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

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The effects of chylomicron remnants enriched in $n-3$ or $n-6$ polyunsaturated fatty acids (derived from fish or corn oil respectively) on the secretion of very-low-density lipoprotein (VLDL) lipid and apolipoprotein B (apoB) by rat hepatocytes in culture was investigated. Remnants were prepared *in vivo* from chylomicrons obtained from rats given an oral dose of fish or corn oil and incubated with cultured hepatocytes for up to 16 h. The medium was then removed and the secretion of cholesterol and triacylglycerol into the whole medium or the $\rho < 1.050$ g/ml fraction during the following 7–24 h was determined. After exposure of the cells to fish-oil as compared with corn-oil remnants, secretion of both cholesterol and triacylglycerol into the whole medium was decreased by 25–35%, and secretion into the $\rho < 1.050$ g/ml fraction was decreased by 20–25%. In addition, the levels of apoB48 found in the $\rho < 1.050$ g/ml fraction were significantly lower in cells treated with fish-oil rather than corn-oil remnants, although the levels of apoB100 remained unchanged. The expression of mRNA for apoB, as determined by reverse-transcriptase PCR, however, was not significantly changed after exposure of the cells to both types of remnants. These results demonstrate that the effects of dietary $n-3$ polyunsaturated fatty acids in depressing hepatic VLDL secretion occur directly when they are delivered to the liver from the intestine in chylomicron remnants, and that the secretion, but not the synthesis, of apoB is targeted.

Key words: apolipoprotein B, cholesterol, cholesteryl ester, triacylglycerol.

INTRODUCTION

It has been known for many years that the type of fat in the diet influences blood lipid levels, and, consequently, the risk of the development of atherosclerosis and related cardiovascular diseases [1,2]. Early studies showed that dietary intake of $n-6$ polyunsaturated fatty acids, which are abundant in vegetable oils, was inversely related to the incidence of cardiovascular disease [1,2], and more recently, it has been established that diets containing oily fish, which is rich in the $n-3$ polyunsaturated fatty acids, eicosapentaenoic ($C_{20:5}$) and docosahexaenoic ($C_{22:6}$) acid, also retard the development of atherosclerosis [3,4].

The relationship between increased plasma cholesterol levels and the development of atherosclerosis has been demonstrated in many studies over a long period [5,6]. The role of hypertriglyceridaemia, however, has been more controversial, but it is now generally accepted as a risk factor for coronary heart disease [7,8]. Both dietary $n-3$ and $n-6$ polyunsaturated fatty acids have been shown to decrease plasma cholesterol and triacylglycerol levels, but the responses to the two types of fat show important differences. It is clear that the major effect of the $n-6$ series is a lowering of plasma cholesterol levels, mainly via a decrease in low-density lipoprotein (LDL) cholesterol [9,10], whereas their effect on plasma triacylglycerols is modest [11]. In contrast, dietary fish oil causes a substantial lowering of plasma triacylglycerol levels through a decrease in very-low-density lipoprotein (VLDL) concentrations, but appears to have little effect on LDL cholesterol levels in most circumstances [12,13].

There is considerable evidence to suggest that the influence of dietary $n-3$ polyunsaturated fatty acids on plasma triacylglycerol levels is caused by the suppression of VLDL secretion by the liver [3,14], an effect that is not observed with dietary $n-6$ polyunsaturated fatty acids [14]. Feeding studies have shown that dietary fish oil decreases hepatic secretion of triacylglycerol [15–19], whereas the secretion of apolipoprotein B (apoB) by hepatocytes from fish-oil-fed rats has been reported to be decreased compared with that found with cells from animals fed corn oil (rich in $n-6$ polyunsaturated fatty acids) or a low fat diet [17–19]. Furthermore, direct addition of eicosapentaenoic acid to hepatocyte cultures *in vitro* has also been shown to decrease the secretion of triacylglycerol and apoB by the cells [18–20]. Fatty acids from the diet, however, do not reach the liver in their non-esterified form, but in triacylglycerol carried in chylomicron remnants. These studies, therefore, cannot resolve the question of whether effects of dietary fatty acids on hepatic VLDL secretion arise directly as a result of their delivery to the liver from the gut, or subsequently as a result of changes in the fatty acid composition of the tissues or endogenous lipoproteins such as LDL.

Ingested fat and cholesterol are initially incorporated into chylomicrons: large, triacylglycerol-rich lipoproteins that enter the circulation via the thoracic duct. These particles initially undergo lipolysis in extra-hepatic capillary beds, a process which removes much of the triacylglycerol and forms remnant particles, which then deliver the cholesterol and the remaining triacylglycerol to the liver [21]. Recent work in our laboratory has

Abbreviations used: apoB, apolipoprotein B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; RT-PCR, reverse transcriptase-PCR; VLDL, very-low-density lipoprotein.

