Changes in fatty acid metabolism in rat hepatocytes in response to dietary n–3 fatty acids are associated with changes in the intracellular metabolism and secretion of apolipoprotein B-48

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Abstract Cultured hepatocytes from rats fed a low fat, chow diet (LF) or diets rich in fish oil (FO, 20% v/w) or olive oil (OO, 20%, v/w) were used to determine how the intracellular metabolism and secretion of apolipoproteins (apo)B-100 and B-48 respond to in vivo and in vitro manipulations of fatty acid esterification, storage, secretion, and oxidation. Hepatocytes from the FO- and OO-fed rats had higher initial triacylglycerol (TAG) contents and higher rates of fatty acid oxidation and ketogenesis than hepatocytes from the LF-fed group. However, only in the cells from the FO-fed animals was there any decrease in the rate of TAG synthesis and in the secretion of VLDL TAG. Decreased secretion of TAG by the FO hepatocytes was accompanied by a decreased synthesis and degradation of apoB, particularly apoB-48, and a decreased secretion of apoB-48 VLDL. In all dietary groups a substantial proportion of the apoB-48 and apoB-100 secreted into the medium was associated with small, lipid-poor particles of density >1.006. FO feeding had no effect on the amounts of apoB-48 and apoB-100 that appeared in this fraction. Dietary composition affected the channelling of exogenous oleate in the culture medium into the oxidative and esterification pathways. While exogenous fatty acid increased the secretion of VLDL TAG in the FO-fed group, VLDL TAG secretion remained lower than that observed in the hepatocytes from the LF- and OO-fed groups cultured under identical conditions. Exogenous oleate did not significantly increase the secretion of either newly-synthesized apoB-48 or apoB-100 by hepatocytes in either of the dietary groups. We conclude that, in rat liver, a decreased capacity to transport TAG out of the hepatocyte after consumption of a diet rich in fish oil is associated with a decreased synthesis and presecretory degradation of apoB-48, and with a decreased secretion of VLDL apoB-48.—**Brown, A-M., P. W. Baker, and G. F. Gibbons.** Changes in fatty acid metabolism in rat hepatocytes in response to dietary n–3 fatty acids are associated with changes in the intracellular metabolism and secretion of apolipoprotein B-48. *J. Lipid Res.* 1997. **38:** 469–481.

Supplemetary key words very low density lipoprotein • triacylglycerol • fatty acid oxidation • ketogenesis • fatty acid esterification • phospholipids • monounsaturated fat

Numerous epidemiological studies have demonstrated an inverse relationship between the incidence of cardiovascular disease (CD) and the consumption of oily fish (e.g., 1,2). Subsequently, the effect of fish oils, rich in long chain n–3 polyunsaturated fatty acids (n–3 PUFA), on the risk factors for CD have been investigated and have been shown to be beneficial in reducing plasma triacylglycerol (TAG) (3–5). Eicosapentaenoic and docosahexaenoic acids (EPA and DHA), both long chain n–3 PUFA, are believed to be the active components of fish oil. Although many studies have shown hypertriglyceridemia to be associated with an increased risk of CD (6–9) controversy still exists as to whether plasma TAG can be classified as an independent risk factor. Nevertheless, intervention studies have shown that TAG-lowering drugs may lead to clinically significant reductions in the risk of CD (10). Hence, dietary supplementation with either fish oils or marine-derived n–3 PUFA has been suggested as a preventative measure against CD (10, 11).

In vivo VLDL is synthesized and secreted by the liver primarily to transport hepatic TAG to adipose tissue for storage or to muscle for oxidation (12, 13). Several studies indicate that the TAG-lowering properties of marine-derived n–3 PUFA are mediated at the hepatic level by reducing the rate of VLDL TAG secretion. This appears to be the case both in human subjects (14, 15) and in

Abbreviations: VLDL very low density lipoprotein; apoB, apolipoprotein B; TAG, triacylglycerol; BSA, bovine serum albumin; CD, cardiovascular disease; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PL, phospholipid; DAG, diacylglycerol.

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animal models (16–18). At present, however, there is no general consensus of opinion as to the primary mechanism underlying the inhibitory effect of n–3 PUFA on hepatic VLDL secretion. In particular, controversy exists as to whether or not the decline in VLDL TAG secretion is accompanied by a decreased output of apoB. For instance, dietary studies in non-human primates (19) showed that consumption of n–3 fatty acids had no effect on apoB secretion by perfused livers. On the other hand, some non-dietary studies in which n–3 fatty acids were added directly to isolated liver preparations in vitro suggested that apoB output declined (16, 20), an effect that was ascribed to an increased rate of apoB degradation (21, 22). Another complication arises in differences in the experimental model used, and, in particular, between those that secrete particles containing apoB-100 and those that secrete particles containing both apoB-100 and apoB-48. For instance, studies in the rat have shown that the hepatic secretion of apoB-48 is more sensitive to dietary manipulation than is that of apoB-100 (12) and evidence is now emerging that the intracellular assembly of apoB-48 particles may differ subtly from that of apoB-100-containing particles (23–25). These differences may be associated with variations in neutral lipid availability resulting from changes in intracellular fatty acid metabolism. Dietary n–3 fatty acids have unique effects across a broad spectrum of hepatic fatty acid metabolic pathways including synthesis (26), mitochondrial oxidation (27), esterification (28), and peroxisomal metabolism (29). The main question we have asked, therefore, is whether or not dietary n–3 fatty acid consumption in rats affects various aspects of apoB metabolism including synthesis, degradation, secretion, and lipoprotein particle size and, if so, whether apoB-100 and apoB-48 are differentially affected. In some cases, rats were fed a diet containing olive oil, rich in triolein, which is generally considered to be relatively inert in terms of hepatic lipid metabolism. This group of animals was used to confirm that the observed effects of dietary n–3 fatty acids were not a result of a nonspecific effect of a high fat diet.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade. Waymouth's medium and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco Ltd. (Paisley, Scotland, U.K.); [9,10(n)-^{3}H]oleate, [^{35}S]methionine, and NCS-II tissue solubilizer were from Amersham International (Aylesbury, Bucks, U.K.); bovine pancreas insulin, dexamethasone, sodium oleate, and anti-sheep IgG antibody were from Sigma (Poole, Dorset, U.K.); 3-hydroxybutyrate dehydrogenase *(Rhodobacter spheroides)* and anti-human apolipoprotein B (apoB) antiserum were obtained from Boehringer-Mannheim (Lewes, Sussex, U.K.). MaxEPA was kindly supplied by Seven Seas Ltd. (Marfleet, Hull, HU9 5NJ, U.K.). Filippo Berrio Extra Virgin Olive Oil was used for this study. Acrylamide/bis-acrylamide (40%, w/v) was purchased from Bio-Rad Laboratories Ltd. (Hemel Hempstead, Herts, U.K.) Autoradiography film (blue-sensitive X-ray film) was obtained from Genetic Research Instrumentation (Dunmow, Essex, U.K.). Anti-rat apoB antiserum was raised in rabbits as described previously (25).

