The Effects of Dietary n-3 Polyunsaturated Fatty Acids Delivered in Chylomicron Remnants on the Transcription of Genes Regulating Synthesis and Secretion of Very-Low-Density Lipoprotein by the Liver: Modulation by Cellular Oxidative State

KATHLEEN M. BOTHAM,[1]* XIAOZHONG ZHENG,* MARIAROSARIA NAPOLITANO,† MICHAEL AVELLA,* CLAUDIO CAVALLARI,‡ ROBERTO RIVABENE,† AND ELENA BRAVO†

Department of Veterinary Basic Sciences, Royal Veterinary College, London NW1 0TU, United Kingdom; †Laboratory of Metabolism and Pathological Biochemistry, Istituto Superiore di Sanitá, 00161 Rome, Italy; and ‡Department of Cellular Biotechnology and Hematology, Molecular Genetics Section, University of Rome "La Sapienza," Rome, Italy

The influence of chylomicron remnants enriched in n-3 or n-6 polyunsaturated fatty acids (PUFA) (derived from fish or corn oil, respectively) on the expression of mRNA for four genes involved in the regulation of the synthesis, assembly, and secretion of very-low-density lipoprotein (VLDL) in the liver was investigated in normal rat hepatocytes and after manipulation of the cellular oxidative state by incubation with N-acetyl cysteine (NAC) or $CuSO_4$. The four genes investigated were those encoding apolipoprotein B (apoB), the microsomal triacylglycerol transfer protein (MTP), and the enzymes acyl coenzyme A:diacylglycerol acyltransferase (DGAT) and acyl coenzyme A:cholesterol acyltransferase 2 (ACAT2), which play a role in the regulation of triacylglycerol and cholesteryl ester synthesis, respectively. mRNA levels for apoB, MTP, and DGAT were unaffected by either fish or corn oil chylomicron remnants, but the amount of ACAT2 mRNA was significantly reduced after incubation of the hepatocytes with fish oil remnants as compared with corn oil remnants or without remnants. These findings indicate that the delivery of dietary n-3 PUFA to hepatocytes in chylomicron remnants downregulates the expression of mRNA for ACAT2, and this may play a role in their inhibition of VLDL secretion. However, when the cells were shifted into a pro-oxidizing or pro-reducing state by pretreatment with $CuSO_4$ (1 m*M*) or NAC (5 m*M*) for 24 hr, levels of mRNA for MTP were increased by about 2- or 4-fold, respectively, by fish oil remnants, whereas corn oil remnants had no significant effect. Fish oil remnants also caused a smaller increase in apoB mRNA in comparison with corn oil remnants in NAC-treated cells (+38%). These changes would be expected to lead to increased VLDL secretion rather than the decrease associated with dietary n-3 PUFA in normal conditions. These findings suggest that relatively minor changes in cellular redox levels can have a major influence on important liver functions such as VLDL synthesis and secretion. Exp Biol Med 228:143–151, 2003

Key words: chylomicron remnants; dietary n-3 PUFA; VLDL secretion; cellular redox state; hepatocytes

The link between increased plasma cholesterol levels and atherogenesis has been established in a large number of studies (1, 2), but it is now generally recognized that hypertriacylglycerolemia is also a risk factor for the disease (3, 4). Dietary intake of n-6 polyunsaturated fatty acids (PUFA), found in vegetable oils, and n-3 PUFA, found in oily fish, is known to decrease blood cholesterol and triacylglycerol levels, and consequently to reduce the risk atherosclerosis development (5, 6). However, dietary n-6 PUFA exert their effects mainly by lowering plasma low-density lipoprotein (LDL) cholesterol levels and cause only a modest reduction in blood triacylglycerols, whereas consumption of n-3 PUFA leads to a substantial decrease in plasma triacylglycerol concentrations, but has little effect on blood cholesterol in most circumstances (7, 8).

Triacylglycerol and cholesterol from the diet are delivered to the liver for processing in chylomicron remnants,

This work was supported by the Wellcome Trust, Grants 0572872/Z/99/Z and 057620/Z/99/Z and by the Programme for the "Ricerca Finalizzato 1999" of the Italian Ministry of Health, Grant ISSD70.

[1] To whom requests for reprints should be addressed at Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London NW1 0TU, United Kingdom. E-mail: botham@rvc.ac.uk

Received June 12, 2002.
Accepted September 16, 2002.

1535-3702/03/2282-0143$15.00

which are lipoproteins that are formed from the larger, intestinally derived chylomicrons by lipolysis of part of their triacylglycerol content during their transport in the blood (9). A considerable amount of evidence has accumulated in recent years to indicate that these remnant particles are strongly atherogenic. We and others (10–12) have shown that they are taken up by the blood vessel wall, and this process occurs as efficiently as the uptake of LDL. Furthermore, delayed clearance of chylomicron remnants from the blood has been found to be associated with progression of the development of atherosclerotic lesions (13–15).

Dietary n-3 PUFA lower plasma triacylglycerols by decreasing plasma levels of very-low-density lipoprotein (VLDL) (7, 8), and this is believed to be caused by suppression of VLDL secretion by the liver, an effect that is not observed with dietary n-6 PUFA (16, 17). Feeding studies have shown that hepatic secretion of triacylglycerol, cholesterol, and apolipoprotein B (apoB) in VLDL secretion is reduced by n-3 PUFA (18–20). Moreover, recent work in our laboratory has demonstrated that these effects are brought about directly when the n-3 PUFA are delivered to hepatocytes in chylomicron remnants (21).

Reactive oxygen species (ROS) are formed in the body as by-products of biological reactions involving electron transfer (22). In addition, however, they also modify the intracellular redox balance, causing oxidative stress, and current evidence suggests that relatively small changes in this parameter can affect cell functions such as gene expression and the post-translational modification of proteins (23), which could have an important influence on the development of atherosclerotic lesions (24). Because of its role in detoxification, liver tissue is highly susceptible to oxidative stress generated by its metabolism of drugs, heavy metals, and alcohol (25). There is also evidence to suggest that the type of fat in the diet can influence the oxidative state of hepatocytes, as n-6 PUFA have been shown to increase oxidation levels in comparison with saturated fat (26). Furthermore, we have reported recently that lipid metabolism is altered after exposure of cultured hepatocytes to mild oxidative stress (27).