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shown that the fatty acid composition of chylomicrons and the remnants derived from them reflects that of the fat in the diet [22]. Thus, in order to study the direct effects of dietary fats on hepatic VLDL secretion, it is necessary to deliver the fatty acids to hepatocytes in chylomicron remnants.

In the present work, we have investigated the hypothesis that dietary $n-3$ polyunsaturated fatty acids influence hepatic VLDL secretion directly when delivered to the liver in chylomicron remnants by determining the effects of chylomicron remnants enriched in $n-3$ or $n-6$ polyunsaturated fatty acids on the secretion of triacylglycerol, cholesterol and apoB by cultured rat hepatocytes, and on the expression of mRNA for apoB in the cells.

EXPERIMENTAL

Animals and materials

Male Wistar rats (300–350 g body mass) were fed a standard pellet diet, housed under conditions of constant day length (12 h) and allowed access to food and water *ad libitum*.

Sodium pentobarbitol, collagenase, BSA, Percoll and Menhaden fish oil were obtained from Sigma–Aldrich Chemical Co. Ltd. (Poole, Dorset, U.K.). RPMI 1640 culture medium, antibiotics, insulin and fetal bovine serum were supplied by Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). Fetal bovine serum was heat-inactivated at 56 °C for 30 min before use.

Preparation of chylomicron remnants

Chylomicrons and chylomicron remnants enriched in $n-6$ or $n-3$ polyunsaturated fatty acids were prepared as described previously [22]. Briefly, rats were given 1 ml of corn or fish oil (containing α-tocopherol as an antioxidant) by stomach tube and approx. 1 h later the animals were anaesthetized with sodium pentobarbitol (60 mg/kg body weight), and their thoracic ducts were cannulated. Chyle was collected for 15–18 h in the presence of ampicillin (0.1 mg/ml), layered under 0.9 % NaCl (ρ 1.006 g/ml), and then centrifuged for 30000 ***g*** for 20 min in a fixed-angle rotor at 12 °C, before large chylomicrons were harvested from the top fraction by tube slicing. For remnant preparation, rats were anaesthetized and functionally hepatectomized by ligation of all major blood vessels supplying the liver and the gut, and chylomicrons (30–40 μmol of triacylglycerol), together with 50 mg of glucose, were injected intravenously. After 45 min, the animals were exsanguinated and blood obtained was centrifuged [2500 ***g*** (3000 rev./min), 25 min, 12 °C] to remove the erythrocytes. Chylomicron remnants were isolated from the serum by centrifugation for 65000 ***g*** for 16 h, followed by collection of the top fraction by tube slicing, and purified further by centrifugation for 104000 ***g*** for 5 h after layering under 0.9 % NaCl (ρ 1.006 g/ml). The remnants were then collected from the top fraction by tube slicing. The triacylglycerol:cholesterol molar ratio (fish-oil remnants 7.9 ± 0.6; corn-oil remnants 7.9 ± 0.5) and the diameter of the remnant particles obtained (fish-oil remnants, 104 ± 45 nm; corn-oil remnants, 109 ± 43 nm) were similar in those derived from fish or corn oil. The apolipoprotein composition of the two types of preparation was also found to be similar to apoB48 and apoE, but not other apolipoproteins, detected after SDS/PAGE. We have shown previously that the chylomicron remnants prepared in this way are enriched in the fatty acids predominating in the oils fed prior to chyle collection, and that their fatty acid composition shows little variation from preparation to preparation [22].

Preparation and culture of hepatocytes

Hepatocytes were isolated from rat livers by perfusion with collagenase, as described previously [23]. The cells were resuspended in RPMI 1640 medium containing sodium bicarbonate (2 g/l), penicillin/streptomycin (100 mg/l), gentamicin (50 mg/l), glucose (2 g/l), pyruvate (110 mg/l), dexamethasone (1 μM), and applied to a 0–70 % (v/v) Percoll gradient to separate the viable from the non-viable cells, as described before [23]. The viable cells were washed twice with RPMI 1640 medium supplemented as above, and finally were resuspended in RPMI medium containing 10 % (v/v) fetal bovine serum and insulin (4 mg/ml). Cell viability, as assessed by Trypan Blue exclusion, was routinely > 90 %. The cells were cultured in Primaria-coated plastic Petri dishes at 37 °C in an atmosphere of air/CO_2 (19:1), as described by Isusi et al. [24]. After adhesion of the cells to the dishes, the medium was then removed and replaced with supplemented RPMI medium containing 60 μg/l insulin, but without fetal bovine serum. The cultures were incubated in the presence of chylomicron remnants derived from corn oil or fish oil (separate remnant preparations of each type were used for each hepatocyte preparation), or with an equal volume of culture medium, at 37 °C in air/CO_2 (19:1) for the times indicated. The medium was then removed, and the cells were washed three times with PBS (in 2 ml) for experiments on VLDL secretion, or with incubation medium (three times in 1 ml) for experiments involving RNA extraction. For mRNA determination, the total RNA was then extracted from the cells as described below. For lipid and VLDL secretion studies, fresh medium without remnants was added and the incubation was continued for the appropriate time. The medium was then collected and either extracted with chloroform:methanol (2:1, v/v) or centrifuged for 104000 ***g*** for 16 h at ρ 1.050 g/ml (the density was adjusted using KBr), and the top fraction containing VLDL was obtained by tube slicing. A density of $\rho < 1.050$ g/ml was chosen to exclude high-density lipoprotein, but to include any intermediate-density lipoproteins (IDLs) or LDL that may have been formed by the action on VLDL of lipases secreted by the cells [25]. The cells were harvested from the dishes, washed twice with PBS, and after the removal of an aliquot for protein determination, the lipids were extracted with chloroform:methanol (2:1, v/v).