Preparation of diets

Rat and Mouse No. 3 Breeding Diet from Special Diet Service (Witham, Essex, U.K.) was used as the low fat (LF) control diet. This diet contained 4.3% fat, 51.2% carbohydrate (mainly starch), 22.3% protein, 4.5% fibre, and 7.7% ash. The 20% v/w olive oil (OO) and MaxEPA (FO) diets were prepared by mixing 200 ml of the respective oil with 800 g of powdered LF diet and 400 ml of deionized water. After thorough mixing, the diets were re-pelleted and allowed to dry in a well-ventilated area with subdued light. All diets contained the following antioxidants; α-tocopherol (110 mg/kg), vitamin E (120 mg/kg), vitamin C (10 mg/kg), and β-carotene (200 μg/kg). After preparation of the diets, extraction of lipids (30) followed by *trans*-methylation of the fatty acids and quantitative GC analysis indicated that these anti-oxidants were sufficient to prevent oxidation of the n–3 fatty acids during preparation. The OO and FO diets were then kept frozen at −20°C until use. The LF diet contained 3.66 cal/g and the OO and FO diets contained 4.69 cal/g.

Dietary regime

Male rats (Wistar strain) were fed either LF, OO, or FO diet for 14 days. For the duration of the feeding period rats were housed individually in cages; wire-based cages were used to separate the animals from urine and feces. Room temperature was kept constant at 20 ± 2°C with a 10-h light/14-h dark cycle. Food and water were given ad libitum. Food was replaced daily with any uneaten portion discarded. These studies were performed over a 12-month period. At any particular time over this period two groups of rats, each containing 2 rats, were fed. The groups were randomly spread to minimize seasonal variation. On the 14th day, one rat from each group was used for hepatocyte isolation (2 h into the light cycle); the remaining rats were

used to obtain blood and tissue. Plasma was obtained on the 14th day 5 to 6 h into the light cycle.

Hepatocyte isolation and incubation

Hepatocytes were isolated under sterile conditions and plated out onto rat tail collagen-coated dishes as a suspension (approximately 1.8×10^6 cells/dish, 3 mL/dish) in Waymouth's medium MB752/1. Rat tail collagen was obtained as described by Michalopoulos and Pitot (31). Falcon Primaria (60 × 15 mm) dishes were used for culture. The plating media contained 10% fetal calf serum, penicillin (90000 U/l), streptomycin (90000 μg/l), and added amino acids (3.60 mM glutamine, 0.36 mM alanine, and 0.45 mM serine). Initial cell viability, as assessed by trypan blue exclusion, was 88–96% (n = 28). There were no differences in hepatocyte viability among the three groups. The cells were plated at an original density of 1.68 ± 0.07, 1.81 ± 0.22, and 1.68 ± 0.17 × 10^6 cells/dish for LF, OO, and FO cells, respectively. After 4 h, the serum-containing medium was removed and the cell monolayer was washed twice with PBS. After the 4-h attachment period DNA levels were 19.6 ± 2.9, 19.6 ± 2.2, and 20.7 ± 1.7 μg/dish for LF, OO, and FO cells, respectively. Hence, there was no significant difference in plating efficiency among the cells in each group. The TAG level in OO cells was significantly higher than that in FO cells ($P < 0.05$). After washing, serum-free Waymouth's medium (3 ml), containing the added amino acids, penicillin, streptomycin (see above), dexamethasone (1 μM), insulin (0.5 nM), lactate (10 mM), and pyruvate (1 mM), was added. At this stage cells were cultured for 24 h either in the presence of bovine serum albumin (0.5% w/v) alone or in the presence of ^{3}H-labeled oleate bound to albumin prepared as described previously (32). The initial oleate concentration was 0.75 mM containing 830 dpm/nmol of [9,10(n), ^{3}H]oleate. After culturing for 24 h the medium was removed and the cells were harvested using a rubber policeman. VLDL was isolated as described previously (33).

Pulse-chase experiments

For measurements of apoB synthesis, degradation, and secretion, the following procedure was adopted. After removal of serum-containing medium and washing the cell monolayer (see above), the cells were cultured for 1 h in supplemented, serum-free Waymouth's medium lacking methionine. After this time, the medium was removed and replaced with 3.0 ml of the same medium after washing twice with PBS. ^{35}S-labeled methionine (100 μCi; 1000 Ci/mmol) and albumin (0.5%) or oleate (0.75 mM) bound to albumin (0.5%) were then added and the cells were cultured for a further 1 h. At this stage the labeled medium was removed and the cells were washed twice with PBS. The cells were harvested from some dishes for measurement of [^{35}S]methionine incorporation into apoB. To the remaining dishes was added 3.0 ml of supplemented medium containing cold methionine (10 mM) and albumin (0.5%) or oleate bound to albumin as above. The medium was removed and the cells were harvested after periods of 0.25, 0.5, 1, 2, and 24 h. At the end of the 1-h pulse period, the amount of immunoprecipitable label in the medium was approximately 1–2% of the amounts associated with the cells, irrespective of dietary treatment. Pilot studies had already suggested that there was no net loss of total newly synthesized apoB-100 plus apoB-48 during the pulse period.

Immunoprecipitation of apoB

At the point of harvesting, the cells were washed twice with PBS and solubilized by the addition of 0.5 ml of hot (75°C) 1% SDS-RIPA buffer. This buffer consisted of 0.05 M Tris (pH 8.0), 1 mM EDTA (pH 8.0), 1% (v/v) Triton-X-100, 1% (w/v) deoxycholate, 1 mM DTT, 0.15 M NaCl, 0.1 mM PMSF, and 1% (w/v) SDS. After addition of the hot SDS-RIPA, the cells were left for 2 min, scraped into a capped tube, and incubated at 75°C for 15 min. This suspension was then spun at 15000 rev/min in a Beckman "Avanti" centrifuge for 30 sec. The samples were then vortexed and further incubated at 75°C until all of the protein appeared to be solubilized. At this point, 2.0 ml of 0% SDS-RIPA buffer (the above buffer without SDS) was added to each of the samples which were then sonicated for 3 sec. A further 2.5 ml of 0% SDS-RIPA was added to each of the samples and 1 ml of the resulting suspension was then used for the apoB immunoprecipitation. For this, 50 μl of a 1:10 dilution of the rabbit polyclonal IgG antiserum was added and the samples were left overnight at 4°C to facilitate antibody binding. ApoB was immunoprecipitated from the whole cell medium, the VLDL, and the d > 1.006 infranate in essentially the same way except that 100 μl of a 10× concentrate SDS-RIPA was added to 0.9 ml of the sample and heated at 75°C for 20 min. The samples were then cooled to room temperature before addition of the antibody. After 14–18 h, the samples were shaken with 40 μl of a protein A-Sepharose bead suspension (1 g/8 ml 0.05% SDS-RIPA), using an IKA Vibrax VXR, for 2 h. The beads were spun down at 15000 rev/min for 1 min, the supernatant was discarded, and the beads were then resuspended in 1 ml of 0.1% SDS-RIPA and shaken for 15 min on the Vibrax. This washing procedure was repeated a further 5 times until no radioactivity was associated with the supernatant wash.