It is now clear that dietary PUFA influence hepatic gene expression by suppressing the transcription of certain genes and inducing that of others, and that this leads to significant changes in lipid and lipoprotein metabolism (28). In previous work, we have demonstrated that PUFA delivered to liver cells in chylomicron remnants downregulates the expression of mRNA for two important genes involved in hepatic lipid metabolism, namely, the LDL receptor-related protein (LRP), which is involved in the uptake of the remnant particles, and the nuclear receptor peroxisome proliferator-activated receptor α (PPARα), which has an important role in the regulation of hepatic lipid metabolism. Moreover, we have shown that these effects are modulated not only by the type of PUFA predominating in the particles, with n-3 being more effective than n-6 PUFA, but also by the oxidative state of the hepatocytes (29). These findings indicate that dietary PUFA can influence gene transcription directly when they are taken up by the liver in chylomicron remnants, and that the redox state of the cells may play an important part in determining the exact response elicited.

The aim of the present study was to test the hypothesis that the direct effects of dietary n-3 PUFA in suppressing hepatic VLDL secretion when delivered to the liver in chylomicron remnants (21) are related to changes in the transcription of genes encoding key proteins involved in the synthesis, assembly, and secretion of the VLDL particles, and that the oxidative state of hepatocytes influences these changes. The assembly of VLDL particles in the liver is believed to occur in two steps; first, lipid is transferred by the microsomal triacylglycerol transfer protein (MTP) to apoB during translation, and second, the apoB-containing precursor particles fuse with triacylglycerol droplets to form mature VLDL (30). Thus, in the absence of MTP, VLDL are not synthesized (31). In addition, hepatic levels of component lipids such as cholesteryl ester and triacylglycerol also play a role in modulating VLDL formation (32). Cholesteryl ester synthesis is regulated by acyl coenzyme A:cholesterol acyltransferase (ACAT), an enzyme that exists in two forms, ACAT1, which is widely expressed in tissues, and ACAT2, which is found mainly in the liver and intestine. However, current evidence suggests that ACAT2 is responsible for supplying cholesteryl ester for VLDL (30, 33–35). The only committed step in triacylglycerol synthesis is the last in the pathway, which is catalyzed by acyl coenzyme A:diacylglycerol acyltransferase (DGAT) (36). In the present study, cultured rat hepatocytes were incubated with chylomicron remnants enriched in n-3 or n-6 PUFA (derived from fish or corn oil, respectively [37]), and the effects on the expression of mRNA for apoB, MTP, ACAT2, and DGAT were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). For investigation of the influence of the cellular redox state, mild nontoxic increases or decreases in this parameter were induced by incubation with $CuSO_4$ or N-acetyl cysteine (NAC), respectively.

Materials and Methods

Male Wistar rats (300–350 g of body weight) fed a standard pellet diet were housed under constant day length (12 hr) and were allowed access to food and water *ad libitum*. The use of animals was conducted in compliance with the applicable laws in the United Kingdom, and was approved by the Royal Veterinary College Ethics and Welfare Committee.

Sodium pentobarbital, collagenase, bovine serum albumin (BSA), Percoll, dexamethasone, NAC, and Menhaden fish oil were obtained from Sigma-Aldrich Chemical (Poole, Dorset, UK). RPMI 1640 culture medium, antibiotics, insulin, and fetal bovine serum (FBS) were supplied by Life Technologies (Paisley, Renfrewshire, Scotland, UK).

Fetal bovine serum was heat inactivated at 56°C for 30 min prior to use.

Preparation of Chylomicron Remnants. Chylomicron remnants enriched in n-3 or n-6 PUFA were prepared as described by Lambert *et al.* (37). Rats were fed a single dose (1 ml) of corn or fish oil (containing 4 mg/ml α-tocopherol as antioxidant) by stomach tube, and approximately 1 hr later, rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight) and the thoracic duct was cannulated. Chyle was collected (16–18 hr) in the presence of ampicillin (0.1 mg/ml), layered under 0.9% NaCl (d = 1.006 g/ml) and centrifuged at 30,000*g* (20 min at 12°C in a 50.4Ti fixed angle rotor (Beckman Instruments, Fullerton, CA). Large chylomicrons (d > 100 nm) were harvested from the top 1–1.5 ml and were injected (30–40 μ*M* chylomicron triacylglycerol + 50 mg of added glucose/rat) intravenously into anaesthetized rats, which were functionally hepatectomized by ligation of all blood vessels supplying the liver and the gut. After 45 min, the blood was withdrawn, the erythrocytes were removed by centrifugation (2,500*g* for 25 min at 12°C), and the serum obtained was centrifuged for 16 hr at 65,000*g*. Chylomicron remnants were collected from the top fraction by tube slicing and were further purified by layering under 0.9% NaCl (d = 1.006 g/ml), centrifuging at 104,000*g* for 5 hr, and collecting of the top 1–1.5 ml by tube slicing. The triacylglycerol:total cholesterol molar ratio was similar in remnants derived from fish or corn oil (fish oil remnants 9.1 ± 1.1; corn oil remnants 8.7 ± 0.9). In addition, our previous studies have demonstrated that chylomicron remnants prepared in this way are enriched in the fatty acids predominating in the oils fed prior to collection of the chylomicrons, and that the fatty acid composition shows little variation between preparations (37). We have also shown that the ratio of n-3:n-6 PUFA in total lipids is approximately 10-fold higher in fish oil (19.8% n-3 PUFA, 15.4% n-6 PUFA, ratio 1.3) as compared with corn oil (4.2% n-3 PUFA, 33.7% n-6 PUFA, ratio 0.12) remnants (36), and that there is no significant difference in the diameter (104 and 109 nm) of the particles or the apolipoprotein composition of the two types of particles (21, 38).

Preparation and Culture of Hepatocytes. Hepatocytes were isolated by perfusion of rat livers with collagenase as described by Ford *et al.* (39). The cells were resuspended in RPMI 1640 medium supplemented with sodium bicarbonate (2 g/l) penicillin/streptomycin (100 mg/l), gentamycin (50 mg/l), glucose (2 g/l), pyruvate (110 mg/l), and dexamethasone (1 μ*M* [incubation medium]), and the viable cells were separated from the nonviable cells using a Percoll gradient (0%–70%, v/v) (37). The viable cells were washed twice (incubation medium) and were resuspended in incubation medium containing FBS (10%, v/v), and insulin (4 mg/ml). Cell viability as assessed by trypan blue exclusion was routinely >90%. The hepatocytes were cultured in Primaria-coated 6-well plates at 37°C in an atmosphere of 95% air/5% CO_2 as before (21). After attachment of the cells to the dishes, the medium was removed and replaced with incubation medium containing 60 μg/l insulin (culture medium). The monolayer cultures were incubated with chylomicron remnants derived from corn or fish oil or with an equal volume of culture medium, as indicated in the text, at 37°C in air/CO_2 (95%/5%). In some experiments, the cells were preincubated with or without $CuSO_4$ (1 μ*M*) or NAC (5 m*M*) for 24 hr, the medium was removed and replaced with fresh culture medium, and the chylomicron remnants were then added. Cell viability as assessed by trypan blue exclusion after the incubations with $CuSO_4$ or NAC was not different from that in control cells (>90%). For mRNA determination, total RNA was extracted from the cells using a Promega kit (Promega UK, Southampton, UK) according to the manufacturer's instructions.