Analytical methods

The triacylglycerol and total or unesterified cholesterol contents of the chylomicron remnants or cell, medium or VLDL extracts were determined using kits supplied by Sigma–Aldrich Co. Ltd, and the esterified cholesterol content was calculated by subtracting the value for unesterified cholesterol from that for total cholesterol. Protein concentrations were determined by the method of Lowry et al. [26].

Apolipoproteins in the $\rho < 1.050$ g/ml medium fractions were separated by SDS/PAGE [27] after preparation of the samples, as described by Mindham and Mayes [28]. The gels were stained with Coomassie Blue followed by silver staining, and the bands corresponding to apoB100 and apoB48 were quantified by optical density volume analysis using a Biorad HP600 scanning densitometer and Molecular Analytical software.

Total RNA was extracted from hepatocytes using a Promega kit (Promega UK, Southampton, U.K.) according to the manufacturer's instructions. For reverse transcriptase (RT)-PCR, first-strand cDNA synthesis was performed using avian myeloblastosis virus ('AMV') reverse transcriptase, and the resulting cDNA was amplified with sense (5′-TACCTCCGGCAGCTCCATTCC-3′) and antisense (5′- TGCGCTTCCTGCTCTTGCTGTT-3′) primers for rat apoB under the following PCR

conditions: in a total volume of 30 μl, initial denaturation at 95 °C for 2 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. The linearity of the assay was established in preliminary experiments, and 35 cycles were found to be suitable for quantification of the product. The 340 bp product was analysed by electrophoresis using agarose gels (1.2 %) containing ethidium bromide (0.5 μg/ml), and the bands were quantified by optical density volume analysis, and normalized using the values obtained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the samples using a similar assay system. GAPDH was assayed at the same time as apoB, but using separate tubes. The assay used measured total apoB mRNA (apoB100 + apoB48) in the cells.

Statistical significance was calculated using Student's paired *t* test.

RESULTS

Effect of chylomicron remnants derived from fish oil or corn oil on the secretion of lipid by cultured rat hepatocytes

In the first set of experiments, cultured rat hepatocytes were incubated with or without chylomicron remnants derived from fish oil or corn oil (0.3 μmol of triacylglycerol/ml) for 5 h, the medium was then removed and the secretion of triacylglycerol and cholesterol into the medium during a further 16 h of incubation was determined. The results (Table 1) showed that the amount of both cholesterol and triacylglycerol secreted into the medium was significantly lower in the presence of remnants derived from fish oil as compared with corn oil, and the secretion of both unesterified and esterified cholesterol was reduced to a similar extent. The levels of all the lipids measured were decreased by an average of 20–25 % in medium from cells incubated with fish oil as compared with corn-oil remnants in all six experiments (values for experiments with fish-oil remnants as a percentage of those with corn-oil remnants: unesterified cholesterol, 81.2 ± 4.8 %; cholesteryl ester, 80.2 ± 2.7 %; total cholesterol, 77.2 ± 4.9 %; triacylglycerol, 77.9 ± 3.7 %; means ± S.E.M., $n = 6$). In addition, hepatocytes incubated with fish-oil, but not corn-oil remnants, secreted less total cholesterol into the medium in comparison with cells incubated without remnants, and these changes were due to decreases in the output of both unesterified and esterified cholesterol (Table 1). No significant changes in the cellular cholesterol content were detected at the end of these experiments (Table 3).