SDS-PAGE electrophoresis

The beads were combined with 40 μl of sample buffer consisting of: 45% (v/v) glycerol, 10% (w/v) SDS, 0.05% (w/v) bromophenol blue, and 10% mercaptoethanol in 0.75 M Tris (pH 6.8). Twenty μl of a 1 mg/ml Triton-treated rat-plasma VLDL apoB solution was then added (to enable visualization of the apoB bands on the gel) and the samples were heated at 75°C for 20 min to denature the protein-antibody complex. The beads were removed by centrifugation and the resulting supernatant was run on a 3–20% gradient, polyacrylamide gel (20 cm × 20 cm). The residual beads typically contained ≤5% of the original immunoprecipitate label, as determined by scintillation counting in Optiphase scintillant. The gel was electrophoresed at 15 mA/gel for 18 h, after which they were stained with Coomassie Brilliant Blue R (0.1% (w/v) in 45% (v/v) methanol–10% (v/v) acetic acid, for 1 h. Destaining of the gels was carried out in 10% (v/v) methanol–10% (v/v) acetic acid until the background of the gel was clear.

Autoradiography and scintillation counting of apoB-48 and apoB-100

Gels were dried down onto 1 mm Whatman paper using an LKB Bromma 2003 slab gel dryer. Blue sensitive X-ray film was then exposed to the gels for 48 h to visualize the labeled apoB-48 and apoB-100. Dried gel slices were solubilized before scintillation counting. The slices were incubated with 30% H_2O_2 (1.0 ml) then NCS-Π tissue solubilizer (1.0 ml) each for 18 h. Glacial acetic acid (150 μl) was then added to neutralize the solubilizer followed by 10 ml of Optiphase scintillant. The samples were then counted on a Beckman LS-6500 scintillation counter. After electrophoresis, there was no effect of dietary manipulation in the recoveries of the amounts of label in the sum of apoB-48 and apoB-100 compared to that in the original immunoprecipitate. In the hepatocytes from the chow-fed animals, these recoveries were 63.2 ± 3.3, 61.9 ± 1.5, and 65.5 ± 2.2% in the cellular, VLDL, and infranate fractions, respectively. The corresponding values in the FO-fed were 67.8 ± 7.2, 63.4 ± 2.6, and 66.2 ± 3.1%, respectively. As approximately 5% of the total label remained with the beads, we could account for about 70% of the immunoprecipitable label. We could not detect any discrete bands of label in immunoprecipitable fragments of molecular mass less than that of apoB-48 (see Fig. 6).

Other analytical methods

Cellular protein was determined calorimetrically by the method of Lowry et al. (34). Total ketone body production was determined enzymatically on the infranate using the method of Williamson, Mellanby, and Krebs (35). The infranate was the fraction of medium ($d > 1.006$) obtained after flotation of VLDL ($d < 1.006$). Folch extraction (30) of the infranate followed by scintillation counting of an aliquot of the organic phase was used to determine the amount of [9,10(n)-^{3}H], oleate remaining, and, thus, the extent of cellular oleate uptake. There is little, if any, esterified lipid in this fraction (25). Moreover, enzymatic determination of residual nonesterified fatty acid in the infranate, at 24 h, agreed with the value obtained through Folch extraction and scintillation counting. Cellular and VLDL TAG mass were determined enzymatically after Folch extraction using the GPO-PAP kit from Boehringer Mannheim (Lewes, U.K.). Newly-synthesized cellular and VLDL TAG and PL were determined by scintillation counting after Folch extraction and thin-layer chromatography of labeled lipids on silica plates (Uniplate, Silica Gel G, 20 × 20 cm, 250 μm) with n-hexane–diethyl ether–glacial acetic acid 70:30:1.6.(v/v/v) (36). Bands were visualized using Rhodamine 6G. PL remained at the origin. VLDL apoB was determined using an enzyme-linked immunoabsorbent assay with anti-sheep IgG antibody and anti-human apolipoprotein B (apoB) anti-serum (37). Infranate nonesterified fatty acids were determined enzymatically using the NEFA C kit from Wako (Neuss, Germany). DNA was determined calorimetrically by the method of Burton (38).

Statistical methods

All statistical tests (ANOVA: two-factor with replication and one-way *t*-tests: two samples assuming equal variances) were performed using the data analysis package in Microsoft Excel for Windows 95, Version 7.0. Significance was determined at the 95% confidence interval, i.e., $P < 0.05$. The data obtained in this work were derived from experiments on between four and six independent hepatocyte preparations in each dietary group.

RESULTS

Effect of dietary n–3 PUFA on food intake and weight gain

Although daily food intake in the three dietary groups was similar (LF, 26.0 ± 0.5 g/day; OO, 23.6 ± 0.2 g/day; FO, 25.0 ± 0.5 g/day) because of the higher energy density of the fat-containing diets, total energy intake was lower in the LF group (95 ± 2 cal/day vs. 111 ± 1 cal/day (OO) and 117 ± 2 cal/day (FO), $P <$ 0.05). However, only the OO-fed group showed a sig-

TABLE 1. Effect of dietary fat composition and exogenous oleate on VLDL TAG secretion by cultured hepatocytes

Diet	Exogenous Oleate	Exogenous Oleate Uptake	VLDL TAG Secretion
		%	*nmol/dish*
Low fat	−	−	71 ± 11
	+	89 ± 1	103 ± 9[c]
Olive oil	−	−	79 ± 22
	+	90 ± 1	132 ± 18
Fish oil	−	−	42 ± 6[a]
	+	90 ± 1	70 ± 7[a,b,c]

Hepatocytes were cultured for 24 h in the absence or presence of oleate (2250 nmol/dish or 0.75 mM; 830 dpm/nmol of [9,10(n)-^{3}H]oleate). At the end of the culture period VLDL TAG (mass determined enzymatically) was determined. Figures in the third column refer to the percentage of exogenous oleate taken up from the media over the culture period. Results are expressed as mean ± SEM (n = 5).

[a]Significant difference from low fat, $P < 0.05$.
[b]Significant difference from olive oil, $P < 0.05$.
[c]Significant difference from −oleate, $P < 0.05$.

nificant increase in body weight during the 14-day feeding period (6.93 ± 0.19 g/day vs. 6.04 ± 0.27 g/day (LF), $P < 0.05$). The increase in the FO-fed group (6.63 ± 0.23 g/day) was not statistically significant.

Changes in hepatocyte lipid metabolism and VLDL secretion

VLDL TAG secretion was decreased in cells from the FO group compared to cells from the LF group (**Table 1**). The increased rate of fatty acid oxidation (as reflected by ketogenesis) (**Table 2**) in the cells from the FO group compared to those from the LF group might suggest that increased lipid flux through the oxidative pathway limited the availability of TAG for VLDL assembly. However, the increased TAG content of cells in the FO group, at the beginning of the culture period, compared to that in the LF group (96.2 ± 9.6 vs. 63.9 ± 6.5 nmol/dish, respectively; $P < 0.05$) and of intact livers following FO consumption (ref. 5 and A. Hebbachi, unpublished results) suggested that this explanation could not be entirely correct. That the decreased output of VLDL TAG was not simply a nonspecific effect of high-fat consumption was suggested by the observation that a similar decrease was not observed in hepatocytes from animals fed the OO diet (Table 1) in which cellular TAG levels were also increased compared to the LF (191.4 ± 28.4 vs. 63.9 ± 6.5 nmol/dish, respectively, $P < 0.05$).

