Effect of fish oil diet on hepatic lipid metabolism in nonhuman primates: lowering of secretion of hepatic triglyceride but not apoB

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Abstract African green monkeys were fed diets containing either 11% (by weight) fish oil or lard for 2.5 yr. To test the hypothesis that fish oil decreases hepatic secretion of triglyceride (TG) and apoB, livers from these animals were perfused with a fatty acid mixture [85% (w/w) oleate containing [^{14}C]oleate and 15% n-3 containing [^{3}H]eicosapentaenoic acid (EPA)] at a rate of 0.1 μmol fatty acid/min per g liver. Liver perfusate was sampled every 30 min during 4 h of recirculating perfusion. The concentration of triglyceride was similar for livers of animals of both groups and there was no difference between groups in the extent of incorporation of [^{3}H]EPA or [^{14}C]oleate into hepatic TG. While the secretion rate for the mass of TG was less in the fish oil-fed group (8.3 ± 2.5 vs 18.3 ± 4.4 mg/h per 100 g liver, $P<0.05$), the apoB secretion rate was similar (0.92 ± 0.15 vs 1.01 ± 0.13 mg/h per 100 g liver). Significantly less [^{3}H]EPA was incorporated into secreted TG in the fish oil group (0.4 ± 0.1 vs 1.0 ± 0.1% infused dose/h; $P<0.01$). The rate of secretion of [^{14}C]TG was similar for both groups (1.3 ± 0.3 vs 1.4 ± 0.1% infused dose/h for fish oil and lard groups, respectively). No significant diet-related differences in [^{3}H]TG or [^{14}C]TG fatty acid specific activity were observed for perfusate TG or hepatic TG. After perfusion, livers from fish oil-fed monkeys contained significantly more [^{3}H]EPA in hepatic phospholipid than livers from lard-fed monkeys (19.5 ± 1.8 vs 11.4 ± 1.7% infused dose; $P<0.01$) although hepatic phospholipid mass concentrations were similar. The liver phospholipids of the fish oil group were enriched in n-3 fatty acid mass and were relatively depleted of oleate and linoleate. We conclude that although apoB secretion was unaffected, dietary fish oil significantly decreased hepatic TG secretion through relatively poor utilization of EPA for the synthesis of TG destined for secretion in VLDL; at the same time, increased incorporation of [^{3}H]EPA into hepatic phospholipid accompanied the decreased incorporation into secreted TG and these events may be coupled. As a result, the livers of monkeys fed fish oil secreted the same number of VLDL particles as their lard counterparts although the VLDL were relatively deficient in TG, an effect that likely contributes to the finding of equal particle concentrations but reduced size of plasma LDL in the monkeys fed dietary fish oil (1987. Parks, J. S., and B. C. Bullock. *J. Lipid Res.* **28:** 173-182). —**Parks, J. S., F. L. Johnson, M. D. Wilson, and L. L. Rudel.** Effect of fish oil diet on hepatic lipid metabolism in nonhuman primates: lowering of secretion of hepatic triglyceride but not apoB. *J. Lipid Res.* 1990. **31:** 455-466.

Supplementary key words VLDL • phospholipids • eicosapentaenoic acid • lard

Interest in dietary fish oil has been stimulated by the observation that Eskimo populations, whose consumption of cold-water marine products is high, had decreased incidence of coronary heart disease and lower plasma lipids compared to a Danish control population (1). The ingredients that could confer such unique properties to fish oil appear to be the n-3 fatty acids, which are abundant in the fat of cold-water fish. Although the beneficial effects of n-3 fatty acids on coronary heart disease are almost certainly multifactorial, and may include decreased platelet aggregation and reduced coronary vasospasm, the effects of fish oil on lipid and lipoprotein metabolism are also likely to be important (2).

It is well documented that consumption of fish oil is associated with a decrease in plasma TG concentrations (3). Variable responses of total plasma cholesterol, LDL, and HDL concentrations to fish oil have been reported and appear to relate to the experimental conditions used (3). In a study of VLDL turnover in humans it was concluded that fish oil decreased hepatic VLDL apoB and TG production (4). Two studies using cultured hepatocytes indicated that n-3 fatty acids decrease TG secretion (5, 6), but in only one of these studies was there a decrease in apoB secretion associated with n-3 fatty acids (5). One aspect that was common to all of these studies was the relatively short term use of n-3 fatty acids (<4 weeks) to modify lipid metabolism. Thus, the longer term effects of fish oil on hepatic lipoprotein production are unknown.

We previously have reported on the effects of fish oil on plasma lipoprotein, apoprotein, and lipid concentrations in African green monkeys (7, 8). When fish oil is isocalorically substituted for lard as 22% of the calories in the

Abbreviations: TG, triglyceride(s); EPA, eicosapentaenoic acid; VLDL, very low density lipoprotein(s); LDL, low density lipoprotein(s); CE, cholesteryl ester(s); HDL, high density lipoprotein(s); DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PL, phospholipid(s); TLC, thin-layer chromatgraphy; FA, fatty acid.

diet (11% by wt), lower plasma concentrations of total cholesterol, LDL, and HDL result (7). Although plasma TG concentrations were low for both the lard and fish oil groups as compared to those in human subjects, they were consistently somewhat higher for the fish oil group [30 vs 20 mg/dl (7)]. The reason that these values are low compared to those in human subjects is likely due to the very efficient clearance of TG from plasma by lipoprotein lipase (L. L. Rudel and R. J. Star, unpublished data). The efficiency of clearance is so high that effects on production are difficult to detect by measurement of plasma concentrations of TG. Therefore, it was of interest to study hepatic triglyceride secretion in these animals to learn the effects of fish oil, compared to lard, on the secretion of VLDL. Thus, the purpose of this study was to investigate the long term effects of dietary fish oil on hepatic TG and VLDL production using the African green monkey model we had previously characterized (7–12). The animals initially were maintained on diets for 2.5 years to study the effect of fish oil on atherosclerosis development before we initiated the liver perfusion studies. We found that hepatic TG, but not apoB, production was decreased for the fish oil group due, in part, to: *1*) the poor utilization of n-3 fatty acids for the synthesis of secreted TG in animals fed fish oil compared to those fed lard; and *2*) the increased incorporation of n-3 fatty acids into hepatic PL.

METHODS

Animals

Twenty-four adult male African green grivets (*Cercopithecus aethiops aethiops*), purchased from Primate Imports (Port Washington, NY), were fed atherogenic diets containing 40% of calories as fat and 0.76 mg chol/kcal for 2.5 years before initiation of the liver perfusion studies. Half of the fat calories of the diets was derived from lard or menhaden oil (11% wt) and half came from egg yolk and egg yolk replacement, the later being a low cholesterol mixture made to resemble the composition of egg yolk. Daily intake of n-3 fatty acids was 3.8 g for the fish oil group and consumption of dietary cholesterol for both groups was 550 mg/day. The lard diet fatty acid composition consisted of 38% saturated, 44% monounsaturated, and 13% n-6 polyunsaturated, while the fish oil diet contained 40% saturated, 34% monounsaturated, 10% n-6 polyunsaturated and 12% n-3 polyunsaturated fatty acids. Approximately 5% of the fatty acids of each diet was unidentified. Detailed compositions of the diets have been published previously (7). A subset (n = 14) of these animals was used for liver perfusion studies; some of the perfusion data have been reported previously (13).

