Upregulation of hepatic LDL transport by n-3 fatty acids in LDL receptor knockout mice

Chandna Vasandani,* Abdallah I. Kafrouni,* Antonella Caronna,* Yuriy Bashmakov,† Michael Gotthardt,† Jay D. Horton,*,† and David K. Spady[1],*

Departments of Internal Medicine* and Molecular Genetics,† The University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390-8887

Abstract **We determined the effects of dietary n-6 and n-3 polyunsaturated fatty acids (PUFA) on parameters of plasma lipoprotein and hepatic lipid metabolism in LDL receptor (LDLr) knockout mice. Dietary n-3 PUFA decreased the rate of appearance and increased the hepatic clearance of IDL/LDL resulting in a marked decrease in the plasma concentration of these particles. Dietary n-3 PUFA increased the hepatic clearance of IDL/LDL through a mechanism that appears to involve apolipoprotein (apo)E but is independent of the LDLr, the LDLr related protein (LRP), the scavenger receptor B1, and the VLDLr. The decreased rate of appearance of IDL/VLDL in the plasma of animals fed n-3 PUFA could be attributed to a marked decrease in the plasma concentration of precursor VLDL. Decreased plasma VLDL concentrations were due in part to decreased hepatic secretion of VLDL triglyceride and cholesteryl esters, which in turn was associated with decreased concentrations of these lipids in liver. Decreased hepatic triglyceride concentrations in animals fed n-3 PUFA were due in part to suppression of fatty acid synthesis as a result of a decrease in sterol regulatory element binding protein-1 (SREBP-1) expression and processing. In conclusion, these studies indicate that n-3 PUFA can markedly decrease the plasma concentration of apoB-containing lipoproteins and enhance hepatic LDL clearance through a mechanism that does not involve the LDLr pathway or LRP.**—Vasandani, C., A. I. Kafrouni, A. Caronna, Y. Bashmakov, M. Gotthardt, J. D. Horton, and D. K. Spady. **Upregulation of hepatic LDL Transport by n-3 fatty acids in LDL receptor knockout mice. *J. Lipid Res.* 2002.** 43: **772–784.**

Supplementary key words polyunsaturated fatty acids • fish oil • liver • lipoproteins • cholesterol • triglyceride • VLDL

The apparent low incidence of cardiovascular disease in populations consuming diets rich in marine lipids has created considerable interest in the use of these lipids to prevent and treat complications of atherosclerosis (1, 2). Marine lipids contain large amounts of the long-chain n-3 PUFA eicosapentenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). These fatty acids are incorporated into membrane phospholipids, alter prostaglandin and leukotriene formation, and regulate gene expression resulting in widespread biological activities, including effects on platelet function, inflammation, and plasma lipids. With respect to effects on plasma lipids, dietary n-3 PUFA consistently lowers plasma triglyceride concentrations in normal and hypertriglyceridemic humans (3, 4) and in many animal models (5). Dietary n-3 PUFA also dramatically blunts the rise in serum triglyceride concentrations that follow a fatty meal (6, 7). The effect of dietary n-3 PUFA on plasma cholesterol concentrations is far more variable (3–5). In subjects with type IV hyperlipidemia, n-3 PUFA markedly lowers VLDL cholesterol (VLDL-C) but usually increases LDL-C concentrations at least in the short term. Plasma LDL concentrations are reduced by high doses of n-3 PUFA in normal persons (3, 4, 7, 8), in those with primary hypercholesterolemia (4), and in nonhuman primates (9), especially if n-3 PUFA replaces saturated fatty acids in the diet. The response of serum cholesterol to n-3 fatty acids appears to depend on several factors, including *i*) whether purified n-3 fatty acids are used or marine lipids containing a variety of saturated fatty acids and sterols, *ii*) whether n-3 fatty acids are taken as a supplement or used to replace other fats in the diet, *iii*) the level of n-3 fatty acid intake, *iv*) the underlying lipoprotein phenotype, and *v*) the specific genotype responsible for a particular lipoprotein phenotype.

The mechanisms underlying the lipid-lowering effects of n-3 PUFA are incompletely understood. Human turnover studies suggest that n-3 PUFA decreases serum triglyceride concentrations in part by inhibiting VLDL triglyceride secretion by the liver (10). Consistent with these human studies are data showing that dietary n-3 PUFA decreases VLDL triglyceride secretion from perfused rat (11) and

Abbreviations: ACC, acetyl CoA carboxylase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPL, lipoprotein lipase; LRP, receptor related protein; MTP, microsomal triglyceride transport protein; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein; SR-BI, scavenger receptor BI.

[1] To whom correspondence should be addressed at 1600 West College, Suite 680, Grapevine, TX 76051.
e-mail: jay.horton@utsouthwestern.edu

monkey liver (12), decrease triglyceride synthesis and VLDL triglyceride secretion in primary cultures of rabbit (13) and rat (14, 15) hepatocytes, inhibit fatty acid synthesis in rat liver (11, 16) and isolated rat hepatocytes (17), decrease the expression of lipogenic genes in rat (18) and mouse (19–22) liver, and upregulate genes involved in fatty acid oxidation (23). Suppression of the expression of lipogenic genes by n-3 PUFA is likely the result of decreased nuclear sterol regulatory element-binding protein-1 (SREBP-1) (18–22, 24), possibly through inhibition of the liver X receptor (25). In hepatocytes, n-6 and n-3 PUFA accelerate the decay of SREBP-1 mRNA (26). Stimulation of genes involved in fatty acid oxidation by n-3 PUFA is likely mediated by peroxisome proliferator-activated receptors (PPAR)α (19). Whether this effect of n-3 PUFA contributes to lipid lowering is unclear since n-3 PUFA decreases plasma lipids nearly as well in PPARα deficient mice as in control mice (27). Most human turnover studies show that dietary n-3 PUFA also decreases the residence time of VLDL in serum (4). Dietary PUFA appears to have little effect on LPL or hepatic lipase activity in post-heparin serum in humans (7, 28, 29). On the other hand, LPL may be more reactive toward VLDL with polyunsaturated triglyceride substrate (7, 30), leading to more rapid lipolysis of triglyceride-rich lipoproteins with dietary PUFA. In addition, dietary n-3 PUFA has been shown to accelerate chylomicron triglyceride clearance in rats (31) and to enhance the conversion VLDL apolipoprotein (apo)B to LDL apoB in pigs (32).

High doses of n-3 PUFA lowers serum LDL concentrations in normotriglyceridemic subjects (4, 7, 8). One human turnover study suggested that this effect of n-3 PUFA is mainly the result of decreased LDL production (33). We found that n-3 PUFA also increases hepatic LDL uptake through an LDL receptor (LDLr)-independent pathway in the rat (34–36). Together these data suggested that n-3 PUFA could decrease plasma triglyceride and cholesterol concentrations in animals lacking LDLrs. In the current studies, we compare the lipid lowering properties of n-6 (18:2) and n-3 (20:5/22:6) fatty acids in mice lacking LDLrs and investigate the mechanistic basis for the observed effects.