To study the extent to which the above effects could be modified by the provision of an external source of fatty acids in vitro, hepatocytes from the three dietary groups were cultured in the presence of oleate at an initial concentration of 0.75 mM. Although under these conditions there was a similar relative increase in the secretion of VLDL TAG from all the dietary groups, the output from the FO group remained lower than that from the LF group (Table 1). Again, the VLDL TAG output by the FO group was also lower than that by the OO group, indicating that the inhibitory effect was restricted to diets containing only n−3 fatty acids and was not a nonspecific effect of a high-fat diet. The observed

TABLE 2. Effect of dietary fat composition and exogenous oleate on TAG storage and secretion (total TAG) and ketogenesis by cultured hepatocytes

Diet	Exogenous Oleate	Total TAG	% Increase in Total TAG (+ oleate)	Ketogenesis	% Increase in Ketogenesis (+ oleate)	Oleate Equivalents in Oxidation Products/Oleate Equivalents in Esterification Products
		nmol/dish	%	μ*mol/dish*	%	
Low fat	−	97 ± 12	135 ± 22	1.64 ± 0.04	22 ± 2	1.20 ± 0.12
	+	219 ± 8[c]		2.00 ± 0.06[c]		0.65 ± 0.03[c]
Olive oil	−	160 ± 39	95 ± 30	2.62 ± 0.37[a]	34 ± 13	1.38 ± 0.25
	+	268 ± 29[c]		3.39 ± 0.35[a]		0.95 ± 0.08[a]
Fish oil	−	79 ± 10[b]	73 ± 22[a]	2.78 ± 0.36[a]	44 ± 16	2.73 ± 0.35[a,b]
	+	128 ± 7[a,b,c]		3.86 ± 0.37[a,c]		2.27 ± 0.25[a,b]

Hepatocytes were cultured for 24 h in the absence or presence of oleate (2250 nmol/dish or 0.75 mM; 830 dpm/nmol of [9,10(n)-^{3}H]oleate). At the end of the culture period residual cellular TAG and VLDL TAG secretion (mass determined enzymatically) and ketone body (acetoacetate and β-hydroxybutyrate) production were determined. Total TAG refers to the sum of the residual cellular TAG and the secreted VLDL TAG. Figures in the fourth and sixth columns represent the percentage increase in total TAG or ketone body production upon addition of oleate. Figures in the seventh column represent the ratio of oleate equivalents oxidized (ketogenesis) to oleate equivalents esterified (total TAG). The quantity of oleate equivalents oxidized was calculated from the rate of ketogenesis given that one molecule of oleate yields 4.5 ketone bodies and one TAG molecule contains three molecules of oleate. Results are expressed as mean ± SEM (n = 5).

[a]Significant difference from low fat, $P < 0.05$.
[b]Significant difference from olive oil, $P < 0.05$.
[c]Significant difference from −oleate, $P < 0.05$.

increases in total TAG on the one hand and ketogenesis on the other, in each cell type cultured with exogenous oleate (Table 2), suggested that oleate was channelled to a greater extent into the esterification pathway and to a lesser extent into the oxidative pathway in the LF group compared to the FO group of cells. The higher ratio (Table 2) in the FO group, in the presence of absence of exogenous oleate, compared to either the LF or OO groups highlights the extent to which the available TAG in the FO cells was channelled into the oxidative pathway and again emphasizes the specificity of n−3 dietary fatty acids in this regard. The decreased incorporation of exogenous oleate into total lipid was also reflected by the significant reduction in TAG synthesis from [^{3}H]oleate by FO cells (**Table 3**). There was no significant change in TAG synthesis by cells from the OO-fed group. It is interesting to note that despite the decreased conversion of exogenous oleate to TAG in cells from the FO-fed group, the rate of phospholipid synthesis actually increased under these conditions (Table 3). These changes were not accompanied by any changes in the cellular uptake of [^{3}H]oleate from the medium (Table 2).

Changes in hepatocellular apoB metabolism

The relative lack of any change in lipid metabolism resulting from consumption of the OO diet suggested that the effects of the FO-containing diet on hepatic lipid metabolism, and particularly on the secretion of VLDL, were specific and did not represent merely the nonspecific consequences of feeding a high-fat diet. In view of this, in the following experiments, the effects of dietary n−3 fatty acid consumption on hepatocellular apoB metabolism was compared directly with that in hepatocytes from animals fed a low-fat diet.

In the FO-fed group, after labeling hepatocellular apoB with [^{35}S]methionine, the secretion of labeled VLDL apoB decreased compared to that observed with the LF-fed group (**Fig. 1a**). Measurement of the total mass of secreted VLDL apoB using an ELISA method showed that this also declined in hepatocytes from the FO-fed group (3.24 ± 0.51 μg/dish/24 h) compared to that in hepatocytes from the LF group (4.86 ± 0.17 μg/dish/24 h; $P < 0.05$). The decline in labeled apoB output into the VLDL fraction could be accounted for, almost exclusively, by a decreased output of apoB-48

TABLE 3. Effect of dietary fat composition on TAG and PL synthesis in cultured hepatocytes

Diet	TAG Synthesis	PL Synthesis	Flux of DAG to PL
	nmol/dish	*nmol/dish*	%
Low fat	121 ± 6	17 ± 1	12 ± 1
Olive oil	98 ± 17	20 ± 3	18 ± 4
Fish oil	54 ± 6[a,b]	25 ± 2[a]	32 ± 3[a,b]

Hepatocytes were cultured for 24 h in the presence of oleate (2250 nmol/dish or 0.75 mM; 830 dpm/nmol of [9,10(n)-^{3}H]oleate). At the end of the culture period total TAG and PL synthesis were determined radiometrically as determined by the incorporation of ^{3}H into TAG and PL in both the cell and VLDL. Figures in the fourth column represent the percentage of PL synthesized relative to the total amount of TAG and PL synthesis. Results are expressed as mean ± SEM (n = 5).

[a]Significant difference from low fat, $P < 0.05$.

[b]Significant difference from olive oil, $P < 0.05$.