Lipoprotein isolation and liver perfusions

Blood samples were taken from animals after an overnight (18 h) fast as described previously (7, 13). LDL molecular weight was measured using the agarose column method after isolation of the $d < 1.225$ g/ml lipoproteins from plasma (14). Total plasma and HDL cholesterol and plasma TG concentrations were determined using the Lipid Research Clinic methodology with an RA500 AutoAnalyzer (13, 15, 16).

Perfusion of the isolated livers was performed essentially as described previously using a medium consisting of Krebs-Henseleit original Ringer bicarbonate buffer, D-glucose, amino acids, insulin, hydrocortisone, streptomycin, penicillin, and washed human erythrocytes at 22% hematocrit, pH 7.4 (10, 12). Recirculating perfusion was performed for 90 min to allow plasma lipoproteins bound or entrapped in the liver to be released, after which time the medium was flushed from the liver with approximately 1 liter of oxygenated erythrocyte-free medium. The liver was then perfused by recirculation for 4 h with fresh medium containing erythrocytes.

The following modifications to our previously published procedure were made. Fatty acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was added to the recirculating medium at a final concentration of 3 g/100 ml. Oleic acid and a mixture of n-3 fatty acids ("EPA mixture 50%") were purchased from Nu-Chek Prep (Elysian, MN); the latter mixture contained 54% 20:5 and 18% 22:6. To prepare the fatty acid mixture used for liver perfusions, 16.8 g of 18:1 and 5.4 g of the n-3 fatty acid mixture were weighed and placed in a 1.0-liter flask. Krebs-Henseleit original Ringer bicarbonate (KRB) buffer without Ca^{2+} (730 ml) was then added to the flask followed by 1.1 equivalents of NaOH. After the solution turned clear, the final volume was adjusted to 771 ml to give a 100 mM solution of fatty acid soaps. Fifty-ml aliquots of this mixture were gassed with N_2 and frozen at $-20°C$ until used. All aliquots of this mixture were used within 4 months. Just prior to liver perfusion the fatty acid mixture was thawed and diluted with Ca^{2+}-free KRB buffer, which had been adjusted to pH 10.3, to give a final concentration of 10–20 mM fatty acid. The fatty acid mixture was infused into the perfusate during the 90 min "wash-out period" as well as during the 4-h second period of recirculating perfusion. During the second perfusion period, 40 μCi eicosapentaenoic acid (5, 6, 8, 9, 11, 12, 14, 15, 17, 18-^{3}H(n); 79 Ci/mmol) and 10 μCi [1-^{14}C]oleic acid (57 mCi/mmol) obtained from New England Nuclear (Boston, MA) were added to the fatty acid infusate. The purity of the radioactive compounds was judged to be 94% for the [^{3}H]EPA and 99% for the [^{14}C]oleate as judged by thin-layer chromatography. The fatty acid composition of the fatty acid

infusate, determined by gas–liquid chromatography, was as follows (mean ± SEM): 82.9 ± 1.5% 18:1, 7.8 ± 0.4% 20:5 (n–3), 1.9 ± 0.1% 22:5 (n–3), 1.5 ± 0.1% 22:6 (n–3), and 5.9% other (n = 12). The final concentration for each perfusion was adjusted to give an estimated infusion rate of 0.1 μmol/min per g liver using a pump rate of 0.475 ml/min, assuming liver weight was 2.2% of the body weight of the animal. Fatty acid infusion rates, normalized for the actual weight of the liver determined at the completion of perfusion, were 0.109 ± 0.004 and 0.098 ± 0.005 μmol fatty acid/min per g liver for lard- and fish oil-fed animals, respectively, and were not statistically different between diet groups.

Aliquots of liver perfusate were taken every 30 min during the 4-h perfusion for quantitation of total perfusate cholesterol, TG, apoB, and incorporation of radioactivity in perfusate lipids. Total cholesterol was assayed by the method of Rudel and Morris (17); TG were assayed enzymatically using the RA 500 AutoAnalyzer (13); and apoB concentrations were measured by an enzyme-linked immunosorbent assay (13). After 4 h of perfusion, the perfusate was collected in tubes chilled to 4°C and adjusted to 0.1% EDTA, 0.1% NaN_3, 0.04% DTNB, pH 7.4. Perfusate plasma was recovered by low-speed centrifugation and perfusate VLDL were isolated by ultracentrifugation at d 1.006 g/ml as previously described (10–12).

Analytical procedures

Lipids were extracted from livers and hepatic VLDL by the method of Folch, Lees, and Sloane Stanley (18). Incorporation of radioactivity into hepatic lipids was determined after separation of lipid classes by thin-layer chromatography, as described below for perfusate. Thin-layer chromatography and fatty acid analyses were performed as described previously (8). Cholesterol content was quantitated by the method of Rudel and Morris (17). Lipid phosphorus was measured by the method of Fiske and SubbaRow (19). Protein was quantitated by the method of Lowry et al. (20), using extraction with hexane to remove turbidity after color development. TG assay of liver lipids and hepatic VLDL was done by the procedure of Sardesai and Manning (21).

Radioactivity incorporation into perfusate lipids was quantitated after lipid extraction of the perfusate (18), thin-layer chromatography of the lipid extract (8), and liquid scintillation counting of the isolated lipid classes. An aliquot of the upper (aqueous) phase of the perfusate extract was counted to determine the amount of oxidized radiolabel at each time point of perfusion. In preliminary studies we found that all of the aqueous radioactivity of the perfusate extract was volatile enough to be lost upon drying the upper phase at 50°C under N_2. An aliquot of the fatty acid infusate for each perfusion was also subjected to extraction and thin-layer chromatography along with the perfusate samples. Incorporation of radioactivity was normalized as a percentage of that infused. Recovery of ^3H and ^{14}C radioactivity from perfusate samples after extraction and TLC averaged 98.5 ± 3.1% and 85.2 ± 1.7%, respectively. The recovery of radioactivity in the lower phase of the Folch extract for the fatty acid infusate was 98.1 ± 0.3% for [^3H]EPA and 99.5 ± 0.1% for [^{14}C]oleate (n = 13). Of the recovered radioactivity of fatty acid infusate in the extract, 94.3 ± 1.0% of the ^3H and 99.3 ± 0.1% of the ^{14}C migrated with authentic fatty acid on TLC. The mean $^3H/^{14}C$ ratio of the fatty acids extracted from infusate and purified by TLC was 3.56 ± 0.19 for all animals (n = 14). Total recovery of [^3H]EPA (liver + perfusate) was 86.0 ± 10.0% and 94.1 ± 8.9% of infused dose for the lard and fish oil groups, respectively. For [^{14}C]oleate total recoveries were 67.5 ± 9.0% and 67.5 ± 10.0%, respectively. The lower total recovery of ^{14}C relative to ^3H presumably was due to the loss of ^{14}C as CO_2 during perfusion.