In order to investigate the effects of longer-term exposure of hepatocytes to chylomicron remnants, a second series of experiments was performed in which the cells were incubated with or without the two types of particles for 16 h, and secretion of lipid into the medium in the following 7 or 24 h was measured (Table 2). In cells exposed to fish-oil remnants rather than corn-oil remnants, the levels of triacylglycerol secreted after 7 h were significantly lower (as a percentage of the value with corn remnants, 50.7 ± 14.6 %), but although cholesterol levels tended to be decreased, the changes did not reach statistical significance. After 24 h, however, the secretion of both triacylglycerol and cholesterol was significantly decreased by about 35 %, with unesterified cholesterol and cholesteryl ester again showing changes of a similar magnitude (percentage value with corn-oil remnants: unesterified cholesterol, 66.7 ± 8.9 %; cholesteryl ester, 65.7 ± 9.4 %; total cholesterol, 67.6 ± 9.2 %; triacylglycerol, 65.5 ± 12.3 %; means ± S.E.M., $n = 7$). In comparison with cells incubated without remnants, there was no change in the secretion of total or unesterified cholesterol in cells exposed to fish- or corn-oil remnants, but there was a significant decrease in the output of cholesteryl ester in those treated with the particles derived from fish oil after both 7 and 24 h (Table 2). In addition, after 7 h, there was a small, but significant, rise in the total cholesterol content of the cells, and this was due to an increase in unesterified cholesterol, although cholesteryl ester levels were

Table 1 Secretion of lipids into the medium of rat hepatocytes after incubation with chylomicron remnants derived from fish or corn oil for 5 h

Hepatocytes were incubated with chylomicron remnants derived from fish or corn oil (0.3 μmol triacylglycerol/ml) for 5 h, the medium was then removed and replaced with fresh medium without remnants, and the incubation was continued for a further 16 h. After this time, the medium was collected, and the total content of triacylglycerol (TG), total cholesterol (TC), unesterified cholesterol (UC) and cholesteryl ester (CE) was determined. Data are expressed as nmol/mg cell protein and are the means ± S.E.M. from six separate hepatocyte preparations. Significance limits; $*P <$ 0.05 vs. corn-oil remnants, $\dagger P < 0.01$ vs. corn-oil remnants; $\ddagger P < 0.05$, vs. incubation without remnants, $\S P < 0.01$ vs. incubation without remnants.

Incubation time with CR (h)	Secretion period (h)	Remnant type	UC	CE	TC	TG
5	16	None	6.65 ± 1.16	3.17 ± 0.39	9.81 ± 1.38	29.75 ± 3.48
		Corn	6.90 ± 1.22	2.56 ± 0.27	9.46 ± 1.34	33.09 ± 2.98
		Fish	5.58 ± 1.07*‡	1.97 ± 0.20†§	7.55 ± 1.07†§	27.65 ± 3.16†

Table 2 Secretion of lipid into the medium after incubation of rat hepatocytes with chylomicron remnants derived from fish or corn oil for 16 h

Hepatocytes were incubated with chylomicron remnants derived from fish or corn oil (0.3 μmol triacylglycerol/ml) for 16 h, the medium was then removed and replaced with fresh medium without remnants, and the incubation was continued for a further 7 or 24 h. After this time, the medium was collected, and the total content of triacylglycerol (TG), total cholesterol (TC), unesterified cholesterol (UC) and cholesteryl ester (CE) was determined. Data are expressed as nmol/mg cell protein and are the means ± S.E.M. from the number of separate hepatocyte preparations shown in parentheses. Significance limits; $*P < 0.05$ vs. corn-oil remnants; $\dagger P < 0.05$ vs. incubation without remnants.

Incubation time with CR (h)	Secretion period (h)	Remnant type	UC	CE	TC	TG
16	7	None	1.90 ± 0.25(4)	0.79 ± 0.12(4)	2.69 ± 0.35(4)	7.72 ± 3.29(4)
		Corn	2.28 ± 0.42(4)	0.95 ± 0.27(4)	3.22 ± 0.67(4)	11.81 ± 2.58(4)
		Fish	1.66 ± 0.31(4)	0.42 ± 0.03(4)†	2.08 ± 0.34(4)	5.61 ± 1.60(4)*
16	24	None	3.77 ± 0.43(7)	1.07 ± 0.21(7)	4.84 ± 0.61(7)	11.66 ± 3.73(6)
		Corn	5.04 ± 0.80(7)	1.27 ± 0.36(7)	6.31 ± 1.12(7)	20.01 ± 3.71(6)
		Fish	3.00 ± 0.34(7)*	0.72 ± 0.14(7)*†	3.72 ± 0.42(7)*	11.67 ± 2.60(6)*

Table 3 Cholesterol content of rat hepatocytes after incubation with chylomicron remnants derived from fish or corn oil

Hepatocytes were incubated with chylomicron remnants derived from fish or corn oil (0.3 μmol triacylglycerol/ml) for 5 or 16 h, the medium was then removed and replaced with fresh medium without remnants, and the incubation was continued for a further 7, 16 or 24 h. After this time the cells were harvested and the content of total cholesterol (TC), unesterified cholesterol (UC) and cholesteryl ester (CE) was determined. Data are expressed as nmol/mg cell protein and are the means ± S.E.M. from the number of separate hepatocyte preparations shown in parentheses. Significance limits; $^*P < 0.05$ vs. corn-oil remnants $^{**}P < 0.01$ vs. corn-oil remnants.