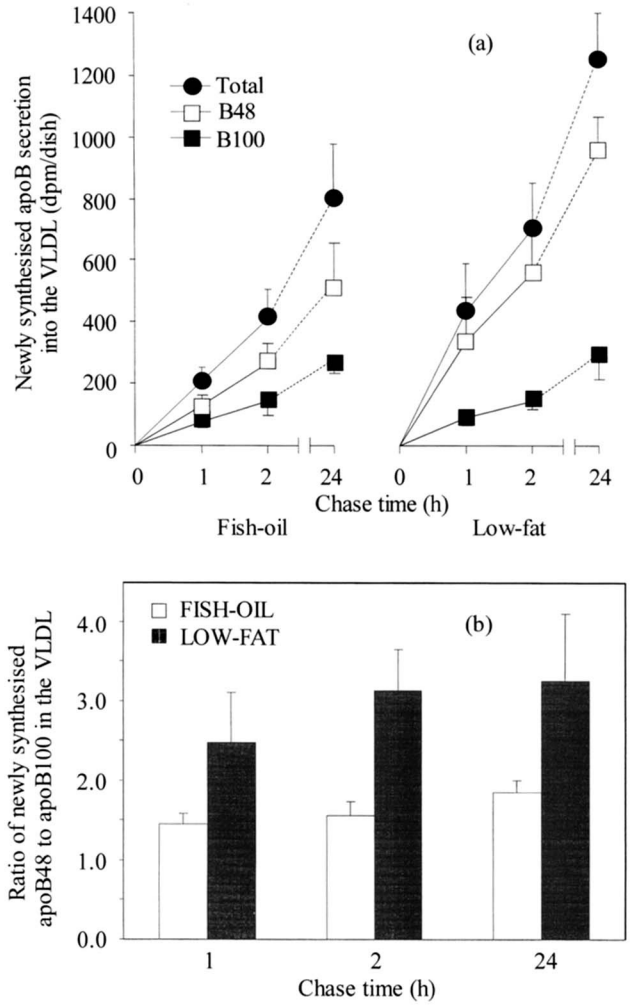

Fig. 1. a: Effect of dietary composition on the secretion of apoB-100, apoB-48, and total apoB into the d < 1.006 (VLDL) fraction of the culture medium. Hepatocytes were pulse-labeled for 1 h with [^{35}S]methionine (100 μCi/dish) and then chased with cold methionine (10 mM) for various times up to 24 h. The secretion of VLDL apoB-48 was significantly suppressed in the hepatocytes from the FO-fed group (n = 4; $P < 0.025$ by ANOVA). b: Effect of dietary composition on the ratio of apoB-48 to apoB-100 secreted into the d < 1.006 fraction of the culture medium. Hepatocytes were pulse-labeled for 1 h with [^{35}S] methionine (100 μCi/dish) and then chased with cold methionine (10 mM) for various times up to 24 h. Ratios in the cells from the FO-fed group were significantly lower ($P < 0.05$) than those in the LF group at 1 and 2 h.

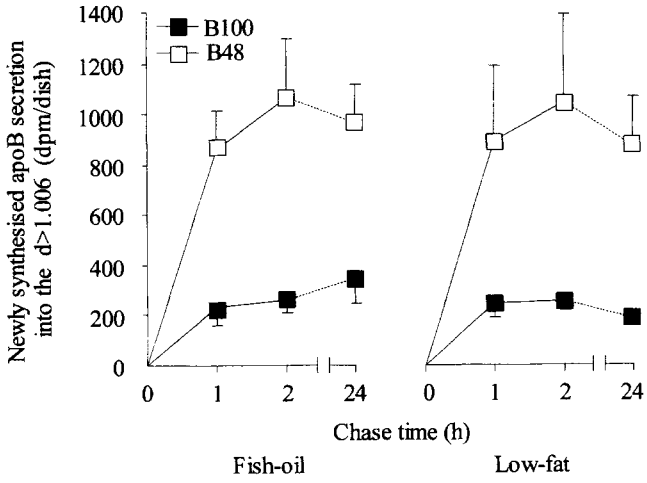

Fig. 2. Effect of dietary composition on the rates of apoB-48 and apoB-100 secretion into the d > 1.006 fraction of the culture medium. Hepatocytes were pulse-labeled for 1 h with [^{35}S]methionine (100 μCi/dish) and then chased with cold methionine (10 mM) for various times up to 24 h (n = 4).

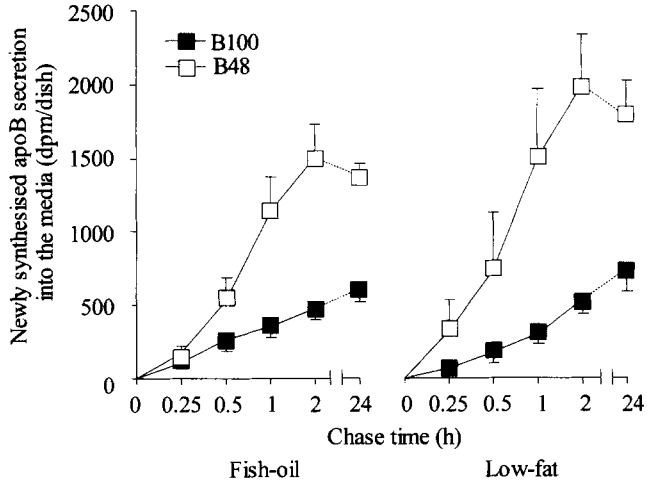

Fig. 3. Effect of dietary composition on the total secretion of apoB-48 and apoB-100 into the culture medium. Hepatocytes were pulse-labeled for 1 h with [^{35}S]methionine (100 μCi/dish) and then chased with cold methionine (10 mM) for various times up to 24 h (n = 6).

(ANOVA, $P < 0.025$). There was little or no change in the secretion of labeled apoB-100 (Fig. 1a).

The selective effect of FO feeding on VLDL apoB-48 output can be more clearly seen in terms of changes in the apoB-48/apoB-100 ratio (Fig. 1b). After 2 h chase, the B-48/B-100 ratios in the VLDL from the hepatocytes in the FO and LF groups were 1.6 ± 0.2 and 3.1 ± 0.5, respectively. At 24 h, these values were 1.8 ± 0.2 and 3.2 ± 0.9, respectively. In this experiment, the output of labeled apoB-containing particles into the VLDL infranate was also determined. These measurements reflect the secretion of labeled apoB in association with smaller, denser, lipid-depleted particles than that associated with the large VLDL. With both dietary groups a somewhat larger amount of labeled apoB appeared in these denser particles. This remained the case even in the presence of 20 μM tetrahydrolipstatin, a potent lipase inhibitor. These results (data not shown) showed that these smaller particles occurred predominantly via direct secretion, rather than by extrahepatic lipolysis of secreted VLDL. The secretion of the small particles was unaffected by FO feeding, in contrast to the inhibitory effect on the secretion of VLDL apoB (**Fig. 2**). Moreover, FO feeding had no effect on the relative proportions of apoB-100 and apoB-48 secreted into this denser fraction of the medium (data not shown). In other experiments, the secretion of apoB into the whole unfractionated medium of the cells was measured. This reflects total apoB output from the cell and **Fig. 3** shows that this is decreased in the hepatocytes from the FO-fed animals. This is due, in the main, to a decreased cellular output of labeled apoB-48 (Fig. 3) although this did not reach statistical significance ($P = 0.08$). The data from Fig. 1 and Fig. 2 suggest that the above pattern arises entirely from a decreased output of apoB-48, in association with the large TAG-rich VLDL. Output of denser apoB-containing particles appears to be constitutive and unaffected by FO-feeding. This conclusion, of course, does not preclude a possible effect of FO feeding on the distribution of apoB among the IDL, LDL, and HDL fractions.