Data analyses

Values are given as the mean ± standard error of the mean (SEM). Statistical comparisons between the two diet groups were made using the Student's *t*-test (22). Analysis of variance and Fisher's least significant difference test were used to test for differences in radioisotope incorporation between diet groups (22). Two-way analysis of variance was used to analyze group differences for perfusate and hepatic TG specific activities. For comparison of the rates of accumulation of constituents between groups in the liver perfusate, repeated measures analysis of variance was used (22).

RESULTS

The plasma lipoprotein and apoprotein concentrations of the two groups of animals undergoing liver perfusion have been described previously (13). Compared to the lard group, the animals fed fish oil had 45% lower total plasma cholesterol (410 ± 56 vs 225 ± 40 mg/dl; $P < 0.01$), 50% lower VLDL + LDL cholesterol (327 ± 58 vs 162 ± 44 mg/dl; $P < 0.01$), and 24% lower HDL cholesterol concentrations (83 ± 10 vs 63 ± 7 mg/dl; $P = 0.11$). Plasma apoB concentrations were not significantly different between two groups [128 ± 19 (lard) vs 100 ± 14 (fish oil)] but apoA-I concentrations were lower for the fish oil group (305 ± 35 vs 192 ± 13 mg/dl; $P < 0.01$). Plasma TG concentrations were low for both groups compared to values for human subjects but were higher for the fish oil group (13 ± 2 vs 26 ± 2 mg/dl; $P < 0.01$).

Liver lipid concentrations for the two groups of animals have been reported elsewhere (13). Livers of animals fed fish oil had significantly lower concentrations of free

(2.7 ± 0.2 vs 3.9 ± 0.2 mg/g) and esterified (4.1 ± 0.8 vs 7.4 ± 0.7 mg/g) cholesterol compared to those of animals fed lard. However, hepatic concentrations of PL (26.1 ± 2.7 vs 30.9 ± 2.0 mg/g,) TG (10.6 ± 2.0 vs 10.0 ± 1.0 mg/g), and protein (170 ± 6 vs 167 ± 7 mg/g) were similar for both the fish oil and lard groups, respectively.

The fatty acid composition of hepatic TG and PL fractions is given in **Table 1**. For the liver TG fraction of the fish oil group there was approximately a twofold enrichment of each of the major n-3 fatty acids (20:5, 22:5, 22:6) compared to the lard group. There were only small variations in the other TG fatty acids between the two diet groups. The hepatic PL fraction of animals fed fish oil had a fourfold enrichment in n-3 fatty acids compared to their lard counterparts. The diet-induced enrichment of 22:6 (n-3) in the PL fraction was greater than that in the TG fraction of the fish oil group (9.5 vs 3.9%). The livers of the fish oil group had one half as much 18:2 ($P<0.01$) and less 18:1 in the PL fraction than those of the lard group. In general, the hepatic PL fraction of both diet groups contained much more 18:0 (27 to 30% vs 4%) and less 18:1 (9–12% vs 45%) than did the hepatic TG fraction.

The accumulation of TG mass and radioactivity in the recirculating perfusate is shown in **Fig. 1**. Both mass and radioactivity increased linearly with time of perfusion in all cases, after about a 10–30 min delay. The reason for this delay is uncertain, but may reflect an adjustment of the liver after the 5–10 min flush period directly preceding initiation of the second period of perfusion. The rates of accumulation for these constituents as well as for perfusate apoB, which we have previously reported (13), are given in **Table 2**. The rate of accumulation was determined as the slope of the linear regression plot of accumulation versus perfusion time from 30 min to 240 min. The rate of accumulation of TG mass in the liver perfusate of animals fed fish oil was half that occurring in the livers from lard-fed animals (Fig. 1, Table 2). However, the rate of accumulation of apoB in liver perfusate was similar for both diet groups. The rate of incorporation of [^{3}H]EPA into perfusate TG in the fish oil group also was half that of the lard group (Fig. 1, Table 2). On the other hand, the incorporation of [^{14}C]oleate into perfusate TG was similar for both diet groups.

The amount of infused radioactive fatty acid remaining in the perfusate during liver perfusion is shown in **Fig. 2**. The data are expressed as percentage of total radioactivity infused at each time point. Values for both isotopes decrease with perfusion time, but at any given time during perfusion there was significantly less [^{3}H]EPA remaining in the perfusate compared to [^{14}C]oleate regardless of diet group ($P<0.001$; repeated measures ANOVA). There was also a slight but significantly lower amount of [^{3}H]EPA remaining in the perfusate of livers from the fish oil group (compare bottom two curves in Fig. 2). There was no significant diet-related difference in the amount of [^{14}C]oleate remaining in the perfusate. The data imply that removal of [^{3}H]EPA from perfusate was more efficient than removal of [^{14}C]oleate, and that the efficiency of removal of both radiolabeled fatty acid preparations with time was increasing.

Because fatty acid oxidation produces water-soluble products, the amount of infused radioactive fatty acid that was oxidized by the liver was monitored by quantification of radioactivity in the aqueous phase of the lipid extract

TABLE 1. Fatty acid composition of hepatic lipids

	Triglyceride		Phospholipid	
Fatty Acid	Lard	Fish Oil	Lard	Fish Oil
	%		%	
14:0	0.6 ± 0.1	1.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.03
16:0	25.3 ± 1.2	23.5 ± 1.8	27.4 ± 1.7	28.1 ± 1.4
16:1	3.2 ± 0.2	4.5 ± 0.4	0.7 ± 0.1	0.7 ± 0.1
18:0	4.4 ± 0.7	4.1 ± 0.4	30.2 ± 1.9	26.9 ± 1.1
18:1	45.8 ± 2.6	43.8 ± 2.7	11.8 ± 0.5	9.4 ± 0.5[a]
18:2	8.8 ± 1.0	7.3 ± 0.6	9.0 ± 1.2	5.3 ± 0.3[b]
20:4	3.5 ± 1.0	1.9 ± 0.4	8.9 ± 1.5	8.7 ± 0.7
20:5 (n-3)	1.9 ± 0.4	4.3 ± 0.6[a]	1.0 ± 0.4	4.8 ± 0.4[a]
22:5 (n-3)	1.7 ± 0.2	2.6 ± 0.3[b]	0.6 ± 0.3	2.0 ± 0.1[a]
22:6 (n-3)	1.9 ± 0.3	3.9 ± 0.5[a]	2.9 ± 0.9	9.5 ± 0.5[a]
Other	2.7 ± 0.3	3.0 ± 0.2	7.5 ± 0.5	4.6 ± 0.2[a]
Total (n-3)	5.5	10.8	4.5	16.3

Values given as mean ± SEM; n = 7 for each diet group.
[a] $P < 0.01$ by t-test (lard vs. fish oil).
[b] $P < 0.03$ by t-test (lard vs. fish oil).