Incubation time with CR (h)	Secretion period (h)	Remnant type	UC	CE	TC
5	16	None	44.09 ± 2.18(6)	4.84 ± 0.29(6)	48.90 ± 1.93(6)
		Corn	43.41 ± 2.22(6)	4.85 ± 0.38(6)	48.3 ± 2.27(6)
		Fish	43.69 ± 1.40(6)	4.48 ± 0.31(6)	48.18 ± 1.18(6)
16	7	None	44.40 ± 4.8(4)	4.14 ± 0.86(4)	48.54 ± 5.50(4)
		Corn	44.01 ± 4.18(4)	4.43 ± 0.99(4)	48.44 ± 5.09(4)
		Fish	47.62 ± 4.72(4)**	4.54 ± 0.91(4)	52.16 ± 5.66(4)**
16	24	None	48.03 ± 3.04(7)	4.63 ± 0.43(7)	52.66 ± 3.21(7)
		Corn	51.10 ± 2.97(7)	5.54 ± 0.65(7)	56.64 ± 3.48(7)
		Fish	48.91 ± 3.12(7)	4.81 ± 0.53(7)*	53.72 ± 3.36(7)

Table 4 Secretion of lipid into the $\rho < 1.050$ g/ml fraction of the medium after incubation of hepatocytes with chylomicron remnants derived from fish or corn oil for 5 h

Hepatocytes were incubated with chylomicron remnants derived from fish or corn oil (0.3 μmol triacylglycerol/ml) for 5 h, the medium was then removed and replaced with fresh medium without remnants, and the incubation was continued for a further 16 h. After this time the medium was collected and the content of triacylglycerol (TG), total cholesterol (TC), unesterified cholesterol (UC) and cholesteryl ester (CE) in the $\rho < 1.050$ g/ml fraction was determined. Data are expressed as nmol/mg cell protein and are the means ± S.E.M. from six separate hepatocyte preparations. Significance limits; $^*P < 0.05$ vs. corn-oil remnants †$P < 0.01$ vs. corn-oil remnants; ‡$P < 0.05$ vs. incubation without remnants; §$P < 0.01$ vs. incubation without remnants.

Remnant type	UC	CE	TC	TG
None	5.00 ± 0.86	2.35 ± 0.18	7.35 ± 0.85	27.76 ± 3.42
Corn	5.17 ± 0.97	1.93 ± 0.19	7.10 ± 0.93	29.11 ± 3.31
Fish	3.94 ± 0.85*‡	1.44 ± 0.14*§	5.38 ± 0.79†§	23.69 ± 3.62*

Table 5 Secretion of apoB into the medium in VLDL after incubation of hepatocytes with chylomicron remnants derived from fish or corn oil for 5 h

Hepatocytes were incubated with chylomicron remnants derived from fish or corn oil (0.3 μmol triacylglycerol/ml) for 5 h, the medium was then removed and replaced with fresh medium without remnants, and the incubation was continued for a further 16 h. After this time the medium was collected, the proteins in the $\rho < 1.050$ g/ml fraction were separated by SDS/PAGE and the bands were quantified by volume density analysis. Data are expressed in arbitrary units and are the means ± S.E.M. from four separate hepatocyte preparations. Significance limits: $^*P < 0.05$ vs. corn-oil remnants; †$P < 0.05$ incubation without remnants.

Remnant type	apoB100	apoB48
None	0.52 ± 0.14	2.68 ± 0.74
Corn	0.34 ± 0.09	2.00 ± 0.55
Fish	0.31 ± 0.13	1.26 ± 0.44*†

unaffected (Table 3). After 24 h, however, this difference was no longer apparent.

Effect of chylomicron remnants derived from fish oil or corn oil on the secretion of lipid and apoB into the $\rho < 1.050$ g/ml fraction of the medium by cultured rat hepatocytes

When the medium of the cells was fractionated by ultracentrifugation, we found that approx. 90 % of the triacylglycerol and approx. 80 % of both the unesterified and esterified cholesterol was found in the top fraction ($\rho < 1.050$ g/ml), thus the results described above suggest that chylomicron remnants derived from fish oil as compared with corn oil decrease the secretion of lipid in VLDL. In the next series of experiments, therefore, the influence of the two types of remnants on the levels of lipid and apoB found in the $\rho < 1.050$ g/ml fraction of the medium was investigated. Hepatocytes were incubated in the presence or absence of chylomicron remnants derived from fish oil or corn oil for 5 h, the medium containing the remnants was then removed, and the amount of cholesterol, triacylglycerol and apoB secreted into the $\rho < 1.050$ g/ml fraction of the medium after a further 16 h was measured. Because rat liver secretes VLDL containing apoB100 and apoB48 [29], both forms of the protein were determined.