During the 1-h pulse-label, cells from the FO rats synthesized less apoB-48 from [^{35}S]methionine than did the cells from the LF-fed (see time-zero in **Fig. 4**) ($P < 0.05$). There was no change in the rate of synthesis of apoB-100. However, during the 24-h chase period, a smaller proportion of the newly-synthesized apoB-48 disappeared from the cells of the FO group (Fig. 4) than from those of the LF group. This pattern of change could be explained by a decreased rate of degradation of apoB-48 in the cells from the FO-fed animals compared to that from the LF group. This was particularly apparent after 24 h in which 71.6 ± 6.1 % of the maximum incorporation remained in hepatocytes from the FO-fed group compared to 51.3 ± 6.1% in those from LF-fed ($P < 0.05$). Degradation of apoB-100 was unaffected by FO feeding (**Fig. 5**). A visual summary of some of the effects of FO-feeding on synthesis and secretion of B-48, is provided by typical autoradiographs of SDS-PAGE gels (**Fig. 6**) where the newly-synthesized apoB-48 present in the cell at the end of the pulse period (t = 0) was clearly less than that present in the LF-fed cells. Also, retention of this B-48 in the cells of the FO-fed animals resulted in slower secretion into the VLDL during the chase time periods.

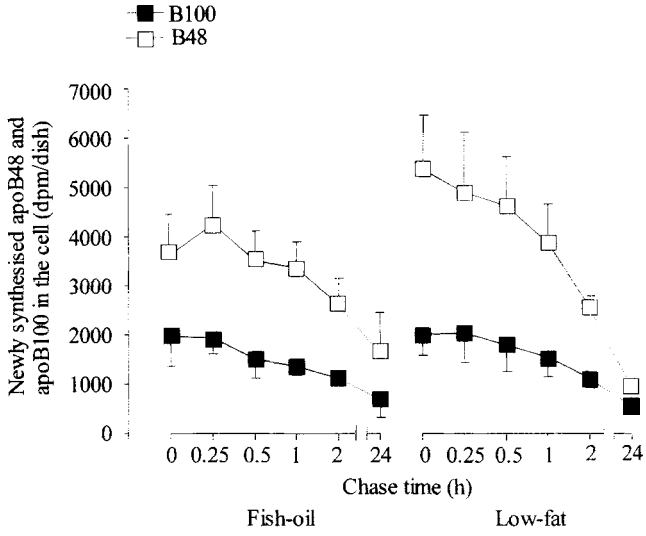

Fig. 4. Disappearance of apoB from the cells during the chase. Hepatocytes were pulse-labeled for 1 h with [^{35}S]methionine (100 μCi/dish) and then chased with cold methionine (10 mM) for various times up to 24 h (n = 6). Points without bars for S.E.M. are those in which the SEM fell within the size of the symbol.

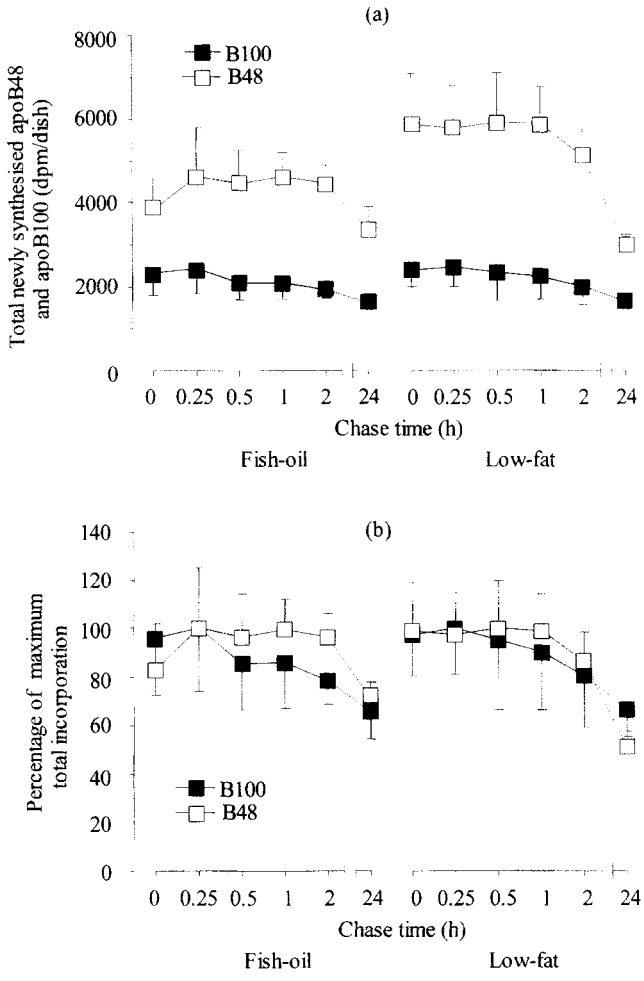

Fig. 5. a: Effect of dietary composition on the degradation of apoB-100 and apoB-48. Hepatocytes were pulse-labeled for 1 h with [^{35}S]methionine (100 μCi/dish) and then chased with cold methionine (10 mM) for various times up to 24 h. Values at each time during the chase period represent the sum of label remaining within the cell and that secreted into the medium (n = 6). Points without bars for SEM are those in which the SEM fell within the size of the symbol. b: Effect of dietary composition on the proportion of apoB-100 and apoB-48 degraded by hepatocytes. Hepatocytes were pulse-labeled for 1 h with [^{35}S]methionine (100 μCi/dish) and then chased with cold methionine (10 mM) for various times up to 24 h. Results are expressed as percentage of maximum values and are based on the absolute values obtained in Fig. 5a.

The presence of exogenous oleate in the medium of cells, pulse-labeled with [^{35}S] methionine, had no significant effect on the secretion of either labeled apoB-48 or labelled apoB-100 in the VLDL fraction over the next 24 in hepatocytes from animals fed the LF diet. Neither did oleate have any effect on these parameters in the cells from the FO-fed animals (**Table 4**). The stability of VLDL apoB secretion under these conditions was in contrast to the stimulatory effects of oleate on the secretion of VLDL TAG in both groups of cells (Table 1). The presence of oleate did not affect the apoB-48/apoB-100 ratio in either group of cells (data not shown).

DISCUSSION

It would appear that, in vivo, the net effect of changes in fatty acid metabolism that accompany dietary consumption of n–3 fatty acids result in an increase in the hepatocellular TAG content. Accumulation of TAG occurs despite a considerable increase in the rate of fatty acid oxidation (Table 2). Clearly, a contributory factor to the elevated hepatic TAG content is the increased flux of lipid to the liver in vivo in the form of intestinal TAG-rich lipoprotein, and, possibly, an increased availability of plasma-free fatty acids.