Analytical Methods. The triacylglycerol and total cholesterol of the chylomicron remnants was determined using kits supplied by Sigma-Aldrich.

The relative abundance of mRNA transcripts for the ACAT2, DGAT, apoB, and MTP were determined by RT-PCR. First-strand synthesis of cDNA was performed using Hotstar Taq reverse transcriptase (Qiagen, Crowley, West Sussex, UK) RT, and amplification was carried out using the following primers for the appropriate rat (apoB) or mouse (ACAT2, DGAT, and MTP) genes: ACAT2, sense, 5′-CCATTGATCTATTCCCTTGTCC-3′, antisense, 5′-GAGTCCTTGGGTAGTTGTCTCG-3′; DGAT, sense, 5′-GTGGTGATGCTGATCCTGAGT-3′, antisense, GAGTATGATGCCAGAGCAAAC-3′, apoB, sense, 5′-TACCTCCGGCAGCTCCATTCC-3′, antisense, 5′-TGCGCTTCCTGCTCTTGCTGTT-3′; MTP, sense 5′-GCTGGAAGGCTTAATTGCAG-3′, antisense, 5′-CGGGTTTTAGACTCGCGATA-3′. Initial denaturation was carried out at 95°C for 15 min, followed by 30 (apoB), 32 (DGAT and MTP) or 35 (ACAT2) cycles consisting of denaturation at 94°C for 1 min, annealing at 56°C (apoB and ACAT2) or 58°C (DGAT and MTP) for 1 min, extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. The products (ACAT2, 300 bp; DGAT, 329 bp; apoB, 340 bp, and MTP 321 bp) were analyzed by electrophoresis using agarose gels (1.2%, w/v) containing ethidium bromide (0.5 μg/ml). Product size was verified using a 100-bp DNA ladder standard (Promega UK). The bands were quantified by optical density volume analysis and were normalized using the values obtained simultaneously for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the samples in the same assay system. The primers were designed to produce products of a different size for each the four genes under investigation. Each primer set was tested by running tubes containing the sense primer + RNA, the antisense primer + RNA, both primers without RNA, and both primers with RNA. In all cases, one band of the expected product size was obtained when both primers and RNA were included, and no bands were found in any other conditions. All samples were diluted appropriately to ensure that they were assayed on the linear part of the curve of

band intensity versus RNA concentration for each gene, which was determined in preliminary experiments (Fig. 1).

Significance limits were calculated using one- or two-way analysis of variance (ANOVA).

Results

Effect of Chylomicron Remnants Derived from Corn or Fish Oil on apoB, MTP, ACAT2, and DGAT mRNA Levels in Cultured Rat Hepatocytes. The levels of mRNA for apoB, MTP, ACAT2, and DGAT were measured after incubation of cultured rat hepatocytes with chylomicron remnants derived from corn or fish oil. The results are shown in Table I. Incubation with corn oil remnants had no significant effect on the amount of mRNA found in the cells for any of the four genes tested after 5 hr of incubation, or on mRNA levels for ACAT2 or apoB after 16 hr of incubation. Experiments with fish oil remnants also showed no change in the amount of mRNA for DGAT, MTP (5 hr of incubation), or apoB (5 or 16 hr of incubation) in the hepatocytes. However, mRNA levels for ACAT2 were decreased markedly (mean change −50%, range of −15% to −80%) in cells exposed to fish oil remnants for 16 hr as compared with those incubated without remnants (Table I and Fig. 2). In addition, the value found with fish oil remnants was significantly lower than that observed with corn oil remnants after the 16-hr time point (mean change, −44%, range of −30% to −69%).

Effect of Treatment of Cultured Rat Hepatocytes with NAC or $CuSO_4$ on mRNA Levels for apoB, MTP, ACAT2, and DGAT. In previous work, we have shown that treatment of cultured hepatocytes with NAC (5 m*M*) or $CuSO_4$ (1 μ*M*) for 24 hr alters the thiol balance so that the GSH:GSSG ratio is approximately 8-fold higher in $CuSO_4$- as compared with the NAC-treated cells, indicating a shift to a pro-oxidizing or pro-reducing state, respectively (29). However, the treatments did not results in any significant loss of viability of the cells as assessed by trypan blue exclusion, general cell morphology under light microscopy, or the propensity of the cells to detach from the culture dishes

The effect of incubation of the cells with NAC or $CuSO_4$ for 24 hr on the levels of mRNA for apoB, MTP, ACAT2, and DGAT are shown in Table II. No effect of either treatment on the amounts of mRNA for ACAT2, MTP, or apoB were observed. However, DGAT mRNA was significantly reduced in NAC-treated as compared with untreated cells (mean change −35%, range of −22% to −60%).

Effect of Chylomicron Remnants Derived from Corn or Fish Oil on apoB, MTP, ACAT2, and DGAT mRNA Levels in Cultured Rat Hepatocytes. Table III shows the effects of corn or fish oil remnants on mRNA levels for apoB, MTP, ACAT2, and DGAT in hepatocytes pretreated with $CuSO_4$ or NAC for 24 hr. ACAT2 mRNA was significantly reduced after 5 hr of incubation without remnants in NAC-treated as compared with untreated and $CuSO_4$-treated cells (NAC versus untreated: mean change of −32%, range of −49% to −18%; NAC versus $CuSO_4$: mean change of −32%, range of −48% to −16%). Parallel decreases were also observed in the presence of corn or fish oil remnants, although the difference between $CuSO_4$- and NAC-treated cells did not reach significance with fish oil remnants. The effect of NAC treatment in lowering DGAT mRNA after the 24-hr pretreatment period (see Table II) was maintained after 5 hr of incubation in the absence of remnants (mean change of −42%, range of −25% to −57%), but these differences were abolished when either corn or fish oil remnants were included in the incubations. In the case of MTP mRNA (Table II and Fig. 3), levels were not significantly different in untreated as compared with $CuSO_4$-treated cells regardless of whether remnants were present or not. However, in NAC-treated cells, fish oil remnants caused an increase in the value observed compared with untreated cells (mean change of +89%, range of +64% to +115%). Furthermore, when the hepatocytes were pretreated with either $CuSO_4$ or NAC, there was a marked rise in MTP mRNA after incubation with fish oil remnants as compared with without remnants ($CuSO_4$: mean change of +118%, range of +58% to +273%; NAC: mean change +277%, range +59% to +385%). mRNA levels for apoB showed no significant change in the presence or absence of remnants after $CuSO_4$ or NAC treatment in comparison with untreated cells. However, there was a small but significant increase in apoB mRNA levels in NAC-treated cells incubated with fish oil remnants as compared with those found with cells incubated with corn oil remnants (mean change of +38%, range of +23% to +44%).