The secretion of lipid into the $\rho < 1.050$ g/ml fraction of the medium is shown in Table 4. The levels of triacylglycerol were significantly reduced by approx. 20 % (percentage value with corn-oil remnants, 81.2 ± 6.8 %), and the levels of total cholesterol by approx. 24 % (76.1 ± 4.4 %; means ± S.E.M., $n = 6$), after incubation with fish-oil as compared with corn-oil remnants, with similar changes observed in both unesterified cholesterol and in cholesteryl ester. The secretion of total cholesterol was also decreased in cells exposed to fish-oil remnants in comparison with those incubated without remnants, and both unesterified cholesterol and cholesteryl ester levels were significantly lowered (Table 4). Table 5 shows the levels of apoB100 and apoB48 found in the $\rho < 1.050$ g/ml fraction of the medium, and Figure 1 shows the results of SDS/PAGE from a typical experiment. In cells exposed to fish-oil remnants, the amount of apoB48 secreted was significantly decreased as compared with the values obtained in experiments with corn-oil remnants, although the levels of apoB100 were not significantly changed. In addition, hepatocytes treated with fish-oil remnants secreted less apoB48 than those incubated in the absence of remnant lipoproteins.

Effect of chylomicron remnants derived from fish oil or corn oil on the expression of mRNA for apoB in cultured rat hepatocytes

Hepatocytes were incubated in the presence or absence of chylomicron remnants derived from fish oil or corn oil for 5 h or 16 h, the total RNA from the cells was then extracted, and the expression of apoB mRNA was determined by RT-PCR. The

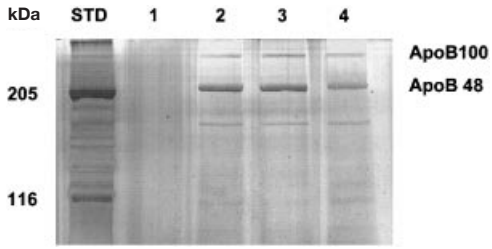

Figure 1 SDS/PAGE analysis of proteins secreted in VLDL after incubation of hepatocytes with chylomicron remnants derived from fish or corn oil

Hepatocytes were incubated with or without chylomicron remnants derived from fish or corn oil (0.3 μmol triacylglycerol/ml) for 5 h, the medium was then removed and replaced with fresh medium without remnants (time zero), and the incubation was continued for a further 16 h. After this time, the medium was collected, the proteins in the $\rho < 1.050$ g/ml fraction were separated by SDS/PAGE. STD, molecular weight standards; 1, incubation medium at time zero; 2, incubation without remnants; 3, +corn-oil remnants; 4, +fish-oil remnants. The image shown is a typical example from four separate experiments.

Table 6 Expression of apoB mRNA in hepatocytes after incubation with chylomicron remnants derived from fish or corn oil

Hepatocytes were incubated with chylomicron remnants derived from fish or corn oil (0.3 μmol triacylglycerol/ml) for 5 h or 16 h, the medium was then removed, the cells were extracted for total RNA and the relative abundance of transcripts for apoB 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:GAPDH and are the means $\pm$ S.E.M. from four (5 h incubation) or 5 (16 h incubation) separate experiments.

	Incubation time (h)	
Remnant type	5	16
None	0.79 ± 0.13	0.83 ± 0.06
Corn	0.92 ± 0.12	0.67 ± 0.17
Fish	0.65 ± 0.11	0.58 ± 0.17

results are shown in Table 6. The relative abundance of mRNA transcripts for apoB was not consistently changed in the hepatocytes treated with fish-oil as compared with corn-oil remnants, and no significant difference was found after optical density scanning of the gels and normalization of the values using GAPDH.

DISCUSSION

Previous studies on the mechanism of the suppression of hepatic VLDL by dietary $n-3$ polyunsaturated fatty acids have involved either feeding of fish oil in the diet, followed by investigations *in vivo* or *in vitro*, or the direct addition of $n-3$ polyunsaturated fatty acids to hepatocyte cultures. Neither of these approaches, however, can be used to determine whether the fats are able to influence VLDL secretion directly when they are delivered from the gut to the liver in chylomicron remnants, and the present study was designed to answer this question by comparing the effects of chylomicron remnants enriched in $n-3$ or $n-6$ polyunsaturated fatty acids on VLDL secretion in cultured rat hepatocytes. The remnants derived from fish oil or corn oil used had a similar size, apolipoprotein content and triacylglycerol:cholesterol ratio. Furthermore, we have shown previously that the ratio of $n-3$:$n-6$ fatty acids in remnants derived from fish oil (1.33) is 10-fold higher than in those derived from corn oil (0.13) [22]. Any differential effects of the two types of particles on lipid and apoB secretion by cultured rat hepatocytes observed in our experiments, therefore, can be attributed to the difference in their fatty acid composition.