MATERIALS AND METHODS

Animals and diets

Most studies were performed in male mice with targeted disruption of the LDLr gene (37). Some experiments were carried out in $LDLr^{-/-}$ mice with liver-specific inactivation of LRP. These animals were generated by breeding mice that were homozygous for floxed LRP and LDLr deficiency ($LRP^{flox/flox}$ $LDLr^{-/-}$) with $LRP^{flox/flox}LDLr^{-/-}albuminCre^{+}$ mice (38). Cre^{+} mice had >85% decrease in hepatic LRP by Western blot. Some studies were performed in $apoE^{-/-}$ mice that were obtained from Jackson Laboratories. Animals were housed in colony cages (five animals/cage) in a room with temperature and humidity control and subjected to light cycling for at least 2 weeks prior to use in studies. All studies were carried out during the mid-dark phase of the light cycle. The control semisynthetic diet used in these studies contained 20% soy protein, 0.3% DL-methionine, 10% cellulose, 8.5% salt mix, 1% vitamin mix, 0.2% choline bitartrate, 2% corn oil, 38% sucrose, and 20% corn starch. The experimental diets were prepared by replacing sucrose with the desired amount of fatty acid ethyl esters on a cal/cal basis to provide 10% of total energy, assuming 4 cal/g of corn starch and 9 cal/g of fatty acid ester. The n-3 and n-6 fatty acid ethyl ester preparations were provided by the NIH/NOAA Biomedical Test Material Program. The n-3 fatty acid ethyl ester preparation contained 392 mg/g eicosapentaenoic acid, 264 mg/g docosahexaenoic acid (764 mg/g n-3 fatty acid), and virtually no cholesterol (<0.01%). The n-6 fatty acid ethyl esters were prepared from safflower oil and contained 771 mg/g linoleic acid. In some experiments highly purified (>95%) ethyl ester preparations of EPA and DHA were used. All diets contained 0.1% tertiary butylhydroquinone and 0.01% vitamin E. Diets were stored under N_2 at −20°C and provided fresh to the animals each day. Diets were fed ad lib for 6 weeks prior to specific experiments. All experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center at Dallas.

Determination of VLDL secretion rates

Rates of VLDL secretion were measured by quantifying the rate of VLDL accumulation in plasma after the administration of Triton WR1339 (Sigma Chemical Co., St. Louis) to block VLDL metabolism (39). On the day of study, experimental diets were replaced with fat-free diet. Four hours later animals were administered 20 mg Triton WR1339 in 100 μl sterile saline (or saline only) intravenously and sacrificed 1 h later. (Preliminary studies showed that triglyceride accumulated in plasma as a linear function of time for at least 2 h.) Plasma lipoproteins were size-fractionated by fast protein liquid chromatography (FPLC) using a Superose 6 HR column (Pharmacia Biotech) and enzymatic kits were used to quantify total (Boehringer Diagnostics, Indianapolis, IN, catalog #1127771) and unesterified (Wako Chemicals, USA, catalog #274-47109) cholesterol and triglyceride (Sigma Diagnostics, catalog #343-25P) in fractions corresponding to VLDL. VLDL-apoB-100 and -apoB-48 were separated on gradient polyacrylamide gels, stained, and quantified by densitometry. The difference in these lipid and apoprotein values between animals injected with Triton WR1339 and vehicle was taken as the hepatic secretion rate for these constituents of VLDL.

Determination of LDL transport rates

LDL was isolated from $LDLr^{-/-}$ mice maintained on a low cholesterol diet or from normolipidemic humans. The LDL was isolated from plasma by preparative ultracentrifugation in the density range of 1.020 to 1.055 g/ml by preparative ultracentrifugation (40) and labeled with ^{125}I- or ^{131}I-tyramine cellobiose as previously described (41, 42). The human LDL was modified by reductive methylation (43–45). Lipoprotein preparations were reisolated by gel filtration chromatography, dialyzed, and filtered through a 0.45 μm Milex HA filter immediately prior to administration to experimental animals.

Rates of hepatic LDL transport were quantified in vivo using a primed-continuous infusion of ^{125}I-tyramine cellobiose-labeled LDL through an internal jugular catheter (46). The infusions of ^{125}I-tyramine cellobiose-labeled LDL were continued for 6 h at which time each animal was administered a bolus of ^{131}I-tyramine cellobiose-labeled LDL as a marker of the volume of plasma contained in each tissue, and killed 10 min later by exsanguination through the inferior vena cava. Tissue samples along with aliquots of plasma were assayed for radioactivity in a γ counter (Packard Instrument Co., Inc., Downers Grove, IL). The amount of labeled LDL in the various tissues at 10 min (^{131}I disintegrations per min per g of tissue divided by the specific activity of ^{131}I in

plasma) and at 6 h (^{125}I disintegrations per min per g of tissue divided by the specific activity of ^{125}I in plasma) was then calculated. The increase in the tissue content of LDL-C or LDL protein with time represents the rate of LDL uptake in micrograms of LDL-C or LDL protein taken up per h per g of tissue.

Determination of HDL cholesteryl ether transport rates

Mouse HDL was isolated in the density range of 1.07–1.21 g/ml using sequential preparative ultracentrifugation and standard techniques (40), and labeled with either the intracellularly trapped [1α, 2α(n)-^{3}H]cholesteryl oleyl ether (47–49) or [cholesteryl-4-^{14}C]oleate by exchange from donor liposomes (46, 50, 51). The labeled HDL were reisolated by ultracentrifugation, dialyzed against saline, filtered through a 0.45 μm Milex HA filter, and used within 24 h. Rates of HDL cholesteryl ether transport were quantified in vivo using a primed infusion protocol as previously described (52).

Determination of hepatic cholesterol and fatty acid synthesis rates

Rates of hepatic cholesterol and fatty acid synthesis were measured in vivo using [^{3}H]water as previously described (46). Animals were administered ~25 mCi of [^{3}H]water intravenously through a tail vein and killed one h later by exsanguination through the inferior vena cava. Aliquots of plasma were taken for the determination of body water specific activity and samples of liver were saponified in alcoholic KOH. Saponified liver samples were first extracted with petroleum ether and aliquots used for the isolation of digitonin-precipitable sterols. The saponified liver samples were then acidified with concentrated HCL and the fatty acids extracted into hexane. Rates of sterol and fatty acid synthesis are expressed as the nmoles or μmoles of [^{3}H]water incorporated into digitonin-precipitable sterols or fatty acids per h per g of liver.

Determination of mRNA levels

Hepatic mRNA levels for apoB, apoE, apoC-1, apoC-2, apoC-3, microsomal triglyceride transport protein (MTP), fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), HMG-CoA synthase, SREBP-1, SREBP-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin (as internal controls) were determined by RNase protection as previously described (53). Species-specific ^{32}P-labeled riboprobes were synthesized using MAXIscript in vitro transcription kits (Ambion Inc, Austin, TX) in the presence of 10 μM (apoB, apoE, MTP, FAS, ACC, HMG-CoA synthase, SREBP-1, SREBP-2) or 50 μM (GAPDH and β-actin) labeled nucleotide. Samples of liver were homogenized in RNA STAT-60 (TEL-TEST, inc., Friendswood, TX). Total RNA (40 μg) was hybridized with ^{32}P-labeled riboprobes simultaneously at 68°C using the HybSpeed RPA protocol (Ambion Inc). Following RNase digestion, the mRNA-protected ^{32}P-labeled probes were separated on 8 M urea, 5% polyacrylamide gels together with ^{32}P-labeled *Msp*I-digested pBR322 size standards. The radioactivity in each band, as well as background radioactivity, was quantified using a phosphorimaging system (Molecular Dynamics Inc, Sunnyvale, CA).

Immunoblot analyses

Membrane fractions were prepared from pulverized liver that had been frozen in liquid N_2 immediately after harvesting and stored at −80°. Immunoblot analysis of hepatic lipoprotein receptors was performed using polyclonal rabbit IgGs directed against mouse SR-BI (54), mouse LDLr-related protein (LRP) (55), and VLDLr (56). For immunoblot analysis of SREBP, nuclear extracts were prepared from fresh liver and 30 μg aliquots subjected to 8% SDS-PAGE electrophoresis, transferred to Hybond C extra membranes (Amersham Corp., Arlington Heights, IL), and incubated with rabbit anti-mouse SREBP-1 or anti-mouse SREBP-2 (57). Membrane-bound antibodies were visualized with Enhanced Chemiluminescence Western Blotting Detection System kit (Amersham) using a horseradish peroxidase-labeled donkey anti-rabbit IgG antibody (Amersham).