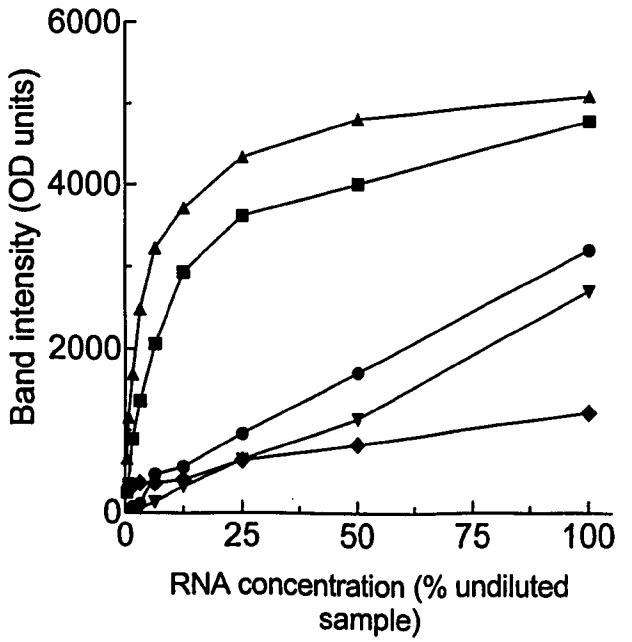

Figure 1. Band intensity versus RNA concentration for GAPDH (▲), apoB (■), ACAT2 (●), DGAT (♦), and MTP (▼). RNA samples were diluted as indicated, and the band intensity after RT-PCR was determined by optical density volume analysis. Data from one experiment of two which gave similar results are shown.

Table I. Effect of Chylomicron Remnants Derived from Corn or Fish Oil on apoB, MTP, DGAT, and ACAT2 mRNA Levels in Cultured Rat Hepatocytes

Treatment	Time	ACAT2	DGAT	MTP	ApoB
None	5 hr	0.28 ± 0.03 (4)	0.45 ± 0.10 (4)	0.46 ± 0.08 (4)	0.83 ± 0.05 (4)
	16 hr	0.34 ± 0.02 (5)	ND	ND	0.78 ± 0.17 (4)
Corn remnants	5 hr	0.32 ± 0.04 (4)	0.35 ± 0.04 (4)	0.63 ± 0.18 (4)	0.83 ± 0.08 (3)
	16 hr	0.30 ± 0.06 (4)	ND	ND	0.95 ± 0.15 (5)
Fish remnants	5 hr	0.21 ± 0.07 (3)	0.42 ± 0.07 (4)	0.70 ± 0.11 (4)	0.72 ± 0.14 (3)
	16 hr	0.17 ± 0.06 (5)*a	ND	ND	0.61 ± 0.13 (4)

Note. Hepatocytes were incubated with chylomicron remnants derived from corn or fish oil (0.3 μmol triacylglycerol/ml) for 5 or 16 hr. Total mRNA was then extracted from the cells and the relative abundance of mRNA for apoB, MTP, DGAT, and ACAT2 was determined by RT-PCR. The bands were quantified by optical density volume analysis, and the values were normalized using those obtained for GAPDH in the same assay system. Data are expressed as the ratio of apoB, MTP, DGAT, or ACAT2:GAPDH and are the mean ± SEM from the number of experiments shown in parentheses. Significance limits: $^*P < 0.05$ vs corresponding control (no remnants); $^aP < 0.05$ vs corn oil remnants (two-way ANOVA, remnant type × time). ND, not determined.

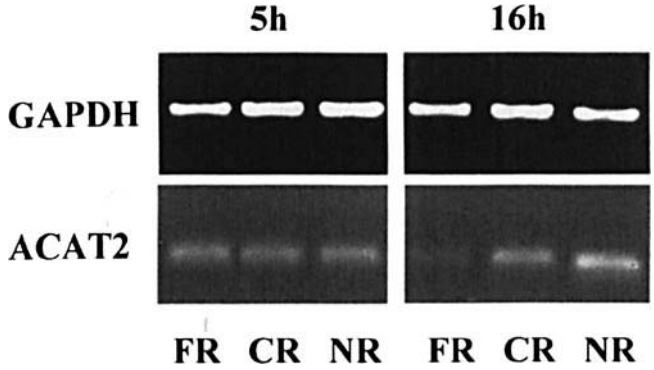

Figure 2. Hepatocytes were incubated without (NR) or with chylomicron remnants derived from corn (CR) or fish oil (FR; 0.3 μ*M* triacylglycerol/ml) for 5 or 16 hr. Total mRNA was then extracted from the cells and the relative abundance of mRNA for ACAT2 was determined by RT-PCR. Bands obtained for ACAT2 and GAPDH in a typical experiment from three to five experiments performed are shown.

Discussion

It has been established in many previous studies that dietary n-3 PUFA decrease plasma triacylglycerol levels by suppressing VLDL secretion by the liver. Feeding fish oil has been shown to decrease the hepatic secretion of triacylglycerol and apoB in humans and experimental animals (16–18, 40), and direct addition of eicosapentaenoic acid to hepatocyte cultures has also been found to have a similar effect (40, 41). However, to study the direct effects of dietary fatty acids on VLDL secretion when they are delivered to the liver from the gut, it is necessary to examine the effects of the physiological carrier, chylomicron remnants, using cultured hepatocytes because the changes observed in feeding experiments could be caused by subsequent changes in the fatty acid composition of the tissues or endogenous lipoproteins such as LDL. In recent work taking this type of approach, we have demonstrated that n-3 PUFA carried in chylomicron remnants directly suppress the secretion of triacylglycerol, cholesterol, and apoB48 in cultured rat liver cells (21). Therefore, the initial objective of the present work was to determine whether the mechanism of these effects involves changes in the transcription of various genes that have an important role in the regulation of hepatic VLDL synthesis, assembly, and secretion.

We have shown previously that chylomicron remnants derived from fish or corn oil prepared *in vivo* from rats as described in "Materials and Methods" are enriched in n-3 (mainly eicosapentaenoic and docosahexaenoic acids) or n-6 (mainly linoleic acid) PUFA, respectively, with the n-3:n-6 ratio being 10-fold higher in the former, and that the two types of particles are similar in all other respects, including their size and lipid and apolipoprotein content (21, 37, 38). Therefore, any differences in the effects of the remnants derived from fish or corn oil must be caused by the difference in their fatty acid composition.