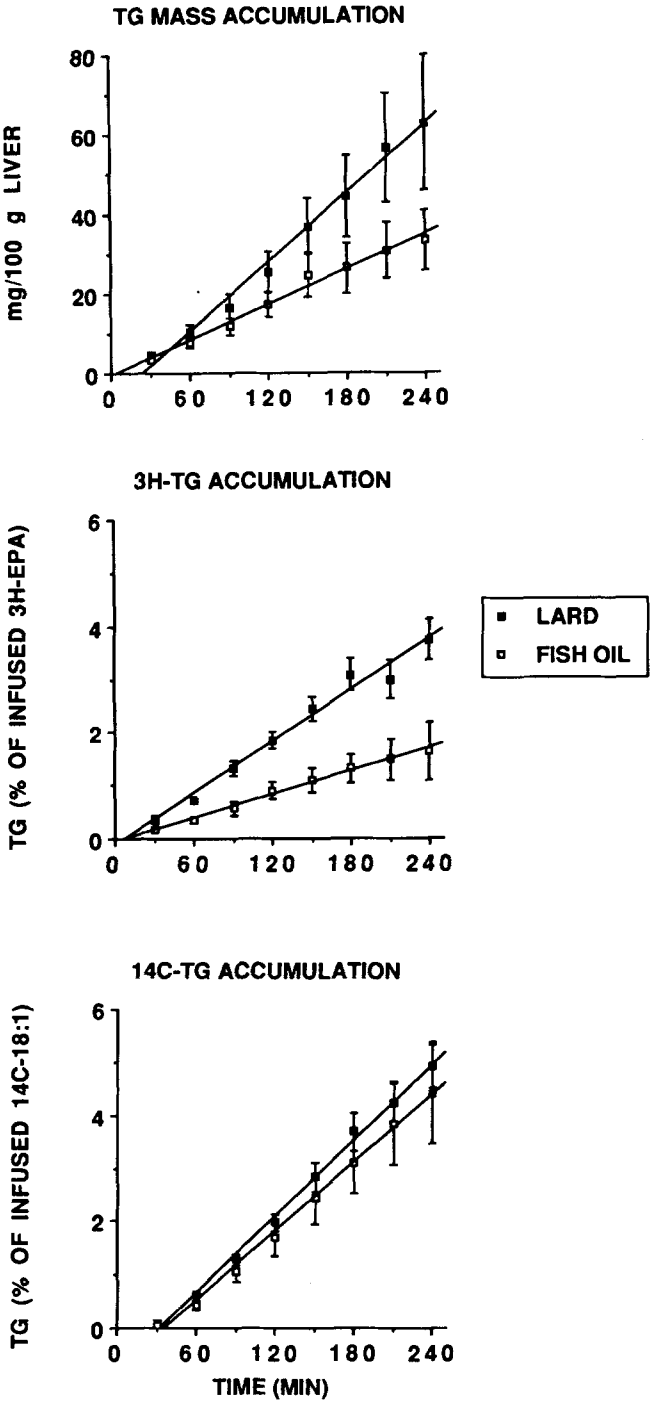

Fig. 1. Hepatic perfusate accumulation of TG radioactivity and mass. A fatty acid mixture (85% 18:1 and 15% n-3) containing [^{3}H]20:5 (40 μCi) and [^{14}C]18:1 (10 μCi) was infused into the recirculating perfusate at a rate of 0.1 μmol fatty acid/min per g liver. At the indicated times aliquots of the perfusate were removed for quantification of TG mass and radioactivity incorporation into perfusate lipids, after lipid extraction and thin-layer chromatographic separation of lipid classes. Radioactivity accumulation is presented as percentage of the total infused radioactive fatty acid at each time point. Each point represents the mean ± SEM of five to seven animals and the line of best fit was determined by linear regression analysis.

of whole perfusate. The radioactivity in the aqueous phase of the extract was volatile enough to be completely lost during evaporation under N_2 at 50°C. **Fig. 3** shows the percentage of total infused radiolabel that was recovered as oxidized material after 4 h of perfusion. There was a somewhat higher percentage recovery of the [^{3}H]EPA as oxidized radioactivity for the fish oil group compared to the lard group (21.5 ± 1.6 vs 17.3 ± 0.6%); however, this difference was not statistically significant. The percent of [^{14}C]oleate recovered as oxidized radioactivity was similar to that for [^{3}H]EPA and was comparable for the two diet groups [16.6 ± 1.3% (fish oil) vs 18.4 ± 1.3% (lard)]. We assume that the values for the amount of [^{14}C]oleate oxidized are underestimates, given the lower recoveries mentioned above for ^{14}C radioactivity that can be attributed to production of $^{14}CO_2$.

A summary of radioactivity incorporation into phospholipid and triglyceride fractions of the liver perfusate and hepatic tissue after 4 h of perfusion is shown in **Fig. 4**. Because radiolabel incorporation into perfusate cholesteryl ester was <0.1% of infused dose in all cases, it was omitted from the graph. There was no significant diet-related difference in the percentage of incorporation of either radioactive fatty acid into perfusate PL (Fig. 4, top panel). There was a significantly lower percentage of incorporation of [^{3}H]EPA into perfusate TG in the fish oil group compared to the lard group and compared to that of [^{14}C]oleate in both diet groups (Fig. 4, top panel). Note that [^{3}H]EPA incorporation into perfusate TG at the end of perfusion was nearly equivalent to that of [^{14}C]oleate in the livers from animals fed the lard diet. The percentages of fatty acid radioactivity incorporation into hepatic lipids are shown in the lower panel of Fig. 4. Since no significant diet-related differences were found for incorporation of radioactivity into hepatic cholesteryl ester, monoglyceride, or diglyceride, and since the amount of radioactivity in any one of these fractions was ≤ 4% of the infused dose, they have been omitted from the graph. Incorporation of fatty acid radioactivity into hepatic TG was a much higher percentage (30–38% of infused dose) than in perfusate TG (1–4% of infused dose; top panel) but there were no significant diet- or radioisotope-related differences in the percentage of incorporation of radioactive fatty acids into hepatic TG. The percentage of infused [^{3}H]EPA incorporated into hepatic phospholipids in the fish oil group was almost twice that of the lard group (19.5 ± 1.8% vs 11.4 ± 1.7%; $P<0.01$). There was no significant difference in the percentage of [^{14}C]oleate incorporated into hepatic PL between the lard and fish oil groups (2.9 ± 0.7% vs 4.1 ± 0.9%, respectively). In addition, the percentage of infused [^{3}H]EPA incorporated into hepatic PL was significantly greater than that of [^{14}C]oleate in both diet groups.

TABLE 2. Rates of accumulation of apoB and triglyceride mass and radiolabeled triglyceride in liver perfusate

Diet	ApoB	TG	% of Infused Fatty Acid Dosage/h[a] [^{3}H]TG	[^{14}C]TG
	mg/h/100 g liver		%	
Lard (n)	1.01 ± 0.13 (7)	18.3 ± 4.4 (5)	0.97 ± 0.12 (7)	1.44 ± 0.13 (7)
Fish oil (n)	0.92 ± 0.15 (7)	8.3 ± 2.5 (6)	0.42 ± 0.11 (6)	1.25 ± 0.26 (6)
P value	NS	<0.05	0.01	NS

Values given as mean ± SEM; NS, not significantly different ($P > 0.05$).
[a]Rate of accumulation of radioactivity in liver perfusate triglyceride from 30 min to 240 min during infusion of a fatty acid mixture containing [^{3}H]20:5 and [^{14}C]18:1. Detailed composition of the fatty acid infusate is given in the Methods section.