Determination of cholesterol and triglyceride concentration in liver and plasma

Liver free and esterified cholesterol (along with internal standards) were extracted in chloroform-methanol, 2:1 (v/v) and separated on Sep-Pak Vac RC silica cartridges (Varion). Eluted free cholesterol was quantified by capillary GC. Eluted cholesteryl esters were saponified and the resulting free cholesterol was extracted in petroleum ether and quantified by capillary GC. Liver triglycerides were extracted in chloroform-methanol, 2:1 (v/v) and washed with 50 mM NaCl × 1 and 0.36 M $CaCl_2$/methanol × 2. Aliquots of the organic phase (along with standards and blanks) were brought to 10% Triton X100, dried under N_2, and assayed for triglyceride (Sigma Diagnostics, catalog #343-25P). The cholesterol distribution in plasma was determined by FPLC using a Superose 6 HR 10/30 column (Pharmacia Biotech). Aliquots were collected and enzymatic kits were used to assay for total cholesterol (Sigma Diagnostics), free cholesterol (Wako Chemicals USA), and triglyceride (Sigma Diagnostics).

Determination of plasma apoprotein concentrations

Plasma apolipoproteins were separated by SDS-PAGE. Total plasma lipoproteins were isolated by ultracentrifugation at d = 1.21 g/ml and size fractionated by FPLC using a Superose 6 HR column. Fractions corresponding to VLDL and IDL/LDL were pooled, delipidated, loaded onto 2–15% gradient polyacrylamide gels (Owl Separation Systems, Portsmouth, NH), and subjected to electrophoresis. Apolipoproteins were visualized using Gelcode Blue Coomasie stain (Pierce, Rockford, IL) and gels were scanned using a densitometer (Molecular Dynamics).

Statistical analysis

The data are presented as means ± 1 SD. To test for differences among the dietary regimens, one-way ANOVA was performed. Significant results were further analyzed using the Tukey multiple comparison procedure.

RESULTS

Effect of dietary PUFA on plasma lipid levels

We previously showed that dietary n-3 fatty acids decreased plasma lipids in the rat through a mechanism that was independent of changes in LDLr expression. We therefore determined the effect of dietary n-3 PUFA on plasma lipids in LDLr-deficient mice. Mice were fed a semipure diet or the same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 2–12% of total energy, assuming 4 cal/g carbohydrate and 9 cal/g PUFA. Initial studies were performed using ethyl ester preparations provided by an NIH Fish Oil Test Materials Program that contained 77% n-3 (20:5n-3 + 22:6n-3) or 78% n-6 (18:2n-6) PUFA. Diets were fed for 6 weeks. Weight gain did not differ significantly among the experimental groups. As shown in **Fig. 1A**, dietary n-3 PUFA decreased plasma triglyceride and cholesterol concentrations in a dose-dependent manner. At lower levels of n-3 intake (2–4% of energy), dietary n-3 PUFA mainly lowered plasma triglyceride concentrations, but at higher in-

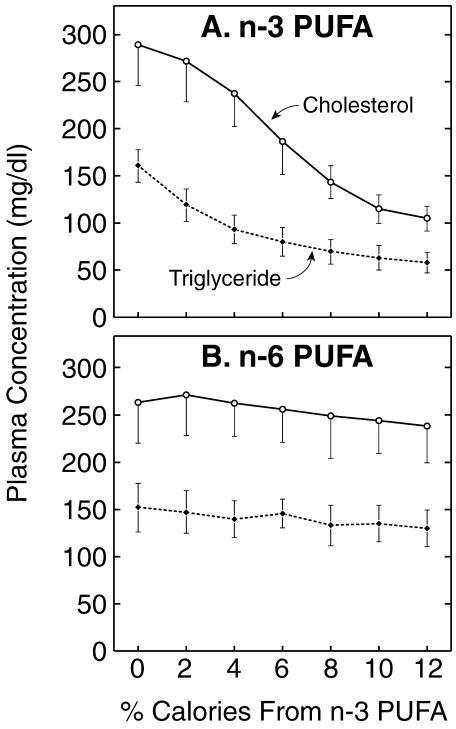

Fig. 1. Dose-dependent effects of dietary PUFA on plasma lipid concentrations in LDL receptor (LDLr)$^{-/-}$ mice. Animals were fed a semipure control diet or this same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 2–12% of total energy. Each value represents the mean ± 1 SD for data obtained in five animals.

takes (8–12% of energy) plasma cholesterol concentrations were also markedly reduced. Dietary n-6 PUFA did not significantly alter plasma lipid concentrations in LDLr$^{-/-}$ mice fed a cholesterol-free diet (Fig. 1B).

Subsequent studies were performed using diets supplemented with PUFA to provide 10% of energy (~4% by wt). The effect of dietary PUFA on the lipoprotein distribution of plasma cholesterol was determined by FPLC and is shown in **Fig. 2A**. Dietary n-3 PUFA markedly reduced the amount of cholesterol carried in the lower density lipoproteins (VLDL and IDL/LDL) compared with animals fed n-6 PUFA or the control diet. Plasma HDL-C concentrations tended to be lower in animals fed n-3 PUFA and higher in animals fed n-6 PUFA compared with the control diet. Equal volumes from FPLC fractions corresponding to VLDL (fractions 2–8) and IDL/LDL (fractions 10–18) were pooled and used for apolipoprotein analysis. Apolipoproteins were separated by polyacrylamide gel electrophoresis. As shown in Fig. 2B, n-3 fatty acids markedly reduced apoB-100, apoB-48, and apoE in VLDL and IDL/LDL relative to n-6 PUFA or the control diet.

We also determined the effect of dietary PUFA on plasma lipids in LDLr$^{-/-}$ mice fed a diet enriched with cholesterol (0.2% by wt). As shown in **Fig. 3A**, supplementation of the semipure control diet with 0.2% cholesterol increased plasma cholesterol and triglyceride concentrations by ~2-fold. Under these conditions, dietary n-3 PUFA (10% of cal) markedly decreased the plasma con-

