 18 hours of incubation with the label, steady-state levels of cellular labeled apo B were estimated and not apo B synthetic rates. It has already been shown that cellular apo B mass does not change in response to either of the fatty acids.18 Thus, it is our conclusion that after more prolonged exposure to eicosapentaenoic acid, apo B synthetic rates are decreased in CaCo-2 cells, and this observation can be related to a decrease in mRNA abundance. It should also be noted that the synthesis of both apo B-100 and apo B-48 was inhibited similarly by eicosapentaenoic acid. The ratio of labeled apo B-100 to apo B-48 was not changed by fatty acid exposure, suggesting that apo B mRNA editing was not regulated by fatty acid influx.

It has recently been reported that the intracellular degradation of apo B may play a significant role in regulating apo B secretion.20-22 For example, Dixon et al21 observed in HepG2 cells that oleate increased the secretion of newly synthesized apo B by inhibiting early intracellular degradation. Sparks and Sparks21 found in rat hepatocytes that insulin inhibited apo B secretion by increasing the rate of intracellular apo B degradation. As stated earlier, in the present study oleic acid influx did not cause an increase in the secretion of newly synthesized apo B by CaCo-2 cells, yet in support of previous observations in HepG2 cells,22 we also observed that oleic acid suppressed the rate of degrada-

tion of newly synthesized apo B. These data would suggest that in the intestinal cell, in response to an influx of oleic acid the newly synthesized apo B enters a larger pool of preformed apo B that is destined for secretion. With the influx of this fatty acid, less newly synthesized apo B is degraded, thereby leaving more available to replenish the preformed pool during lipoprotein assembly and secretion.

The data derived from cells incubated with eicosapentaenoic acid also support the postulate of a preformed pool of secretory apo B. Unlike oleic acid, the long-term administration of eicosapentaenoic acid did not drive lipoprotein secretion, as evidenced by similar
amounts of apo B mass being secreted by control cells versus cells incubated with the n-3 fatty acid. Despite similar amounts of apo B mass being secreted compared with control cells, eicosapentaenoic acid caused a significant decrease in the rate of methionine incorporation into newly synthesized apo B within cells and that found in the basolateral medium. This suggests that under our experimental conditions and in this time frame, newly synthesized apo B is not in equilibrium with the preformed pool. With continued eicosapentaenoic acid administration, it is likely that apo B mass secretion would eventually fall below the levels secreted by control cells, as less newly synthesized apo B would enter the preformed pool. The question as to why the influx of eicosapentaenoic acid would cause a decrease in apo B mRNA abundance and hence apo B synthetic rates remains under investigation.

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Apolipoprotein B mRNA abundance is decreased by eicosapentaenoic acid in CaCo-2 cells.
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