ApoB exists in two forms, apoB100 and apoB48, which corresponds to the N-terminal 48% of apoB100 (42). The two proteins are encoded by the same gene, and apoB48 is produced after post-transcriptional modification of the full-length mRNA transcript by the introduction of a stop signal at codon 2153 (43). ApoB is required for the assembly and secretion of VLDL by the liver, and rat VLDL contains both apoB100 and apoB48 (44, 45). It has been reported previously that the secretion of apoB48, but not apoB100, by rat liver is inhibited by fish oil feeding (20, 40), but that the levels of apoB mRNA are unaffected. Moreover, studies in our laboratory have demonstrated that the suppression of apoB48 secretion occurs directly when n-3 PUFA are delivered to hepatocytes in chylomicron remnants (21), but is not accompanied by changes in the transcription of apoB, and this latter finding was confirmed in the present study (Table I). Therefore, we conclude that dietary n-3 PUFA do not affect the synthesis of apoB at the transcriptional level.

Hepatic MTP mRNA levels have been shown to be influenced by dietary fats, with levels being increased by saturated as compared with monounsaturated and n-6 PUFAs (46). However, the effects of dietary n-3 PUFA do not appear to have been reported previously. Our finding that there was no significant change in MTP mRNA abundance after incubation of hepatocytes with fish oil chylomicron remnants as compared with corn oil remnants or without remnants (Table I) indicates that the effects of remnants

Table II. Effect of $CuSO_4$ and NAC Treatment on apoB, MTP, DGAT, and ACAT2 mRNA Levels in Cultured Rat Hepatocytes

Treatment	ACAT2	DGAT	MTP	ApoB
None	0.85 ± 0.07	0.52 ± 0.08	0.44 ± 0.08	1.26 ± 0.17
$CuSO_4$	0.87 ± 0.05	0.43 ± 0.07	0.43 ± 0.08	1.26 ± 0.18
NAC	0.73 ± 0.07	0.34 ± 0.13*	0.46 ± 0.05	1.14 ± 0.12

Note. Hepatocytes were incubated without (no treatment) or with $CuSO_4$ (1 μ*M*) or NAC (5 m*M*) for 24 hr. Total mRNA was then extracted from the cells and the relative abundance of mRNA for apoB, MTP, DGAT, and ACAT2 was determined by RT-PCR. The bands were quantified by optical density volume analysis, and the values were normalized using those obtained for GAPDH in the same assay system. Data are expressed as the ratio of apoB, MTP, DGAT, or ACAT2:GAPDH and are the mean ± SEM from four experiments. Significance limits: $^*P < 0.05$ vs untreated cell (one-way ANOVA).

Table III. Effect of Chylomicron Remnants Derived from Fish or Corn Oil on apoB, MTP, DGAT, and ACAT2 mRNA Levels in NAC- and $CuSO_4$-Treated Hepatocytes

Gene	Remnant type	No treatment	$CuSO_4$	NAC
ACAT2	None	0.82 ± 0.13 (4)	0.82 ± 0.12 (4)	0.56 ± 0.15 (4)^{ab}
	Corn	0.68 ± 0.15 (4)	0.72 ± 0.16 (4)	0.54 ± 0.17 (4)^{ab}
	Fish	0.63 ± 0.09 (4)	0.60 ± 0.05 (4)	0.52 ± 0.07 (3)^{a}
DGAT	None	0.45 ± 0.10 (4)	0.33 ± 0.08 (4)	0.26 ± 0.07 (4)^{a}
	Corn	0.35 ± 0.04 (4)	0.31 ± 0.05 (4)	0.26 ± 0.03 (4)
	Fish	0.42 ± 0.07 (4)	0.48 ± 0.04 (4)*	0.47 ± 0.07 (4)*
MTP	None	0.46 ± 0.08 (4)	0.50 ± 0.09 (4)	0.35 ± 0.09 (4)
	Corn	0.63 ± 0.18 (4)	0.80 ± 0.25 (4)	0.66 ± 0.20 (4)
	Fish	0.70 ± 0.11 (4)	1.09 ± 0.26 (4)*	1.32 ± 0.33 (4)*#^{a}
ApoB	None	1.37 ± 0.20 (4)	1.36 ± 0.13 (4)	1.32 ± 0.14 (4)
	Corn	1.20 ± 0.05 (4)	1.31 ± 0.13 (4)	1.12 ± 0.14 (4)
	Fish	1.16 ± 0.17 (4)	1.29 ± 0.19 (4)	1.55 ± 0.33 (3)#

Note. Hepatocytes were incubated without (no treatment) or with $CuSO_4$ (1 μ*M*) or NAC (5 m*M*) for 24 hr and the medium was then replaced with fresh medium containing chylomicron remnants derived from corn or fish oil (0.3 μmol triacylglycerol/ml) and the incubation continued for an additional 5 hr. Total mRNA was extracted from the cells and the relative abundance of mRNA for apoB, MTP, DGAT, and ACAT2 was determined by RT-PCR. The bans were quantified by optical density volume analysis, and the values were normalized using those obtained for GAPDH in the same assay system. Data are expressed as the ratio of apoB, MTP, DGAT, or ACAT2:GAPDH and are the mean ± SEM from the number of experiments shown in parentheses. Significance limits: $^*P < 0.05$ vs no remnants with same redox treatment: $^\#P < 0.05$ vs corn remnants with same redox treatment; $^aP < 0.05$ vs untreated cells incubated with the same remnant type or without remnants: $^bP < 0.05$ vs $CuSO_4$-treated cells incubated with same remnant type or without remnants (two-way ANOVA, remnant type × redox treatment).

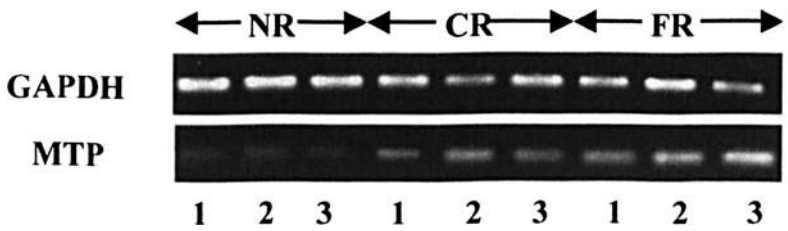

Figure 3. Hepatocytes were incubated without (no treatment) or with $CuSO_4$ (1μ*M*) or NAC (5 m*M*) for 24 hr and the medium was then replaced with fresh medium containing chylomicron remnants derived from corn (CR) or fish oil (FR; 0.3 μ*M* triacylglycerol/ml) or without remnants (NR) and the incubation continued for an additional 5 hr. Total mRNA was extracted from the cells, and the relative abundance of mRNA for MTP was determined by RT-PCR. Bands obtained for MTP and GAPDH in a typical experiment from four experiments performed are shown. 1, no treatment; 2, $CuSO_4$ treated; 3, NAC treated.