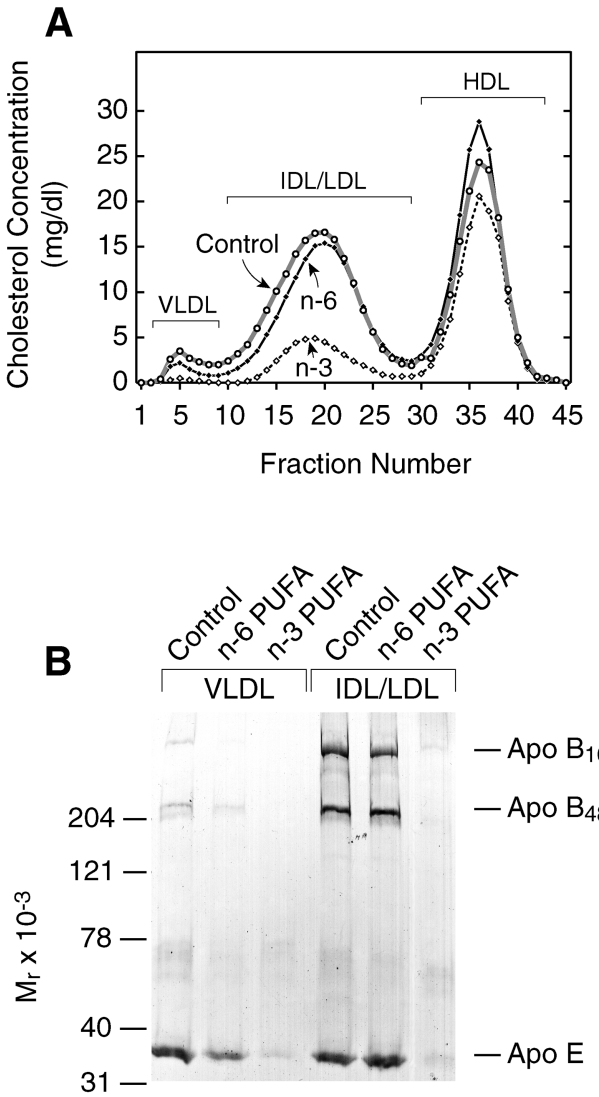

Fig. 2. Effect of dietary PUFA on plasma lipids and apolipoproteins in LDLr$^{-/-}$ mice. Animals were fed a semipure control diet or this same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 10% of total energy (~4% by wt). Equal volumes of plasma from five animals/group were pooled and subjected to fast protein liquid chromatography (FPLC) analysis (A). FPLC fractions corresponding to VLDL and IDL/LDL were delipidated and the apolipoproteins separated by polyacrylamide gel electrophoresis (B).

centration of triglyceride (50%) and cholesterol (62%). Dietary n-6 PUFA also decreased plasma triglyceride (31%) and cholesterol (21%) concentrations in LDLr$^{-/-}$ mice fed 0.2% cholesterol. Figure 3B shows the FPLC profile of plasma cholesterol in these animals. Dietary n-3 PUFA markedly decreased the amount of cholesterol carried in lower density fractions (VLDL and IDL/LDL). Dietary n-6 PUFA also decreased plasma VLDL and IDL/LDL cholesterol concentrations, although less so than n-3 PUFA. As on the cholesterol-free diet, n-3 PUFA tended to decrease, whereas n-6 PUFA tended to increase plasma HDL-concentrations compared with the control diet.

Because the n-3 preparation contained both EPA and DHA we determined the effect of purified (>96%) ethyl es-

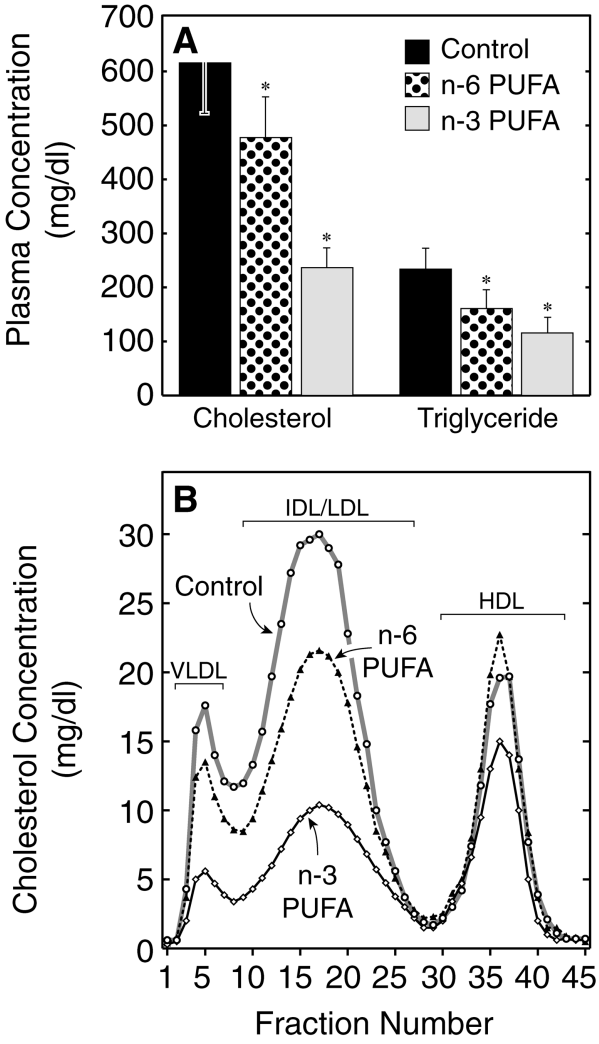

Fig. 3. Effect of dietary PUFA on plasma lipid concentrations and lipoprotein cholesterol distribution in LDLr$^{-/-}$ mice fed a cholesterol-enriched diet. Animals were fed a semipure control diet containing 0.2% cholesterol or this same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 10% of total energy (~4% by wt). A: Total plasma cholesterol and triglyceride concentrations. B: FPLC analysis of plasma pooled from five animals per group. *Significantly differs from the control group, $P < 0.05$.

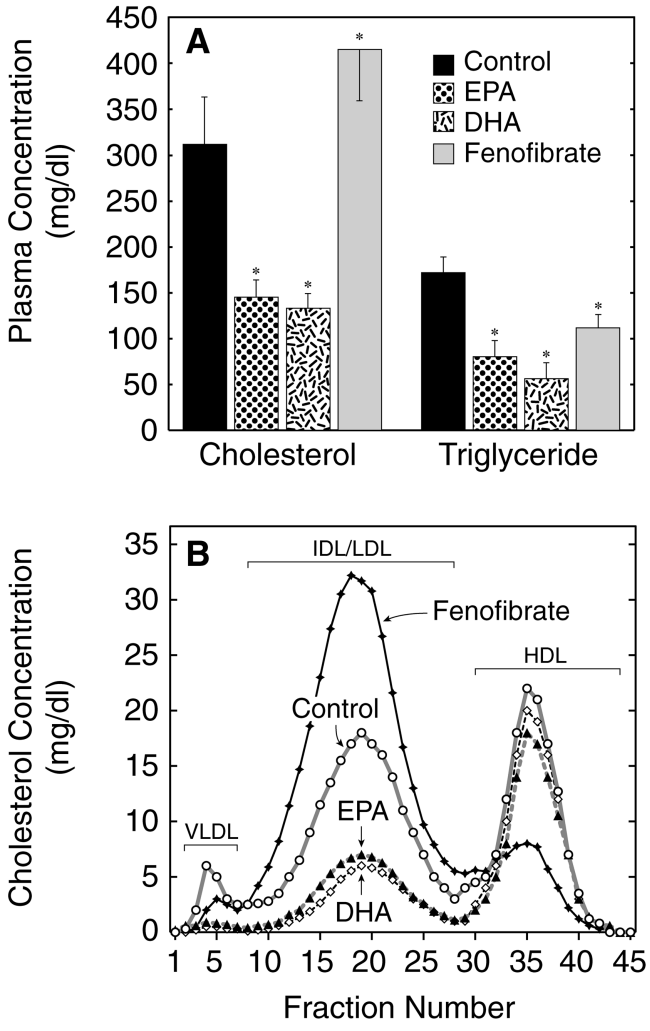

Fig. 4. Effect of dietary eicosapentaenoic acid, docosahexaenoic acid, and fenofibrate on plasma lipid concentrations and lipoprotein cholesterol distribution in LDLr$^{-/-}$ mice. Animals were fed a semipure control diet or this same diet in which EPA or DHA replaced carbohydrate on a cal/cal basis to provide 10% of total energy (~4% by wt). Fenofibrate was added to the control diet (0.25% by wt). A: Total plasma cholesterol and triglyceride concentrations. B: FPLC analysis of plasma pooled from five animals per group. *Significantly differs from the control group, $P < 0.05$.

ter preparations of these two fatty acids on plasma lipid concentrations. Because EPA and DHA activate PPARα we also determined the effect of the PPARα agonist fenofibrate on plasma lipid levels. LDLr$^{-/-}$ mice were fed the semipure control diet or the control diet in which ethyl esters of EPA or DHA were substituted for carbohydrate on a cal/cal basis to provide 10% of total energy. Fenofibrate (0.25% by wt) was added to the semipure control diet. Diets were fed for 6 weeks during which time weight gain did not differ among the experimental groups. As shown in **Fig. 4A**, dietary EPA and DHA were both hypolipidemic, reducing plasma triglyceride and cholesterol by >50%. Fenofibrate at the dose used was less active in lowering plasma triglyceride concentrations (27% decrease) and significantly raised plasma cholesterol concentrations. As shown in Fig. 4B, dietary EPA and DHA markedly decreased the amount of cholesterol carried in VLDL and IDL/LDL. The ratio of HDL to non-HDL-C tended to be higher in animals fed DHA than in animals fed EPA. Fenofibrate decreased the amount of cholesterol carried in VLDL but increased the amount of cholesterol carried in IDL/LDL, and also decreased HDL-C.