The results shown in Tables 1 and 2 indicate that the secretion of lipids into the medium of cultured rat hepatocytes is decreased when the cells are exposed to chylomicron remnants derived from fish rather than corn oil, and that this effect is enhanced when the exposure time is increased from 5 h to 16 h. As a large proportion of both the secreted triacylglycerol and cholesterol was found in the $\rho < 1.050$ g/ml fraction of the medium, which contains the VLDL and any IDL or LDL formed from it by the action of lipases secreted by the cells, these findings suggest that remnants enriched in $n-3$ as compared with $n-6$ polyunsaturated fatty acids decrease the output of VLDL lipid by the cells, and this was confirmed when the lipid content of the $\rho <$ 1.050 g/ml fraction was determined (Table 4). There was a general decline in lipid secretion during prolonged incubation of the hepatocytes *in vitro* (Tables 1 and 2); one possible explanation for the relative effects of the two types of remnants, therefore, is that those enriched in $n-6$ polyunsaturated fatty acids slow the rate of this change.

A large number of previous studies have consistently shown that $n-3$ polyunsaturated fatty acids decrease the hepatic secretion of triacylglycerol in VLDL. Triacylglycerol output has been reported to be lower in perfused livers [15,30] and isolated hepatocytes [17–19] from animals fed with fish oil as compared with corn oil, lard or a low-fat diet. In addition, $n-3$ polyunsaturated fatty acids have been shown to decrease the secretion of VLDL triacylglycerol by hepatocytes of human, rabbit and rat origin [19,20,31–38] in comparison with other fatty acids, including linoleic, oleic, palmitic and stearic acids. Fewer studies have investigated the effects of $n-3$ polyunsaturated fatty acids on the secretion of cholesterol in hepatic VLDL. However, Parks et al. [30] demonstrated that the output of cholesteryl ester in the perfused monkey liver was decreased by fish oil feeding, and Rustan and co-workers [34,39] have reported that $n-3$ polyunsaturated fatty acids depress the secretion of cholesteryl ester by rat hepatocytes as compared with oleic or linoleic acid, although, in contrast with our findings, the secretion of unesterified cholesterol was unchanged. Overall, therefore, our results provide further evidence to indicate that $n-3$ as compared with $n-6$ polyunsaturated fatty acids decrease the secretion of VLDL lipid by hepatocytes, and more importantly, they demonstrate for the first time that this differential effect is brought about acutely when they are carried to the liver from the gut in chylomicron remnants.

The unesterified and esterified cholesterol content of the hepatocytes showed small, but significant, changes after incubation with fish oil, as compared with corn oil, chylomicron remnants (Table 3). This increase in unesterified cholesterol levels seen 7 h after removal of the remnants might be explained by a more rapid uptake of fish-oil remnants, which has been demonstrated in our previous work [40]. However, the decrease in cellular cholesteryl ester found 24 h afterwards seems more likely to be related to effects on intracellular cholesterol metabolism. Rustan and co-workers [34,39] have reported that eicosapentaenoic acid decreases cholesteryl ester synthesis in rat hepatocytes as compared with oleic or linoleic acid, and this is consistent with the latter finding (Table 3).

apoB is essential for the assembly and secretion of hepatic VLDL [41]. The protein exists in two forms, apoB100 and apoB48, the latter of which corresponds to the N-terminal 48 % of apoB100 [42]. Rat hepatic VLDL contains both apoB100 and

apoB48 [29]. Because of the importance of apoB in VLDL production, the effects of $n-3$ polyunsaturated fatty acids on its secretion have been investigated extensively. Parks et al. [15,30] found no effect of dietary fish oil as compared with lard on the secretion of apoB by the perfused monkey liver, whereas Brown et al. [18,19] showed that the secretion of apoB48, but not apoB100, is decreased in hepatocytes from rats fed on fish oil rather than on a low-fat diet. This contrasts with the findings of the same group in experiments where eicosapentaenoic acid was added directly to rat hepatocyte cultures, where the secretion of both apoB100 and apoB48 was inhibited [19], and similar direct effects of $n-3$ polyunsaturated fatty acids on rat liver have been reported by other workers [43,44]. Moreover, direct addition of eicosapentaenoic as compared with oleic or linoleic acid to human hepatocytes [20,36,37] has been found to decrease the secretion of apoB100. $n-3$ Polyunsaturated fatty acids, therefore, have different effects on hepatic apoB secretion, depending on whether they are given via the diet or whether liver tissue is exposed to them directly. Our findings indicate that $n-3$ polyunsaturated fatty acids delivered to the liver in chylomicron remnants have a direct effect on hepatic apoB48, but not apoB100, secretion (Table 4). These findings are in agreement with results of the feeding experiments of Brown et al. [18,19], but contrast with those found by these workers and others on direct addition of the fatty acids to the perfused rat liver or hepatocyte cultures [19,43,44]. We conclude, therefore, that the mode of delivery of $n-3$ polyunsaturated fatty acids to the liver in the rat alters the way in which they suppress apoB secretion, with the free form suppressing the output of apoB100 and apoB48, whereas delivery in chylomicron remnant triacylglycerol, the form in which they reach the liver directly from the diet, inhibits only apoB48 secretion.