enriched in n-3 PUFA in suppressing VLDL secretion demonstrated in our earlier work do not involve effects on MTP at the transcriptional level. Decreased activity of DGAT, causing a reduction in the availability of triacylglycerol, has been suggested as another possible contributory factor in the suppression of VLDL secretion by dietary n-3 PUFA (47). However, our experiments show that DGAT mRNA levels are not affected by exposure of hepatocytes to chylomicron remnants enriched in either n-3 or n-6 PUFA (Table I), and this is consistent with previous work showing that hepatic DGAT activity was not changed in rats fed fish oil (48). Because VLDL secretion is thought to be dependent on cholesteryl ester synthesis (33), the expression of ACAT is another possible target for the action of n-3 PUFA. In an earlier study, we found that the activity of ACAT in cultured rat hepatocytes is decreased by chylomicron remnants derived from fish oil. Levels of mRNA for ACAT1 were unaffected, but the amount of ACAT2 mRNA was decreased by fish oil remnants (49), and a similar effect was observed in the present study (Table I and Fig. 2). Thus, the regulation of the transcription of ACAT2, but not apoB, MTP, or DGAT, appears to play a role in the direct suppression of hepatic VLDL secretion by n-3 PUFA delivered to the liver in chylomicron remnants.

The role of the liver in detoxification and lipid processing renders it susceptible to oxidative stress (25), which may have important effects on hepatocyte function. Mild, nontoxic manipulation of hepatocyte redox levels has been shown in our laboratory and others to influence lipid synthesis, mitochondrial and microsomal cholesterol levels, and the activity of enzymes such as the lysosomal cholesteryl ester hydrolase (27, 50, 51). The GSH:GSSG ratio within cells is known to play an important role in regulating intracellular redox balance, and we have shown previously that treatment of rat hepatocytes with $CuSO_4$ or NAC for 24 hr alters this parameter without detectable loss of cell viability, with the ratio being 8-fold higher in $CuSO_4$- as compared with NAC-treated cells (29). Thus, the treatments shift the cells into pro-oxidizing or pro-reducing states, respectively. Our studies indicated that although these types of redox changes in themselves do not alter the transcription of the genes encoding the LDL receptor, the LDL receptor-related protein (LRP), or PPARα, they do influence the response of mRNA levels of these genes to dietary PUFA taken up by the liver from chylomicron remnants. For example, the abundance of mRNA for all three genes in the presence of remnants enriched in n-3 PUFA was increased in pro-reducing as compared with pro-oxidizing cells (29). However, the effects of the hepatic redox state on the expression of mRNA for the genes involved in VLDL synthesis under investigation in the present work are not known.

The results presented here demonstrate that levels of mRNA for apoB and MTP were unaffected by either NAC or $CuSO_4$, both immediately after 24 hr of exposure, and after an additional 5 hr of incubation in the absence of the compounds (Tables II and III), indicating that the transcription of these genes is unaffected by the cellular redox state. In contrast, the amount of DGAT mRNA (at both time points) and ACAT2 mRNA (5 hr after removal of the $CuSO_4$ or NAC) was decreased in NAC-treated as compared with untreated cells (and $CuSO_4$-treated cells in the case of ACAT2), suggesting that triacylglycerol and cholesteryl ester synthesis is downregulated in pro-reducing conditions. In addition to these changes, the response of transcription of all the genes tested, except ACAT2, to chylomicron remnants was altered by manipulation of the cellular redox state. Moreover, the effects observed varied depending on whether the remnants were enriched in n-3 or n-6 PUFA. In general, the main differences were observed in pro-reducing conditions and with fish oil as compared with corn oil remnants (Table III).

The downregulation of ACAT2 mRNA expression in NAC-treated cells was maintained in the presence of both corn oil and fish oil chylomicron remnants, but the effect on DGAT was abolished, suggesting that the influx of fatty acids from chylomicron remnant triacylglycerol counteracts the suppressive effect of the reducing conditions on the transcription of the gene. However, the most striking effects of the changes in the cellular redox state were observed on the response of MTP mRNA to fish oil remnants (Table III and Fig. 3). In $CuSO_4$- or NAC-treated cells, fish oil remnants caused increases of about 2- or 4-fold, respectively, in hepatocyte MTP mRNA levels, although they had no significant effect in untreated cells (Table III). In marked contrast, no changes in MTP mRNA expression were observed in incubations with corn oil remnants in any redox conditions. In addition, a parallel, but much smaller, change in apoB mRNA was found when NAC-treated cells were exposed to fish oil remnants as compared with corn oil remnants. Assuming that these changes result in increased expression of MTP and apoB protein, they would be expected to lead to a rise in VLDL secretion in hepatocytes exposed to remnants enriched in n-3 as compared with n-6 PUFA in pro-reducing and to a lesser extent in pro-oxidizing cells. Because this is exactly opposite to the effects of the two types of remnants in normal cells, these findings suggest that the cellular redox state is profoundly important in determining the response of hepatic VLDL output to dietary n-3 or n-6 PUFA.

In previous work, we have demonstrated that in pro-reducing conditions, the expression of the LDL receptor, LRP, and PPARα in rat hepatocytes is upregulated by chylomicron remnants derived from fish, but not corn oil (29). Therefore, it appears that a shift to more reducing conditions in the cells enables dietary n-3 PUFA to influence the transcription of a variety of genes involved in the processing of dietary lipids. The highly unsaturated n-3 components of fish oil, eicosapentaenoic (C20:5) and docosahexaenoic (C22:6) acids, are easily peroxidized, and their intake in the diet is known to increase lipid peroxidation in the liver in comparison with the less highly unsaturated n-6 PUFA, linoleic acid (C18:2), which is the major fatty acid in corn oil (52, 53). EPA and DHA have also been shown to increase lipid peroxidation to a greater extent than linoleic acid in cultured rat hepatocytes (54). However, in response, liver cells appear to upregulate their antioxidant defenses. The expression of genes for various antioxidant enzymes, including superoxide dismutase and glutathione transferase, has been shown to be induced by dietary n-3 as compared with n-6 PUFA in mice using high-density oligonucleotide arrays, whereas the expression of genes related to the production of ROS was decreased (55). Furthermore, at the functional level, the activities of enzymes such as catalase and glutathione peroxidase have been found to be increased (56) by dietary fish oil. Therefore, it is clear that complex interactions occur between n-3 PUFA of dietary origin and the cellular redox state, and that these often result in changes at the gene level.