Fig. 5 shows the [^{3}H]EPA to [^{14}C]oleate ratio of the fatty acid infusate, of the perfusate TG, and of the perfusate FA as a function of perfusion time. The ^{3}H to ^{14}C ratio of perfusate FA was approximately 30% that of the infused radioactivity for both diet groups and this ratio remained constant throughout the perfusion. These data are consistent with the data of Fig. 2 and demonstrate the [^{3}H]EPA was more efficiently removed from the perfusate than [^{14}C]oleate. The ^{3}H to ^{14}C ratio of perfusate TG started high, then progressively decreased throughout the perfusion for both diet groups demonstrating a decreased incorporation of [^{3}H]EPA into secreted TG compared to [^{14}C]oleate. This occurred even though the rate of TG mass appearance in perfusate was constant and linear throughout the perfusion and was lower in the animals fed fish oil. The ^{3}H to ^{14}C ratio of perfusate TG was approximately 50% lower for the fish oil group compared to that of the lard group throughout the perfusion. This possibly was due to the greater pool size of EPA compared to that of oleate in the livers of animals fed fish oil versus lard. The hepatic TG fatty acid percentage composition data in Table 1 support this possibility since the percentage of oleic acid was similar for both diet groups (~45%) but the percentage of n-3 fatty acid in the fish oil group was twice that of the lard group (10.8% vs 5.5%, respectively).

The specific activities of TG fatty acids in liver perfusate and liver are given in **Table 3**. For this comparison, we used the liver perfusate taken after 240 min of perfusion to compare with that in liver tissue, which was sampled at the same time. The specific activity of liver perfusate TG was two- to sixfold greater than that of liver TG in both diet groups. Even though there was not a statistically significant diet-related difference in TG specific activities, the perfusate values for the fish oil group were twofold higher for [^{14}C]TG (12.9 vs 6.7 dpm/nmol) compared to the lard group.

The data in Fig. 4 demonstrated a greater proportion

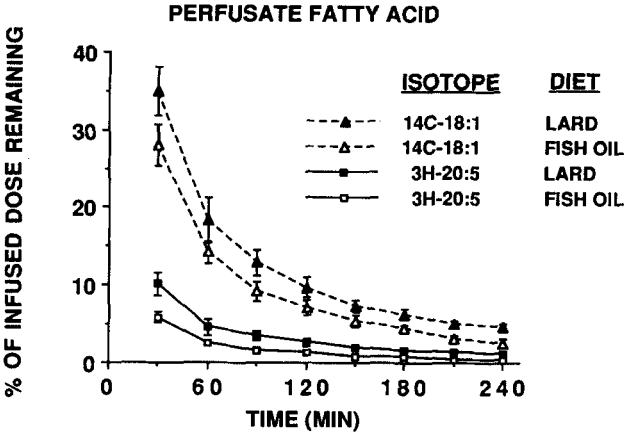

Fig. 2. Amount of radioactive fatty acid ([^{3}H]20:5, [^{14}C]18:1) remaining in the recirculating liver perfusate as a function of perfusion time for monkeys fed lard or fish oil diets. Experimental conditions are given in Fig. 1. Each value represents the mean ± SEM (n = 7) of the percentage of the infused radioactive fatty acid at each time point.

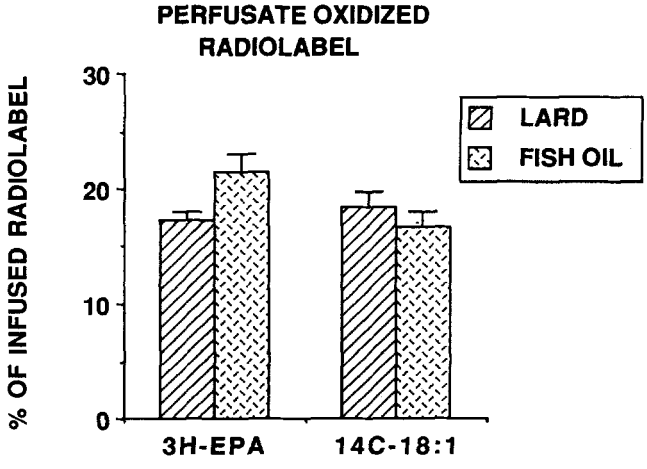

Fig. 3. Oxidation of radioactive fatty acids by perfused livers of monkeys fed lard or fish oil diets. An aliquot of the upper (aqueous) phase of the perfusate lipid extraction was counted to determine the amount of oxidized radioactivity contained in the perfusate after 4 h of perfusion. Data are expressed as percentage of total radioactivity infused. Values are the mean ± SEM for seven animals in each group.

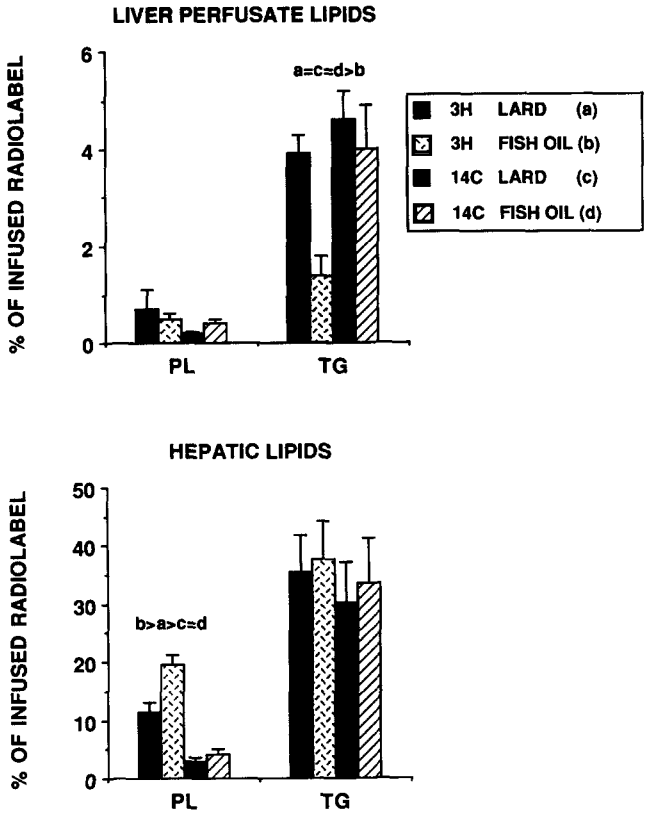

Fig. 4. Distribution of [^{3}H]20:5 and [^{14}C]18:1 between liver perfusate (top) and hepatic (bottom) phospholipid and triglyceride fractions after 4 h liver perfusion of monkeys fed lard or fish oil diets. Data are expressed as percentage of total radioactivity infused during the 4 h of perfusion. Values are mean ± SEM (n = 7). Significant differences ($P<0.05$) are indicated by the small letters.