apoB100 and apoB48 are coded for by the same gene [45]; mRNA for the full-length protein is post-transcriptionally converted into mRNA for apoB48 by an editing enzyme that converts codon 2153 into a stop signal [46]. In the present study, the reduction in apoB48 secretion in VLDL caused by chylomicron remnants derived from fish as compared with corn oil was not accompanied by a significant decrease in the abundance of mRNA transcripts for the protein (Table 6), suggesting that the delivery of $n-3$ polyunsaturated fatty acids to the liver in the remnants does not decrease the synthesis of apoB at the transcriptional level, but acts on the processes regulating apoB secretion to suppress selectively the output of apoB48. This is in agreement with previous studies, which found no change in the levels of apoB mRNA in the liver after feeding rats with fish or corn oil for 3 weeks [17], or after the incubation of HepG2 cells with eicosapentaenoic acid as compared with oleic acid [37].

The findings of the present study clearly show that chylomicron remnants enriched in $n-3$ rather than $n-6$ polyunsaturated fatty acids decrease the secretion of lipid and apoB in VLDL in cultured rat hepatocytes. Previous work in our laboratory using the rat *in vivo*, the perfused rat liver and isolated rat hepatocytes has demonstrated that chylomicron remnant cholesterol, triacylglycerol and protein from particles derived from fish oil are taken up by the liver more rapidly than from those derived from corn oil [40,48,49]. Thus this effect cannot be explained by a decreased rate of uptake of the particles enriched in $n-3$ polyunsaturated fatty acids. It is possible, however, that the different types of remnants may affect the synthesis of triacylglycerol in the cells, altering its availability for VLDL secretion. Feeding experiments have consistently shown that hepatic triacylglycerol synthesis is lower in animals fed on fish oil than in those fed on corn or olive oil [17–19,47], and some workers have reported that direct addition of eicosapentaenoic acid to cultured hepatocytes decreases its production [20,31,33,34,37], although others have found no effect [19,35,38,43]. In earlier work in our laboratory on the metabolism of chylomicron remnants by the perfused liver, we have shown that a greater proportion of the triacylglycerol taken up by the tissue from chylomicron remnants derived from fish as compared with corn oil is used both for oxidation and phospholipid synthesis [48]; thus, it is possible that less fatty acid from the fish-oil remnants is available for triacylglycerol production. These findings suggest that delivery of $n-3$ polyunsaturated fatty acids from the diet to the liver in chylomicron remnants might influence triacylglycerol synthesis in the cells, but further investigation is required to clarify this effect.

As well as decreasing VLDL secretion in hepatocytes in comparison with corn-oil remnants, fish-oil remnants also significantly decreased the output of VLDL cholesterol and apoB48 in comparison with that in cells incubated without remnants (Tables 1, 2 and 4). This effect was more marked after a 5 h incubation than after 16 h, when a significant change was seen only in the cholesteryl ester fraction. However, this might be related to the lower rate of VLDL secretion after the longer incubation, which makes any changes more difficult to detect. Thus the cholesterol taken up from fish-oil remnants does not appear to be secreted in VLDL. This is consistent with our earlier work using the rat *in vivo*, which demonstrated that relatively high levels of cholesterol from this type of remnant are excreted via the bile, with very little returning to the circulation [49]. Furthermore, as VLDL secretion is believed to be dependent on cholesteryl ester synthesis [50], our results suggest that the $n-3$ polyunsaturated fatty acids from the remnants also influence intracellular cholesteryl ester metabolism, and this is currently under further investigation in our laboratory.

In summary, the results reported here demonstrate for the first time that dietary $n-3$ as compared with $n-6$ polyunsaturated fatty acids suppress the secretion of triacylglycerol, cholesterol and apoB in VLDL directly, when they are delivered to the liver in chylomicron remnants. The decrease in apoB secretion involves a selective reduction in the output of apoB48, rather than apoB100, in rat liver cells, but is not associated with a decrease in apoB synthesis at the transcriptional level. The selective effect mimics the influence of $n-3$ polyunsaturated fatty acids on hepatic apoB secretion in feeding studies [18,19], and is in contrast with their inhibitory effect on both apoB48 and apoB100 secretion when added directly to hepatocytes *in vitro* [19,43]. Thus the difference observed between the two experimental models can be attributed to the different mode of delivery of the fatty acids to the hepatocytes, either in chylomicron remnant triacylglycerol in the feeding studies or in the free form in the *in vitro* experiments.

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