The results of the present study demonstrate that the suppression of VLDL secretion by dietary n-3 PUFA delivered to hepatocytes in chylomicron remnants is not associated with changes in the transcription of the genes for apoB, MTP, or DGAT in cells in their basal redox state, but that downregulation of the expression of mRNA for ACAT2 may have a role in this effect. However, mild, nontoxic

changes in the oxidative state of the liver cells, particularly to pro-reducing conditions, caused striking changes in the response of levels of MTP mRNA (and to a lesser extent, apoB mRNA) to remnants enriched in n-3 PUFA, which suggest that VLDL production may be increased rather than decreased in this case. These findings indicate that relatively minor changes in cellular redox levels can have a major influence on important liver functions such as VLDL synthesis and secretion, and they highlight the importance of further investigation of the mechanism of these effects.

1. Martin MJ, Hulley SB, Browner WS, Kuller IH, Wentworth D. Serum cholesterol, blood pressure, and mortality, implications from a cohort of 361,662 men. Lancet **2:**933–936, 1986.
2. Castelli WP, Garrison RJ, Wilson PWF, Abbot RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels: the Framingham study. J Am Med Assoc **256:**2835–2838, 1996.
3. Miller M. Current perspectives on the management of hypertriglyceridemia. Am Heart J **140:**232–240, 2000.
4. Brewer HB. Hypertriglyceridemia: changes in the plasma lipoproteins associated with an increased risk of cardiovascular disease. Am J Cardiol **83:**3F–12F, 1999.
5. Shepherd J, Packard CJ, Grundy SM, Yeshurun AM, Gotto AM, Taunton OD. Effects of saturated and polyunsaturated fat diets on the chemical composition and metabolism of low density lipoproteins in man. J Lipid Res **21:**91–99, 1980.
6. Thornberg JT, Rudel LL. How do polyunsaturated fatty acids lower lipids? Curr Opin Lipidol **3:**17–21, 1992.
7. Von Lossonczy TO, Ruiter A, Brosengeest-Schoute HC, Von Gent CM, Hermus RJJ. The effect of a fish oil diet on serum lipids in healthy subjects. Am J Clin Nutr **31:**1340–1346, 1978.
8. Philipson BE, Rothrock DW, Connor WE, Harris WS, Illingworth DR. Reduction of plasma lipids, lipoproteins and apoproteins by dietary fish oils in patients with hypertriglyceridemia. N Engl J Med **312:**1210–1216, 1985.
9. Redgrave TG. Formation and metabolism of chylomicrons. Int J Physiol **28:**103–130, 1983.
10. Mamo JCL, Wheeler JR. Chylomicrons or their remnants penetrate rabbit thoracic aorta as efficiently as do smaller macromolecules, including low density lipoprotein, high density lipoprotein and albumin. Coronary Artery Dis **5:**695–705, 1994.
11. Proctor SD, Mamo JCL. Retention of fluorescent-labelled chylomicron remnants within the intima of the arterial wall - evidence that plaque cholesterol may be derived from post-prandial lipoproteins. Eur J Clin Invest **28:**497–503, 1998.
12. Grieve DJ, Avella MA, Elliott J, Botham KM. The influence of chylomicron remnants on endothelial cell function in the isolated perfused rat aorta. Atherosclerosis **139:**273–281, 1998.
13. Mahley RW, Weisgraber KH, Innerarity TL, Rall SC. Genetic defects in lipoprotein metabolism. J Am Med Assoc **265:**78–83, 1991.
14. Groot PH, van Stiphoot WJ, Krauss XH, Janssen H, van Tol A, van Ramshorst E, Chin-On S, Hofman A, Cresswell SR, Havekes L. Postprandial lipoprotein metabolism in normolipidemic men without coronary artery disease. Arterioscler Thromb **11:**653–662, 1991.
15. Patsch JR, Miesenbock G, Hopferwieser T, Muhlberger V, Knapp E, Dunn JK, Gotto AM, Patsch W. Relation of triglyceride metabolism and coronary artery disease. Arterioscler Thromb **12:**1336–1345, 1992.
16. Harris WS. Fish oils and plasma lipids and lipoprotein metabolism in humans: a critical review. J Lipid Res **30:**785–807, 1989.
17. Wong SH, Nestel PJ, Trimble RP, Storer GB, Illman RJ, Topping DL. The adaptive changes of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver. Biochim Biophys Acta **792:**103–109, 1984.
18. Harris WJ, Connor WE, Illingworth DR, Rothrock DW, Foster DM. Effects of fish oil on VLDL triglyceride kinetics in humans. J Lipid Res **31:**1549–1558, 1990.
19. Ribiero A, Mangeney M, Cardot P, Loriette C, Rayssiguier Y, Chambaz J, Bereziat G. Effect of dietary fish oil and corn oil on lipid metabolism and apolipoprotein gene expression by rat liver. Eur J Biochem **196:**499–507, 1991.
20. Brown A-M, Baker PW, Gibbons GF. 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 B48. J Lipid Res **38:**469–481, 1999.
21. Zheng X, Avella M, Botham KM. Comparison of the effects of dietary n-3 and n-6 polyunsaturated fatty acids on very-low-density lipoprotein secretion when delivered to hepatocytes in chylomicron remnants. Biochem J **357:**481–487, 2001.
22. Halliwell B. Free radicals, antioxidants and human disease: curiosity, cause or consequence? Lancet **344:**721–724, 1994.
23. Arrigo A-P. Gene expression and thiol redox state. Free Radic Biol Med **27:**916–921, 1999.
24. Rosenblat M, Aviram M. Macrophage glutathione content and glutathione peroxidase activity are inversely related to cell-mediated oxidation of LDL: in vitro and in vivo studies. Free Radic Biol Med **24:**305–317, 1998.
25. Bhattacharya S. Mechanisms of signal transduction in the stress response of hepatocytes. Int Rev Cytol **184:**109–156, 1998.
26. Slim RM, Toborek M, Watkins BA, Boissoneault GA, Hennig B. Susceptibility to hepatic oxidative stress in rabbits fed different animal and plant fats. J Am Coll Nutr **15:**289–294, 1996.
27. Napolitano M, Rivabene R, Avella MA, Amicone L, Tripodi M, Botham KM, Bravo E. Oxidation affects the regulation of hepatic lipid synthesis by chylomicron remnants. Free Radic Biol Med **30:**506–515, 2001.
28. Jump DB, Thelen A, Mater M. Dietary polyunsaturated fatty acids and hepatic gene expression. Lipids **34**(Suppl)**:**S219–S212, 1999.
29. Zheng X, Rivabene R, Cavallari C, Napolitano M, Avella M, Bravo E, Botham KM. The effects of chylomicron remnants enriched in n-3 or n-6 polyunsaturated fatty acids on the transcription of genes regulating their uptake and metabolism by the liver: influence of cellular oxidative state. Free Radic Biol Med **32:**1123–1131, 2002.
30. Shelness GS, Sellers JA. Very-low-density lipoprotein assembly and secretion. Curr Opin Lipidol **12:**151–157, 2001.
31. White DA, Bennett AJ, Billett MA, Salter AM. The assembly of triacylglycerol-rich lipoproteins: an essential role for the microsomal triacylglycerol transfer protein. Br J Nutr **80:**219–229, 1998.
32. Mason TM. The role of factors that regulate the synthesis and secretion of very-low-density lipoprotein by hepatocytes. Crit Rev Clin Lab Sci **35:**461–487, 1998.
33. Joyce C, Skinner K, Anderson RA, Rudel LL. Acyl-coenzyme A: cholesterol acyltransferase 2. Curr Opin Lipidol **10:**89–95, 1999.
34. Lee RG, Willingham MC, Davis MA, Skinner KA, Rudel LL. Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates. J Lipid Res **41:**1991–2001, 2000.
35. Buhman KK, Accad M, Novak S, Choi RS, Wong JS, Hamilton RL, Turley S, Farese RV. Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2 deficient mice. Nat Med **6:**1341–1347, 2000.
36. Cases S, Smith SJ, Zheng YW, Myers HM, Lear SR, Sande E, Novak S, Collins C, Welch CB, Lusis AJ, Erickson SK, Farese RV. Identification of a gene encoding an acyl CoA: diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. Proc Natl Acad Sci U S A **95:**13018–13023, 1998.
37. Lambert MS, Botham KM, Mayes PA. Modification of the composition of dietary oils and fats upon incorporation into chylomicron remnants. Br J Nutr **76:**435–445, 1996.