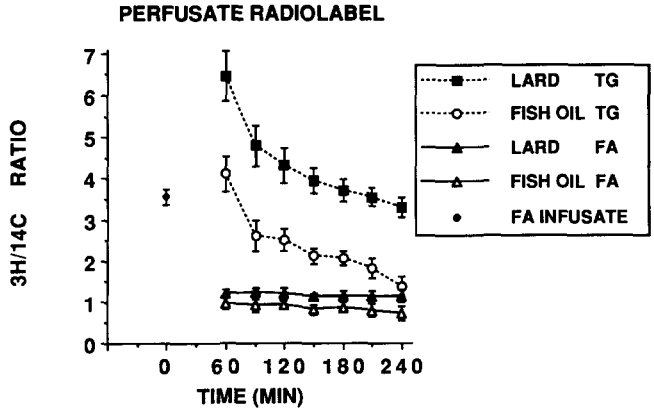

Fig. 5. [^{3}H]EPA to [^{14}C]oleate ratio of liver perfusate triglyceride (TG) and fatty acid (FA) in recirculating liver perfusions of monkeys fed a lard or fish oil diet. Experimental details of the liver perfusion are given in Fig. 1 and the Methods section. Values are the mean ± SEM (n = 7). The ratio of the infused fatty acid mixture was 3.56 ± 0.19 (n = 14) and is shown on the plot for reference (FA infusate).

of [^{3}H]EPA than of [^{14}C]oleate was incorporated into hepatic PL, and a greater proportion [^{3}H]EPA was incorporated into PL in livers of animals that had been fed fish oil versus lard. We compared the ^{3}H/^{14}C ratio in hepatic and perfusate PL to that of TG in **Table 4**. The ratio was 2.5- to 5-fold higher for hepatic PL than for hepatic TG and for secreted PL versus TG in both diet groups. These data clearly show that, compared to oleate, there is preferential incorporation of EPA into PL in the liver. In the fish oil-fed group, the radioactivity ratio in liver tissue PL was higher than in secreted PL (11.3 vs 6.8), although in both cases, PL ratios were much greater than those in TG. This outcome suggests that secreted PL represents only a subfraction of newly synthesized cellular PL containing EPA.

DISCUSSION

Our interpretation of the experimental data is summarized in **Fig. 6**. We have previously shown that livers

TABLE 3. Specific activity of liver and liver perfusate triglyceride

	[^{3}H]TG		[^{14}C]TG	
	Lard	Fish Oil	Lard	Fish Oil
	dpm/nmol TG fatty acid			
Liver	8.0 ± 1.5	7.2 ± 1.1	2.0 ± 0.4	2.2 ± 0.4
Perfusate (240 min)	19.4 ± 4.7	13.5 ± 3.7	6.7 ± 1.6	12.9 ± 5.7
ANOVA				
Liver vs. perfusate	0.004		0.01	
Lard vs. fish oil	NS		NS	

Mean ± SEM (n = 5-7). Values for TG specific activity are presented as dpm/nmol of fatty acid in the TG fraction assuming an average molecular weight of 290 for fatty acid and 900 for TG. TG specific activity was measured after 4 h of liver perfusion in which [^{3}H]20:5 and [^{14}C]18:1 were included in a fatty acid mixture infused at a rate of 0.1 μmol/min per g liver. Specific activities of the infused radiolabels were (mean ± SEM) [^{3}H]20:5 = 31.3 ± 1.7 dpm/nmol total fatty acid and [^{14}C]18:1 = 7.9 ± 0.6 dpm/nmol total fatty acid. Aliquots of liver tissue or liver perfusate (240 min time point) were extracted and TG was isolated by TLC and quantified for mass and radioactivity as described in the Methods section. NS, not significant at $P = 0.05$ by two-way ANOVA.

TABLE 4. Radioactivity ratios of hepatic and liver perfusate lipids after 4 h of recirculating liver perfusion

Lipid Fraction	Diet	
	Lard (n = 7)	Fish Oil (n = 7)
	$^3H/^{14}C$ ratio	
Hepatic TG	3.1 ± 0.2	2.2 ± 0.3
Liver perfusate TG (240 min time point)	3.3 ± 0.2	1.4 ± 0.2[a]
Hepatic PL	9.7 ± 0.5	11.3 ± 1.5
Liver perfusate PL (240 min time point)	8.4 ± 1.5	6.8 ± 1.1[b]

Radioactivity ratios were measured after 4 h of liver perfusion in which [^{3}H]20:5 and [^{14}C]18:1 were included in a fatty acid mixture infused at a rate of 0.1 μmol/min per g liver. Aliquots of liver tissue or liver perfusate (240 min time point) were extracted and lipids were separated by thin-layer chromatography. The $^3H/^{14}C$ ratio of the infused fatty acid mixture was 3.56 ± 0.19 (n = 14).

[a] $P < 0.01$ (lard vs fish oil).

[b] $P < 0.03$ (hepatic tissue vs liver perfusate for fish oil group).

of African green monkeys chronically fed diets containing lard or fish oil secrete the same number of VLDL particles, although the fish oil group had smaller VLDL that contain fewer cholesteryl ester and TG molecules per particle (13). Since there was a strong correlation (r = 0.9; ref. 13) between hepatic cholesteryl ester content and hepatic VLDL cholesterol secretion, the lesser amount of cholesteryl ester accumulation in the liver of animals fed fish oil is likely to lead to the lower cholesteryl ester content in hepatic VLDL. The decreased secretion of TG by livers of animals fed fish oil is apparently related to their inability to efficiently utilize n-3 fatty acids for this purpose. In Fig. 6, the linewidth of the arrows indicates the relative extent of incorporation of n-3 fatty acids into hepatic lipids between the lard and fish oil groups. Because there was no significant diet-related difference in [^{14}C]18:1 incorporation into hepatic and perfusate lipids, these data were omitted from the figure to make it less complicated. The percentage of [^{3}H]EPA incorporation for the major lipid fractions also is given. The percentage of incorporation of [^{3}H]EPA into hepatic TG storage pools was similar for both diet groups, although the livers derived from animals fed fish oil (vs lard) incorporated a relatively higher percentage of [^{3}H]EPA into hepatic phospholipid (20% vs 11%) and incorporated a lower percentage into secreted TG (1% vs 4%). These differences result in the secretion by the liver of VLDL with a lower triglyceride content and a smaller size in the fish oil group (13).