Effect of dietary PUFA on VLDL secretion

The rate of VLDL secretion by the liver is a key determinant of plasma VLDL concentrations. We determined the effect of dietary PUFA on the rate of accumulation of VLDL in plasma after the administration of Triton WR1339 to block VLDL metabolism. LDLr$^{-/-}$ mice were fed the control semisynthetic diet or the same diet in which n-3 or n-6 PUFA were substituted for carbohydrate to provide 10% of energy. As shown in **Fig. 5**, dietary n-3 PUFA modestly decreased the secretion of VLDL apoB

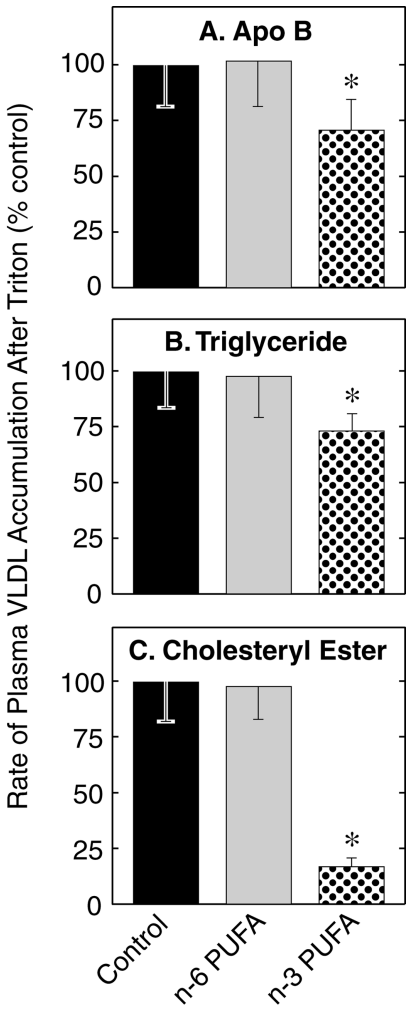

Fig. 5. Effect of dietary PUFA on VLDL secretion in LDLr$^{-/-}$ mice. Animals were fed a semipure control diet or this same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 10% of total energy (~4% by wt). VLDL, apoB, triglyceride, and cholesteryl ester secretion was measured in vivo after the administration of Triton WR1339 to block VLDL metabolism as described in Materials and Methods. Each value represents the mean ± 1 SD for data obtained in 15 animals. *Significantly differs from the control group, $P < 0.05$.

and triglyceride, whereas the secretion of VLDL cholesteryl ester was reduced by 78%. Assuming one molecule of apoB per VLDL particle, these data suggest that dietary n-3 PUFA modestly decreased the number of VLDL particles secreted by the liver and that these particles were depleted of cholesteryl ester. We measured hepatic mRNA levels for apoB, apoE, apoC-1, apoC-2, apoC-3, and MTP using RNase protection assay. When the data from 10 animals per group were quantified using an isotopic image analysis system as described in Materials and Methods, neither n-6 or n-3 PUFA significantly altered hepatic mRNA levels for these genes (data not shown).

Effect of dietary PUFA on hepatic lipid concentrations and synthesis

Studies were performed to determine if decreased VLDL secretion in animals fed n-3 PUFA was associated with decreased hepatic lipid levels and synthesis. LDLr$^{-/-}$ mice were fed the control or PUFA-containing diets for 6 weeks. As shown in **Fig. 6A**, dietary n-3 PUFA decreased hepatic triglyceride concentrations by 50% compared with the control diet, whereas dietary n-6 PUFA had little effect. The decrease in hepatic triglyceride concentrations in animals fed n-3 PUFA was associated with a 40% reduction in the rate of hepatic fatty acid synthesis (Fig. 6B). Decreased rates of fatty acid synthesis were, in turn, accompanied by decreased levels of mRNA for the lipogenic enzymes FAS and ACC as illustrated in Fig. 6C, which shows examples of autoradiograms from RNase protection analyses. When the data from 10 animals per group were quantified using an isotopic image analysis system as described in Materials and Methods, dietary n-3 PUFA decreased hepatic mRNA for FAS by 50% and mRNA for ACC by 40% compared with the control diet. Because the expression of FAS and ACC is regulated by SREBP-1, we quantified the amount of this transcription factor in nuclear extracts prepared from animals fed n-6 or n-3 PUFA. As shown in Fig. 6D, dietary n-3 PUFA markedly decreased nuclear SREBP-1 levels, whereas n-6 PUFA tended to increase nuclear SREBP-1. The marked decrease in nuclear SREBP-1 levels, shown in Fig. 6D, was associated with a 35% decrease in SREBP-1 mRNA levels as illustrated in Fig. 6E.

Dietary n-3 PUFA, and to a lesser extent n-6 PUFA, decreased hepatic total and esterified cholesterol levels as shown in **Fig. 7A**. In particular, dietary n-3 PUFA decreased hepatic cholesteryl ester concentrations by 75% compared with the control diet. As shown in Fig. 7B, rates of hepatic cholesterol synthesis were increased ~2.5-fold in animals fed n-3 or n-6 PUFA. Increased rates of hepatic sterol synthesis were associated with increased expression of HMG-CoA synthase as illustrated by the autoradiograms from RNase protection analyses shown in Fig. 7C. When the data from 10 animals per group were quantified using an isotopic image analysis system as described in Materials and Methods, dietary n-6 and n-3 PUFA increased hepatic mRNA for HMG-CoA synthase by 35% and 55% respectively. Since expression of enzymes in the cholesterol biosynthetic pathway is regulated by SREBP-2, we quantified the level of this transcription factor in nuclear extracts prepared from the livers of animals fed n-6 or n-3 PUFA. Dietary PUFA had no major effect on nuclear SREBP-2 levels (Fig. 7D) and no significant effect on SREBP-2 mRNA levels (Fig. 7E) when compared with the control diet.