38. Lambert MS, Avella MA, Berhane Y, Shervill E, Botham KM. The fatty acid composition of chylomicron remnants influences their binding and internalization by isolated hepatocytes. Eur J Biochem **268:**3983–3992, 2001.
39. Ford RP, Botham KM, Suckling KE, Boyd GS. Characterisation of rat hepatocyte monolayers for investigation of the metabolism of bile salts. Biochim Biophys Acta **836:**185–191, 1985.
40. Brown A-M, Castle J, Hebbachi A-M, Gibbons GF. Administration of n-3 fatty acids in the diet of rats or directly to hepatocyte cultures results in different effects on hepatocellular apoB metabolism and secretion. Atrerioscler Thromb Vasc Biol **19:**106–114, 1999.
41. Lin Y, Smit MJ, Havinga R, Verkade HJ, Vonk RJ, Kuipers F. Differential effects of eicosapentaenoic acid on glycerolipid and apolipoprotein B metabolism in primary human hepatocytes compared to HepG2 cells and primary rat hepatocytes. Biochim Biophys Acta **1256:**88–96, 1995.
42. Olofsson SO, Asp L, Borèn J. The assembly and secretion of apolipoprotein B-containing lipoproteins. Curr Opin Lipidol **10:**341–346, 1999.
43. Lau PP, Xiong W, Zhu H-J, Chen S-H, Chan L. Apolipoprotein B editing is an intranuclear event that occurs post-transcriptionally coincident with splicing and polyadenylation. J Biol Chem **266:**20550–20554, 1991.
44. Shelness GS, Ingram MF, Huang XF, Delozier AJ. Apolipoprotein B in the rough endoplasmic reticulum: translation, translocation and the initiation of lipoprotein assembly. J Nutr **129:**456S–462S, 1999.
45. Windmueller HG, Spaeth AE. Regulated biosynthesis and divergent metabolism of three forms of hepatic apolipoprotein B in the rat. J Lipid Res **26:**70–81, 1985.
46. Bennett AJ, Billett MA, Salter AM, White DA. Regulation of hamster hepatic microsomal triglyceride transfer protein mRNA levels by dietary fats. Biochem Biophys Res Commun **212:**473–478, 1995.
47. Madsen L, Rustan AC, Vaagenes H, Berge K, Dyroy E, Berge RK. Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference. Lipids **34:**951–963, 1999.
48. al-Shurbaji A, Larsson-Backstrom C, Berglund L, Eggertsen G, Bjorkhem I. Effect of n-3 fatty acids on the key enzymes involved in cholesterol and triglyceride turnover in rat liver. Lipids **26:**385–389, 1991.
49. Botham KM, Maldonado E, Chico Y, Zheng X, Avella M, Ochoa B. The influence of chylomicron remnants on cholesteryl ester metabolism in cultured rat hepatocytes: comparison of the effects of particles enriched in n-3 or n-6 polyunsaturated fatty acids. Biochim Biophys Acta **1534:**96–109, 2001.
50. Huertas JR, Battino M, Barzanti V, Maranesi M, Parenti-Castelli G, Littarru GP, Turchetto E, Mataix FJ, Lenaz G. Mitochondrial and microsomal cholesterol mobilization after oxidative stress induced by adriamycin in rats fed with dietary olive and corn oil. Life Sci **50:**2111–2118, 1992.
51. Tanaka M, Iio T, Tabata T. Effect of cupric ions on serum and liver cholesterol metabolism. Lipids **22:**1016–1019, 1987.
52. Yasuda S, Watanabe S, Kobayashi T, Okuyama H. Effects of dietary unsaturated fatty acids and chronic carbon tetrachloride treatment on the accumulation of oxidation products, α-tocopherol and liver injury in mice. Biol Pharm Bull **21:**1050–1056, 1998.
53. Skuladottir GV, Shia-Hua D, Brodie AE, Reed DJ, Wander RC. Effects of dietary oils and methyl ethyl ketone peroxide on in vivo lipid peroxidation and antioxidants in rat heart and liver. Lipids **29:**351–357, 1994.
54. Sugihara N, Tsuruta Y, Date Y, Furuno K, Kohashi K. High peroxidative susceptibility of fish oil polyunsaturated fatty acid in cultured rat hepatocytes. Toxicol Appl Pharmacol **126:**124–128, 1994.
55. Takahashi M, Tsuboyama-Kasaoka N, Nakatani T, Ishii M, Tsusumi S, Aburatani H, Ezaki O. Fish oil feeding alters liver gene expressions to defend against PPARα activation and ROS production. Am J Physiol Gastrointest Liver Physiol **282:**G338–G348, 2002.
56. Ruiz-Gutierrez V, Perez-Espinosa A, Vazquez CM, Santa-Maria C. Effects of dietary fats (fish, olive and high-oleic acid sunflower oils) on lipid composition and antioxidant enzymes in rat liver. Br J Nutr **82:**233–241, 1999.