Studies on the effect of fish oil on hepatic apoB production are few and have not yielded consistent results. Based on data from in vivo turnover studies in human beings fed fish oil for 4 weeks, Nestel et al. (4) concluded that fish oil decreased hepatic VLDL apoB production. Using HepG2 cells in culture Wong and Nestel (5) found that n-3 fatty acids substituted for oleate in the incubations decreased apoB synthesis; in a similar study, Nossen et al. (6) found no difference in secretion of newly synthesized apoB-100 using cultured rat hepatocytes. We found that chronic feeding of fish oil to African green monkeys did not significantly affect any measured parameter of apoB metabolism including plasma concentrations, hepatic secretion (Table 2), apoB distribution among liver perfusate lipoproteins, and hepatic apoB mRNA concentrations (M. Sorci-Thomas, F. L. Johnson, L. L. Rudel, and J. S. Parks, unpublished data). The different outcomes among studies may be related to the vastly different experimental systems since the types of studies (in vivo apoB turnover vs liver perfusion vs cell culture) and the lengths of exposure to n-3 fatty acids were different as were other experimental details. Since our animals were fed diets with fish oil for an extended period of time, and direct measurements of hepatic apoB-100 production and of hepatic apoB-100 mRNA abundance were made, we feel that a strong case exists for the likelihood that dietary fish oil does not affect hepatic apoB production in our ani-

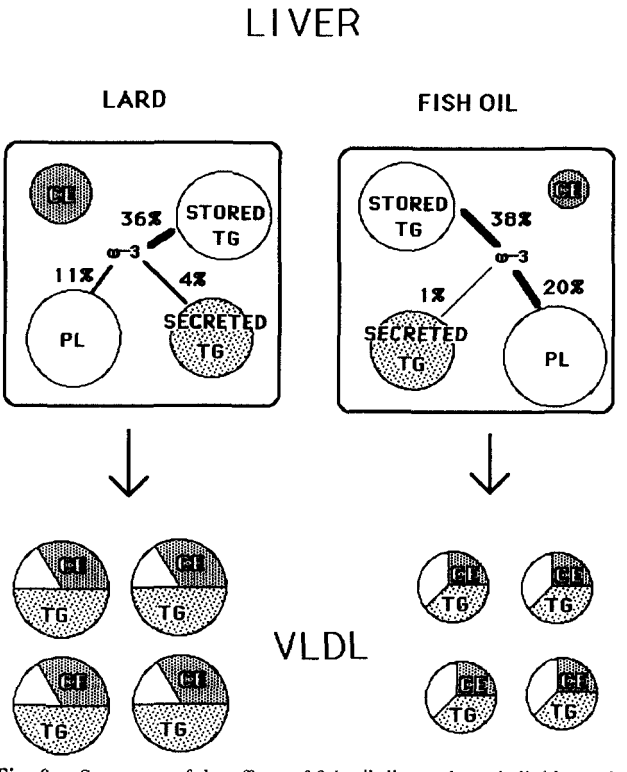

Fig. 6. Summary of the effects of fish oil diet on hepatic lipid metabolism in the African green monkey. Width of lines indicates the relative incorporation of [^{3}H]20:5 into hepatic lipid pools. Numbers beside the lines indicate percentage of total infused [^{3}H]20:5 incorporated into hepatic lipid pools. Because there were no diet-related differences in the incorporation of [^{14}C]18:1 into hepatic or perfusate lipids, these data were not included in this summary. See Discussion section for explanation of the figure.

mal model. At the same time and in contrast, dietary fish oil markedly reduced hepatic lipid secretion.

The animals fed fish oil had decreased TG accumulation in liver perfusate compared to the lard group at equivalent apoB accumulation rates. These results suggest that hepatic TG synthesis was less for the fish oil group. This diet-related difference in synthesis of secreted TG was reflected in secretion of VLDL with a lower TG content and a smaller size in the fish oil group (13). Using radioactive precursors (glycerol, H_2O, fatty acid) others have concluded that n-3 fatty acids decrease hepatic TG synthesis and secretion (5, 6, 23-25). Similarly, we found a decreased extent of incorporation of [^{3}H]EPA into secreted TG of the fish oil versus lard groups (Fig. 1). The low incorporation of [^{3}H]EPA into secreted TG in both groups was not apparently due to low TG synthesis in general, since significant incorporation into stored TG was observed for both diet groups (Fig. 4). In addition, during the perfusion we observed a decreasing [^{3}H]EPA to [^{14}C]18:1 ratio in perfusate TG in both diet groups while the ratio of perfusate fatty acids remained constant (Fig. 5), indicating that [^{14}C]18:1 was increasingly being selected over [^{3}H]EPA for TG secretion throughout the perfusion.

During the entire perfusion, the [^{3}H]EPA to [^{14}C]18:1 ratio in perfusate TG was significantly lower in the fish oil group compared to the lard group, perhaps due to a greater ratio of EPA to oleate in the livers of the fish oil group. However, infusion of the same mixture of free fatty acids into livers of animals in both diet groups would be expected to reduce such diet-induced differences in fatty acid pool sizes and would be expected to bring the ratio of hepatic EPA and oleate pools to a value closer to that of the infused fatty acid mixture. Since a diet-related difference in the [^{3}H]EPA to [^{14}C]18:1 ratio in perfusate TG was maintained throughout the perfusion, the liver was able to discriminate between EPA and oleate, and perhaps other fatty acids, for the synthesis of secreted TG as well as hepatic PL.

Previous studies have suggested that there are at least two fatty acid precursor pools for the synthesis of TG in the liver, one for secreted TG and one for stored TG (26-28). Our data also support this conclusion since livers from the animals fed the fish oil diet showed decreased synthesis of TG in the secretory pool but not in the storage pool when compared to animals fed the lard diet (Figs. 1 and 4). In addition, the specific activity data in Table 3 suggest that liver perfusate TG was synthesized from a different precursor pool of fatty acids than that of stored hepatic TG since in all cases the specific activity of the TG fatty acid was two- to sixfold higher for the liver perfusate compared to hepatic TG. However, an alternative explanation that cannot be excluded by our data is that stored TG in the liver may precede the labeling period of the experiment and lower that apparent TG fatty acid specific activity because labeled TG enters an unlabeled pool of slowly turning over TG in the liver.

Although there was not a statistically significant diet-related difference in TG fatty acid specific activities, some interesting trends were noted for the perfusate TG specific activities (Table 3). The specific activity of perfusate [^{14}C]TG in the fish oil group was nearly twice that of the lard group (12.9 vs 6.7 dpm/nmol TG fatty acid). This difference occurred together with a 50% reduction in perfusate TG mass accumulation in the fish oil group compared to lard group (Fig. 1, Table 2). Taken together, these findings likely explain why the appearance of [^{14}C]TG, as percentage of infused dose, was similar for both diet groups (Fig. 1, Table 2, Fig. 4) in the face of a decreased accumulation of TG mass in the perfusates of the fish oil group. Since the percentage incorporation of [^{14}C]18:1 into hepatic and perfusate lipids was similar for both diet groups (Fig. 4), the fish oil group must have had a smaller hepatic pool of oleate (and perhaps other fatty acids) that could be used for the synthesis of TG destined for secretion, resulting in the higher perfusate [^{14}C]TG specific activity in the fish oil group.