Effect of dietary PUFA on LDL transport

The marked decrease in plasma LDL concentrations in animals fed n-3 PUFA could be due to a change in the rate of LDL entry into the plasma space (resulting from the metabolism of VLDL or direct LDL secretion by the liver) or to a change in the rate of LDL clearance by one or more tissues of the body. To address these possibilities we performed LDL transport studies using [^{125}I]tyramine cellobiose-labeled homologous LDL as described in Materials and Methods. **Figure 8** shows the effect of dietary n-6

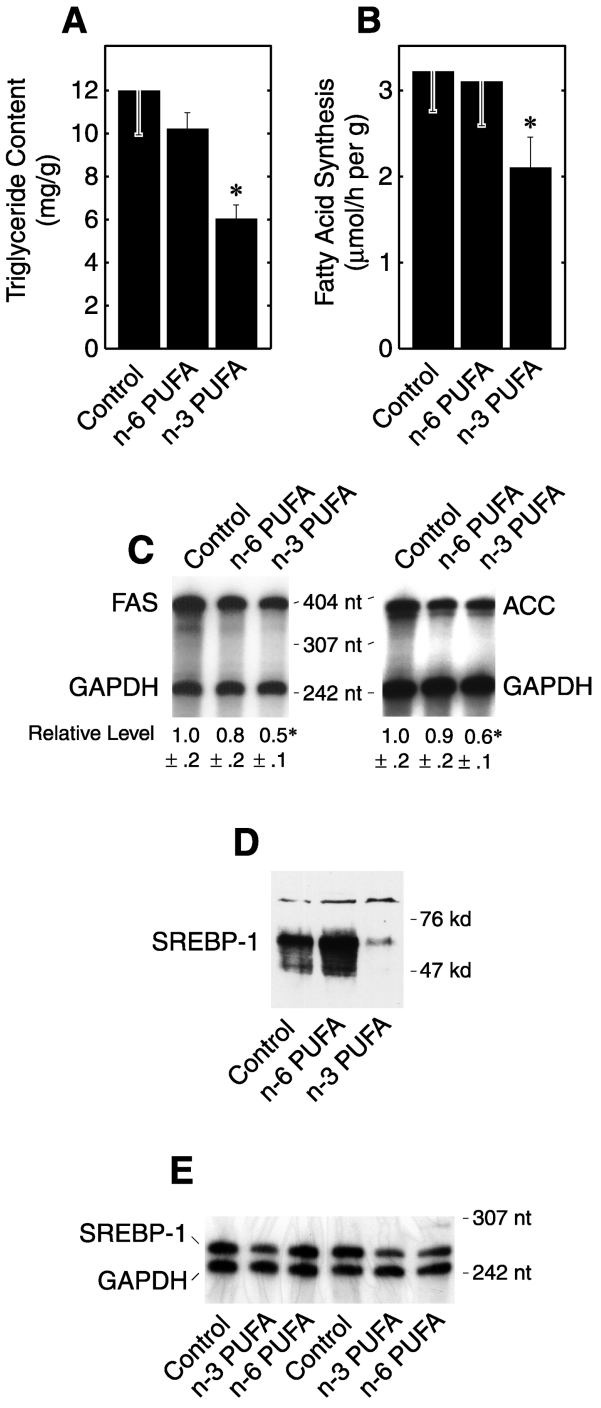

Fig. 6. Effect of dietary PUFA on hepatic triglyceride levels and parameters of lipogenesis in LDLr$^{-/-}$ mice. Animals were fed a semipure control diet or this same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 10% of total energy (~4% by wt). A: Hepatic triglyceride concentration. B: Hepatic fatty acid synthesis rates. C: Representative autoradiograms from RNase protection analyses of hepatic fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) mRNA. D: Autoradiogram from immunoblot analysis of nuclear sterol regulatory element-binding protein (SREBP)-1. E: Autoradiogram from nuclease protection analysis of hepatic SREBP-1 mRNA. *Significantly differs from the control group, $P < 0.05$.

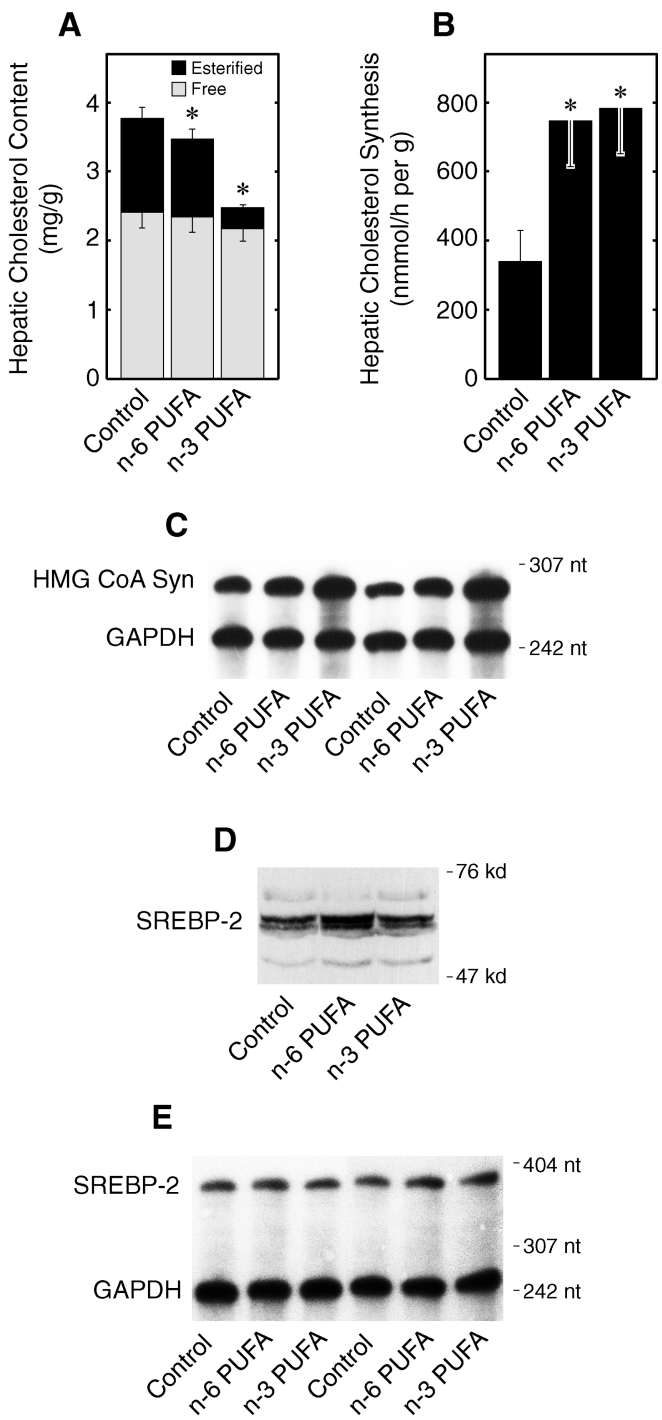

Fig. 7. Effect of dietary PUFA on hepatic cholesterol levels and parameters of sterol synthesis in LDLr$^{-/-}$ mice. Animals were fed a semipure control diet or this same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 10% of total energy (~4% by wt). A: Hepatic free and esterified cholesterol concentrations. B: Hepatic cholesterol synthesis rates. C: Autoradiogram from RNase protection analysis of hepatic HMG-CoA synthetase mRNA. D: Autoradiogram from immunoblot analysis of nuclear SREBP-2. E: Autoradiogram from RNase protection analysis of hepatic SREBP-2 mRNA.

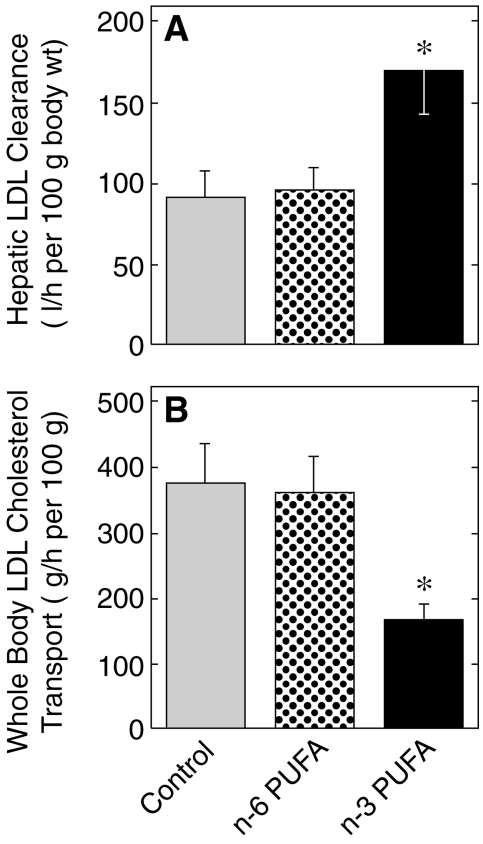

Fig. 8. Effect of dietary PUFA on hepatic LDL clearance (A) and whole body LDL transport (B) in LDLr$^{-/-}$ mice. Animals were fed a semipure control diet or this same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 10% of total energy (~4% by wt). Each value represents the mean ± 1 SD for data obtained in 15 animals. *Significantly differs from the control group, $P < 0.05$.