Despite the increase in hepatocellular TAG, it seems that, for some reason, this VLDL substrate cannot access the secretory pool. It thus remains unavailable for the assembly of VLDL and VLDL TAG secretion declines (Table 1). This situation is not a nonspecific consequence of a high-fat diet as cells from the OO-fed animals secrete an amount of VLDL TAG similar to that from the LF group. It should be pointed out that, despite the increase in the total pool of TAG in the cells from the OO-fed animals (Table 2), there was no corresponding increase in the secretion of VLDL TAG (Table 1). Nevertheless, increasing the total pool of TAG by providing extracellular oleate in vitro produced an increase in VLDL TAG secretion in all three dietary

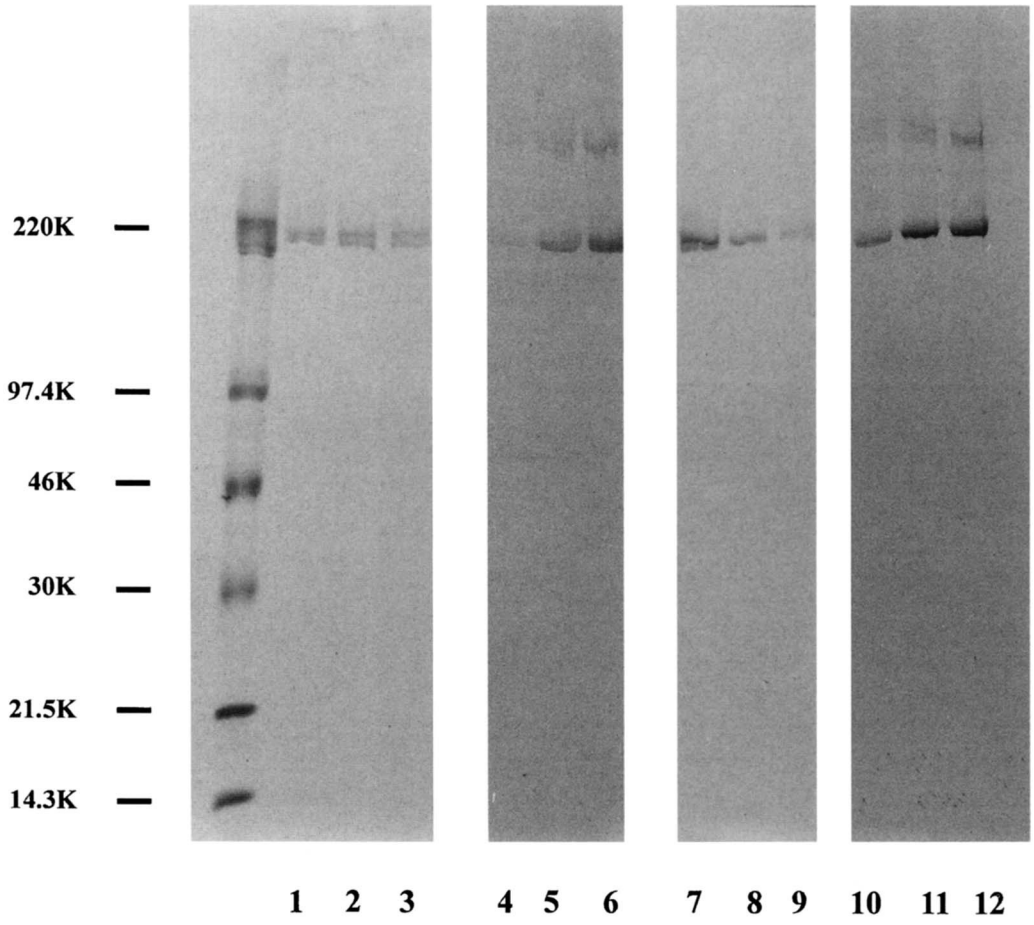

Fig. 6. SDS polyacrylamide gel electrophoresis of labeled immunoprecipitable apoB. Hepatocytes were pulse-labeled for 1 h with [^{35}S]methionine (100 μCi/dish) and then chased for various times up to 24 h. Lanes 1–3 and 7–9 represent the cellular apoB in the hepatocytes from the FO- and chow (LF)-fed animals, respectively, at 0, 1, and 2 h of chase. Lanes 4–6 and 10–12 represent the VLDL apoB from the corresponding hepatocytes at 1, 2, and 24 h, respectively, of the chase period.

groups. Clearly, therefore, there is no simple relationship between TAG pool size and the rate of TAG secretion. Factors that determine the accessibility of the cellular TAG pool to the site(s) of VLDL assembly may play an important role in this respect; for example, the rate of transfer of TAG to apoB mediated by MTP.

In addition to the decreased transport of TAG out of the liver, FO feeding also resulted in a decreased synthesis of TAG. This effect may arise from a decrease in the capacity of the enzymes of TAG synthesis (18, 28, 39) or from an increase in the rate of fatty acid oxidation (Table 2; 40, 41), or, indeed, from a combination of both. The present results show an increase in PL synthesis, and a decrease in TAG synthesis (Table 3). As both

TABLE 4. Effect of exogenous oleate and dietary composition on secretion of apoB-100 and apoB-48 into the d<1.006 (VLDL) fraction

	ApoB-100		ApoB-48	
Diet	−Oleate	+Oleate	−Oleate	+Oleate
	dpm		*dpm*	
Low fat	299 ± 84	230 ± 59	951 ± 114	800 ± 249
Fish oil	278 ± 46	281 ± 44	523 ± 133	560 ± 142

Hepatocytes were pulsed for 1 h with [^{35}S]methionine (100 μCi/dish) in the presence or absence of exogenous oleate (0.75 mM). Results are expressed as dpm per dish in the newly synthesized apoB recovered in the d<1.006 fraction of the culture medium at the end of a 24-h culture period (n = 4).

of these pathways share a common precursor, diacylglycerol, the increase in PL shows that there is decreased competition for this common substrate and suggests that the decreased TAG synthesis results, in part, from a fall in the activity of diacylglycerol acyl transferase. Irrespective of the precise mechanism involved, it is apparent from the present results that dietary consumption of n–3 fatty acids, over a period of time, reduces the capacity of the cells for TAG production from exogenous fatty acids. Clearly, therefore, cells from FO-fed animals behave differently in this respect from cells derived from normal animals treated for short periods with n–3 fatty acids in vitro (16) or from HepG2 cells treated similarly (20, 42) in which TAG synthesis does not decline. The possibility remains, therefore, that a decline in TAG synthesis resulting from a decreased exogenous fatty acid esterification contributes to the diminished VLDL TAG secretion in hepatocytes from the FO-fed animals and amplifies the effects resulting from decreased TAG mobilization noted above.