Our data also support the hypothesis that the storage and secretory pools of liver TG are differentially regulated by dietary factors. As mentioned previously, fish oil, compared to lard, decreased the synthesis of secreted TG but not stored hepatic TG. An analogous situation occurred when rats were fed chow diets supplemented with sucrose or fish oil (29). Both diets resulted in similar increases in liver TG concentration compared to that in rats fed chow, but plasma TG concentrations decreased in rats fed fish oil and increased in rats fed sucrose. There were also changes in the activity of hepatic Mg^{2+}-dependent phosphatidate phosphohydrolase for both diet groups that were proportional to the changes in plasma triglyceride concentration (29). Phosphatidate phosphohydrolase is a pivotal enzyme in TG and PL biosynthesis that catalyzes the conversion of 1,2-diacylglycerol phosphate to 1,2-diacylglycerol. Fatty acids can activate phosphatidate phosphohydrolase by translocation of the enzyme from the cytosolic to endoplasmic reticulum fraction of the cell (30). It is possible that n-3 fatty acids do not function well in this translocation step and thereby result in less phosphatidate phosphohydrolase bound to the ER fraction which, in turn, could result in less TG synthesis for VLDL particle assembly. Direct studies of the effect of n-3 fatty acids on the translocation of phosphatidate phosphohydrolase are needed to test this hypothesis.

While there was no diet-related difference in the percentage of infused [^{3}H]EPA or [^{14}C]oleate incorporated into hepatic TG, there was a significantly higher percentage of [^{3}H]EPA incorporated into hepatic PL in the fish oil group (Fig. 4) suggesting a higher phospholipid synthesis rate in this group. This conclusion is further supported by the [^{3}H]EPA to [^{14}C]18:1 ratio in hepatic

PL, which was three- to fivefold higher than that of the infused fatty acid mixture and that of hepatic and perfusate TG (Table 4). Since hepatic PL mass was similar for both diet groups, the increased incorporation of [^{3}H]EPA in hepatic PL was likely due to the deacylation–reacylation pathway for PL. In this cycle PL is acted upon by phospholipase A_2 which cleaves the fatty acid from the *sn*-2 position resulting in lysoPL. The lysoPL can then be reacylated by lysophosphatidylcholine acyltransferase, an enzyme that prefers polyunsaturated fatty acids (31). The influx into the liver of n-3 fatty acids that are relatively poorly utilized for TG secretion would result in a pool of fatty acids enriched in n-3 polyunsaturated fatty acids to be incorporated into hepatic PL. Evidence for such an event appeared in the fourfold mass enrichment of n-3 fatty acids in the hepatic PL of the fish oil group compared to the lard group (4.5 vs 16.3%; Table 1). Although the hepatic TG fraction of the fish oil group also was enriched with n-3 fatty acids compared to the lard counterparts (5.5 vs 10.8%), this enrichment was not as great as that of the hepatic phospholipid fraction (16.3%). This outcome may have resulted because the enzyme responsible for the acylation of diglycerides (acyl CoA:1,2-diacylglycerol acyltransferase) does not utilize n-3 fatty acids as well as other fatty acids (25) compared to lysoPL acyltransferase (31).

Our data demonstrated that [^{3}H]EPA was more efficiently removed from the perfusate and taken up by the liver than [^{14}C]oleate (Figs. 2 and 5). Furthermore, this differential uptake was maintained throughout the perfusion as shown by the [^{3}H]EPA to [^{14}C]18:1 ratio in perfusate fatty acid remaining constant at 30% of that of the infused fatty acid mixture throughout the perfusion (Fig. 5). Although we cannot rule out the possibility that recyling of the radiolabel occurs with selective retention of EPA by the liver, this possibility seems unlikely since the ratio of ^{3}H/^{14}C in perfusate fatty acid was constant throughout most of the perfusion (i.e., 60–240 min, Fig. 5). Nossen et al. (6) also have shown that cultured rat hepatocytes take up more EPA from the medium than oleate over 3-h incubations. The reason for this outcome is unclear but may relate to different binding affinities of EPA and oleate for albumin in the perfusate.

There was very little radioactive fatty acid incorporation into the hepatic or perfusate CE fraction in this study. Similar findings have been reported for the liver perfusion and hepatocyte cell culture studies in rats (32, 33). This may be because there is a separate poorly radiolabeled pool of fatty acids used for the esterification of cholesterol and/or it could reflect a slower rate and decreased amount of fatty acid used for cholesterol esterification compared to esterification of TG and PL. It is interesting to note that we have found a strong correlation between hepatic CE concentration and rate of hepatic VLDL cholesterol secretion for these same animals (r = 0.9; ref. 13). Given this outcome, the hepatic CE destined for secretion must either be incorporated intact into nascent VLDL or be hydrolyzed to free cholesterol and re-esterified by a pool of fatty acids different from that of the radiolabeled fatty acids being infused. Using cultures of hepatocytes from rats fed a high cholesterol diet, Mathe, Botham, and Boyd (34) also have found a strong correlation between hepatic CE concentration and the initial secretion rate of cholesterol from the cells (r = 0.98). In addition they found no evidence of CE hydrolysis over a 4-h period with hepatocytes prelabeled with [^{3}H]cholesterol and incubated in the presence of a cholesterol acceptor (rat HDL). Drevon, Engelhorn, and Steinberg (35) also have shown that stimulation of acyl CoA:cholesterol acyltransferase (ACAT) resulted in increased secretion of VLDL CE in cultured rat hepatocytes. Our data show that there was little difference in the fatty acid composition of hepatic CE and the CE secreted in hepatic VLDL during liver perfusion even when a fatty acid mixture that contained predominantly oleic acid (~85% 18:1, 11% n-3) was infused (13). Taken together, these data suggest that cholesteryl esters in the liver can be secreted without hydrolysis and resynthesis; however, more direct studies are needed to firmly establish this possibility.

We also found that hepatic CE mass was reduced in animals fed fish oil. Rustan et al. (32) have recently reported that EPA and EPA-CoA reacted poorly with hepatic ACAT compared to other fatty acids. We also have shown that plasma LCAT utilizes n-3 fatty acids poorly for the synthesis of CE compared to other fatty acids (36). These studies suggest that n-3 fatty acids are poorly utilized by the enzymes that synthesize CE and this may be one explanation for the reduced hepatic and plasma CE concentrations found for the monkeys fed fish oil diets.

Fish oil has consistently been shown to result in lower plasma triglyceride in humans (2, 3). Yet in our study plasma TG concentrations were slightly elevated compared to the lard group. Nonhuman primates fed atherogenic diets typically have plasma TG concentrations much lower than those of human beings (20–30 mg/dl vs ~80–150 mg/dl) presumably due to a more efficient clearance of TG by the monkeys, making it difficult to detect differences in TG production by measurement of plasma concentrations of TG. We previously have found that African green monkeys have a greater total plasma lipolytic activity and a greater proportion of total post-heparin plasma lipolytic activity as lipoprotein lipase compared to human beings and this may explain the lower plasma TG concentrations of monkeys (37). In spite of the increased fasting plasma TG concentrations for the fish oil oil group, the rate of TG secretion was approximately one half as much for the livers of the fish oil group compared to the lard group. Therefore, slower clearance

of plasma TG in the fish oil group may actually have occurred, although several other possibilities could explain this outcome as well.

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