and n-3 fatty acids on rates of hepatic LDL clearance and whole body LDL transport. In animals fed the control diet, the liver cleared mouse LDL at a rate of 96 μl/h per 100 g body wt (Fig. 8A). Dietary n-3 PUFA increased hepatic LDL clearance by 88%, whereas n-6 PUFA had no significant effect. Dietary n-3 PUFA had no significant effect on LDL clearance by extrahepatic tissues (data not shown). The effect of dietary PUFA on whole body LDL transport is shown in Fig. 8B. Whole animal LDL transport, which equaled 387 μg/h per 100 g body wt in animals fed the control diet, was reduced 60% by n-3 PUFA and was unaffected by n-6 PUFA. It is possible that n-3 PUFA-induced alterations in the composition of LDL enhanced the clearance of these particles by the liver. To address this possibility we prepared IDL/LDL from LDLr$^{-/-}$ mice fed control, n-6, or n-3 diets. These LDL preparations were trace-labeled and used to measure hepatic uptake rates in LDLr$^{-/-}$ mice fed control diet. IDL/LDL from mice fed the three diets were transported by the liver at the same rate (data not shown).

To determine if the hepatic uptake of other particles was also increased by n-3 fatty acids we measured hepatic uptake rates for mouse albumin and methylated human LDL in LDLr$^{-/-}$ mice. Both particles were cleared by the liver at about one-third the rate of mouse LDL, and clearance rates were not altered by n-6 or n-3 PUFA (data not shown). The observation that the hepatic clearance of methylated human LDL was not increased in animals fed n-3 PUFA raised the possibility that apoE may be necessary for enhanced hepatic clearance of IDL/LDL in animals fed n-3 PUFA. We therefore determined the effect of dietary PUFA on hepatic and plasma lipids in apoE$^{-/-}$ mice. As shown in **Fig. 9**, dietary n-3 PUFA tended to increase plasma cholesterol and significantly increased plasma tri-

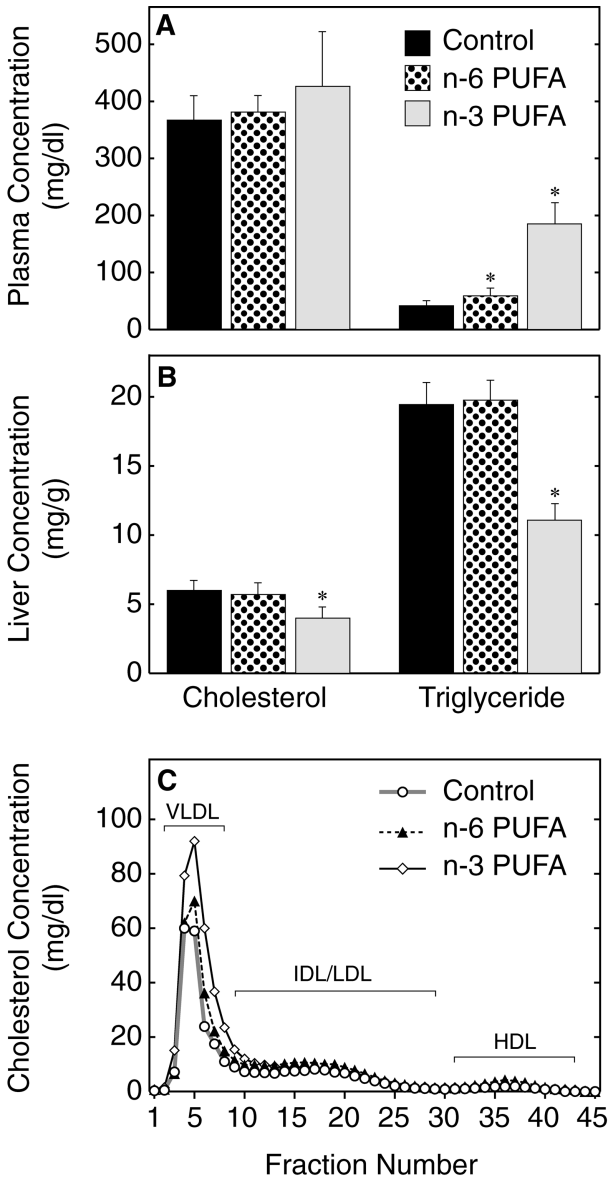

Fig. 9. Effect of dietary PUFA on (A) plasma cholesterol and triglyceride concentrations, (B) liver cholesterol and triglyceride concentrations, and (C) the lipoprotein distribution of plasma cholesterol in apolipoprotein (apo)E$^{-/-}$ mice. Animals were fed a semipure control diet or this same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 10% of total energy (~4% by wt). Each value in A and B represents the mean ± 1 SD for data obtained in 10 animals. *Significantly differs from the control group, $P < 0.05$. FPLC analysis shown in C was obtained using equal volumes of plasma pooled from 10 animals per group.

glyceride concentrations in apoE$^{-/-}$ mice (Fig. 9A) even though liver cholesterol and triglyceride concentrations were decreased as observed in LDLr$^{-/-}$ mice (Fig. 9B). The increase in plasma cholesterol concentrations in animals fed n-3 PUFA was entirely within the VLDL fraction, as shown in Fig. 9C, consistent with a recent report (58).

Other hepatic lipoprotein receptors include SR-BI, LRP, and the VLDLr. We measured SR-BI, LRP, and VLDLr protein levels in the livers of LDLr$^{-/-}$ mice fed control, n-6, or n-3 PUFA diets. As shown in **Fig. 10**, hepatic SR-BI levels were not affected by n-3 or n-6 PUFA. Similarly, n-3 fatty acids had no effect on hepatic LRP, although n-6 PUFA tended to decrease LRP protein. The VLDLr was expressed at very low levels in the liver and was not affected by the experimental diets. To investigate further the possibility that SR-BI might be mediating the hypocholesterolemic effects of n-3 PUFA, we measured HDL cholesteryl ester transport rates in the livers of animals fed the control or PUFA diets. HDL was isolated from LDLr$^{-/-}$ mice fed control diet and trace-labeled with [^{3}H]cholesteryl ether. Hepatic HDL cholesteryl ether clearance was not affected by dietary n-3 or n-6 fatty acids (data not shown). To further assess the potential role of LRP we determined the effect of the PUFA diets on plasma lipoprotein levels in LDLr$^{-/-}$ mice that also had liver-specific deletion of the LRP gene. As shown in **Fig. 11**, n-3 PUFA lowered the plasma concentration of IDL/LDL in LDLr$^{-/-}$ mice by >50% (Fig. 11A) and increased the hepatic clearance of IDL/LDL by 65% (Fig. 11B) even in the absence of hepatic LRP.

DISCUSSION

Dietary n-3 (20:5/22:6) PUFA markedly decreased plasma lipid levels in LDLr deficient mice when compared with n-6 (18:2) PUFA or a low fat control diet. The triglyceride-lowering effect of n-3 PUFA was apparent at relatively low dietary intakes (2–4% of calories) whereas the cholesterol-lowering effect was observed at higher dietary intakes (4–12% of calories). The decrease in plasma lipid levels in animals fed n-3 PUFA was accounted for mainly by a decrease in the plasma concentration of apoB-containing lipoproteins consistent with a previous report (59).