The decreased secretion of newly synthesized apoB (Fig. 1) and the total mass of apoB associated with VLDL in the cells from the FO-fed group forms only a small part of the subtle pattern of changes in apoB metabolism that occur under these conditions. First of all, the decreased VLDL apoB output is due almost entirely to a decrease in the secretion of apoB-48, as shown by changes in the secretion of newly-synthesized apoB labeled with [^{35}S] methionine (Fig. 1). Second, there was no change in the output of labeled apoB, either B-48 or B-100, associated with dense lipid-poor particles (Fig. 2). Thus the observed decrease in output of apoB, especially apoB-48, into the whole, unfractionated medium of the cells from the FO-fed animals was due entirely to changes in the lipid-rich, VLDL-containing fraction. Third, pulse-labeling of hepatocellular apoB with [^{35}S] methionine showed that FO feeding was associated with a slower rate of synthesis of apoB-48 but not of apoB-100 (Fig. 4). Finally, a smaller proportion of newly synthesized apoB-48 underwent pre-secretory degradation in the cells from the FO-fed animals compared to those from the LF group (Fig. 5). A consequence of this was the prolonged retention within the FO cells of a higher proportion of the newly synthesized apoB-48 (Fig. 4). Corresponding changes in the metabolism of apoB-100 in the cells from the FO-fed group were not so pronounced. Most of the above-mentioned effects of FO-feeding on hepatocellular apoB metabolism are apparent in Fig. 6, which shows a representative SDS-PAGE gel of the immunoprecipitable label associated with cellular and VLDL-associated apoB-48 and apoB-100 during the early part of the pulse-chase period.

As far as we are aware, this study is the first to provide direct quantitative information about the effects of dietary n–3 fatty acids on hepatic apoB synthesis and degradation in any species. By contrast, studies using a different experimental model in which n–3 fatty acid action on apoB metabolism was simulated by short-term addition of eicosapentaenoic acid (C20:5) to cultures of primary hepatocytes from LF-fed rats (16,21) or HepG2 (20,42) concluded that these types of fatty acid inhibited apoB secretion by enhancing apoB degradation. It is generally accepted that, in many nutritional states, freshly prepared cultures of primary hepatocytes retain the metabolic characteristics of the intact liver in the donor animals (for a review, see Ref. 43). Thus, whilst acknowledging that it is virtually impossible to precisely simulate nutritional changes in an in vitro environment, we believe that the present model is more likely to accurately reflect the hepatic consequences of nutritional change than by short-term manipulation of the environment of hepatocytes from normal-fed animals in vitro. Nevertheless, it is probable that, in vivo, the livers of FO-fed animals are exposed to a higher concentration of n–3 fatty acids than the livers of the LF-fed. This potential variable was not simulated in vitro in the current model.

The slower rate of synthesis and pre-secretory degradation of apoB-48 in the cells from the FO-fed animals obviously reflects a decreased turnover or "cycling" of apoB-48 that is associated with a slower overall rate of transport of apoB-48 and of TAG out of the cell. This decline in apoB-48 turnover may reflect a long-term adaptation to a decreased requirement to transport TAG, a change which may itself form only a part of the coordinated changes in lipid metabolism by which rodent liver adjusts to a diet rich in n–3 fatty acids. In this respect, it is interesting to note that, in the rodent, other nutritional variations that require changes in hepatic lipid balance, including TAG secretion, are accommodated by changes in apoB-48 metabolism. These changes are associated with, and may be mediated by, changes in the rate of de novo fatty acid synthesis (44–46). In this respect, it may be of interest to note that, of all dietary fatty acids studied, those of the n–3 class are the most potently active supressers of lipogenesis via the acetyl CoA pathway (26). From a mechanistic viewpoint, it has been shown previously that circumstances that result specifically in changes in apoB-48 synthesis result from a change in apoB mRNA editing activity (47, 48). This is accompanied by corresponding variations in the rate of TAG transport out of the hepatocyte. Whether signals resulting from dietary n–3 fatty acid consumption decrease editing activity cannot be answered at the present time. If this were the case, it might have been expected that the corresponding increase in the amounts of apoB-100 mRNA would lead to an increased synthesis of apoB-100 which we did not observe. However, a previ-

ous study (47) showed that fasting, which also decreases editing activity and which led to a 3.9-fold increase in apoB-100 mRNA, did not affect apoB-100 synthesis. It would appear, therefore, that there is a tight regulation of apoB-100 synthesis involving, perhaps, changes in translational efficiency.

Another question that cannot be satisfactorily answered is why the cell continues to secrete relatively large amounts of apoB as dense, lipid-poor particles irrespective of the amount of TAG that is transported from the cell (Fig. 2). It may be that there are both constitutive and regulated elements of hepatic apoB secretion; the constitutive aspect results in the secretion of dense particles whilst the regulated pathway produces VLDL, the amounts of which are determined by longer-term variations in the need to transport TAG and which is characterized by variations in apoB-48 synthesis and degradation.

Extracellular provision of hepatocytes with a TAG-precursor, oleate, stimulated TAG production and secretion in hepatocytes from both the LF and FO groups (Table 1) but VLDL apoB output did not increase (Table 4). It would appear, therefore, that signals which, in vivo, result from longer-term dietary manipulation and which affect VLDL TAG output also affect apoB secretion. However, apoB output cannot be rapidly changed by short-term manipulation in vitro even though this results in increased VLDL TAG output. Nevertheless, longer-term increases in cellular TAG resulting from exposure of hepatocytes to extracellular oleate for 3 days in vitro resulted in a specific increase in the secretion of apoB-48 VLDL and an increase in apoB-48 synthesis. No corresponding changes were observed for apoB-100 (25).

One of the best-planned studies of the effects of FO feeding in experimental animals was that carried out by Parks and colleagues (19) using perfused livers from African green monkeys fed fish oil over a long period of time. One of the major conclusions of this study was that, although hepatic TAG output declined, there was no change in the secretion of apoB. Monkeys, in common with humans, secrete only hepatic apoB-100, in contrast to the rat, which secretes a combination of apoB-48 and apoB-100. It is interesting to note that, in the present study, FO feeding had little or no effect on any measured parameter of apoB-100 metabolism; output, particle size distribution, synthesis, and degradation all remained unchanged. In monkeys, although FO feeding had no effect on hepatic apoB-100 metabolism, the secretion of VLDL TAG was suppressed (19). This resulted in the secretion of smaller, denser VLDL particles similar to those secreted in human subjects fed diets rich in fish oil (14). The hypolipidemic effects of FO feeding in humans, therefore, most probably arise as a result of decreased secretion of VLDL TAG, but not necessarily of VLDL apoB-100.

All the changes in hepatic lipid metabolism that occurred after feeding FO to the rat were associated with variations in the metabolism, specifically, of apoB-48. Lipid metabolism in rodent liver, in contrast to that of some other species, is generally able to respond more flexibly to fluctuations in dietary lipid content, thereby providing a more efficient buffering system. This compensatory mechanism protects against whole-body lipid imbalance which might otherwise occur in the face of dietary challenges. For instance, fluctuations in dietary cholesterol, or in certain types of dietary fatty acid, may be accommodated, in rodent liver, by a relatively unrestricted synthesis of bile acids on the one hand or peroxisomal proliferation (β-oxidation) on the other. It may be possible that the ability of rodent liver to manipulate apoB-48 metabolism forms part of the coordinated response by which these species maintain whole-body lipid balance in the face of major long-term dietary fluctuations.

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