These studies were prompted by previous work in rats where we found that dietary n-3 PUFA accelerated LDL clearance by the liver without altering hepatic LDLr expression (36). These observations raised the possibility that hepatic LDL transport could be upregulated through a mechanism independent of the LDLr pathway. The current studies demonstrate that dietary n-3 PUFA increases hepatic IDL/LDL uptake in LDLr-deficient mice and that

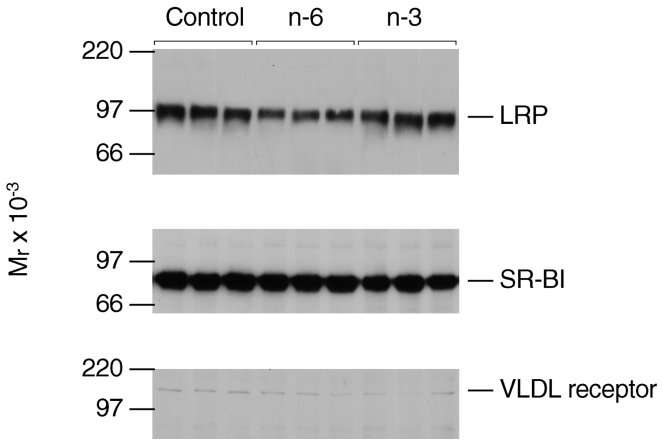

Fig. 10. Immunoblot analysis of hepatic LDLr related protein (LRP), scavenger receptor BI (SR-BI) and VLDLr in LDLr$^{-/-}$ mice. Animals were fed a semipure control diet or this same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 10% of total energy (~4% by wt).

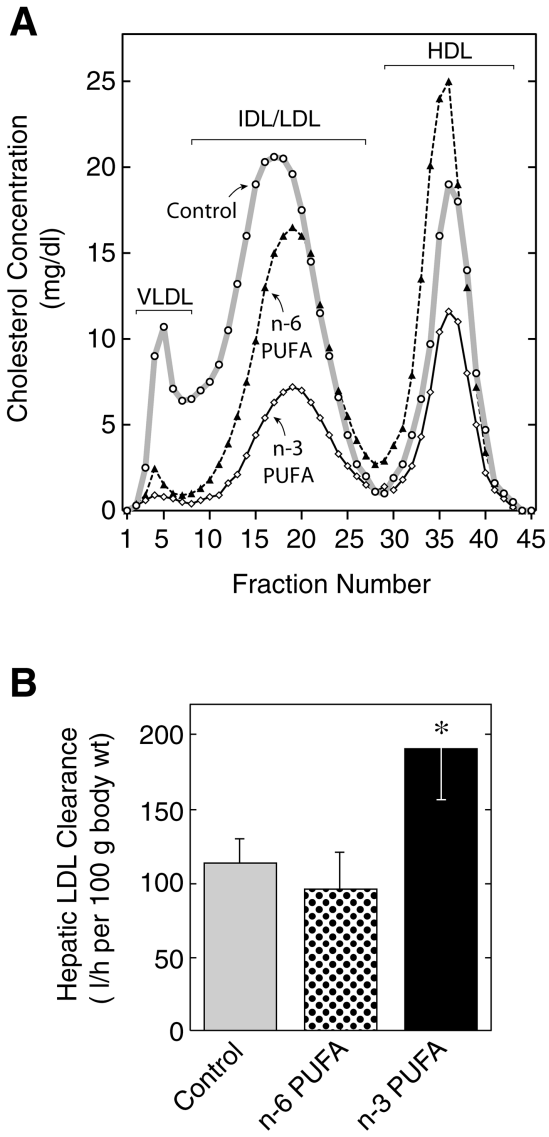

Fig. 11. Effect of dietary PUFA on plasma lipoprotein distribution and the clearance of IDL/LDL by the liver in LDLr$^{-/-}$ mice with liver-specific deletion of LRP. Animals were fed a semipure control diet or this same diet in which n-6 or n-3 PUFA replaced carbohydrate on a cal/cal basis to provide 10% of total energy (~4% by wt). *Significantly differs from the control group, $P < 0.05$.

this effect accounts for ~33% of the decrease in the plasma concentration of these particles. Exactly how n-3 PUFA increases hepatic IDL/LDL uptake in LDLr-deficient mice remains unclear. LRP may contribute to the hepatic clearance of apoB-containing lipoproteins, especially in LDLr deficient animals (60); however, hepatic LRP expression was not upregulated by dietary n-3 PUFA. Moreover, dietary n-3 PUFA increased the hepatic uptake of IDL/LDL and decreased the plasma concentration of IDL/LDL equally well in LDLr deficient mice with and without liver-specific deletion of LRP. Dietary PUFA also had no effect on the hepatic expression of the VLDLr or SR-B1 and no effect on selective cholesteryl ester uptake from HDL. Cell-surface proteoglycans play a key role in mediating remnant uptake by the liver (61). However, n-3 PUFA appears to decrease the interaction between LDL and cell-surface proteoglycans (62). Dietary n-3 PUFA had no effect on the hepatic uptake of mouse serum albumin, arguing against a nonspecific effect of n-3 PUFA on fluid phase endocytosis. The failure of dietary n-3 PUFA to increase the hepatic clearance of methylated human LDL or to decrease plasma cholesterol levels in apoE$^{-/-}$ mice suggests that apoE may mediate the enhanced clearance of IDL/LDL in LDLr$^{-/-}$ mice. Although apoE expression by the liver was not altered by dietary PUFA, plasma concentrations of apoE were markedly reduced in LDLr$^{-/-}$ mice fed n-3 PUFA. Nevertheless, hepatic clearance rates in control LDLr$^{-/-}$ mice of IDL/LDL harvested from animals fed n-3 PUFA, n-6 PUFA, or control diet were identical, suggesting that differences in the composition of the particles do not account for the enhanced hepatic clearance of IDL/LDL in LDLr$^{-/-}$ mice. Additional studies will be required to define the precise mechanism responsible for the accelerated hepatic clearance of IDL/LDL in animals fed n-3 PUFA.

Dietary n-3 PUFA also decreased the rate of IDL/LDL entry into the plasma space and this accounted for ~67% of the decrease in the plasma concentration of IDL/LDL. The decreased rate of appearance of IDL/LDL in plasma is presumably the result of the marked decrease in precursor VLDL in animals fed n-3 PUFA. Previous work suggests that multiple mechanisms may contribute to the decrease in plasma VLDL concentrations associated with n-3 PUFA ingestion. Human turnover studies (10), liver perfusion studies (11, 12), and studies in hepatocyte-derived cells in culture (13–15, 63) indicate that n-3 PUFA inhibit VLDL lipid secretion. We determined the effect of dietary PUFA on VLDL secretion after the administration of Triton to block VLDL metabolism. These studies showed a modest decrease in VLDL apoB secretion, presumably due to enhanced intracellular apoB degradation (64, 65). VLDL triglyceride secretion was also modestly reduced, whereas VLDL cholesteryl ester secretion was markedly decreased. These results suggest that n-3 PUFA modestly decreases the number of VLDL particles secreted by the liver and that these particles are relatively depleted of cholesteryl esters. Because the decrease in VLDL triglyceride secretion cannot fully account for the decrease in plasma VLDL triglyceride concentrations in animals fed n-3 PUFA, it is likely that enhanced VLDL metabolism may also contribute VLDL triglyceride lowering. We found no effect of dietary n-3 PUFA on total or hepatic lipase activity measured in post-heparin plasma (Vasandani and Spady, unpublished observation) consistent with most previous studies in humans (6, 7, 29, 66).

The decrease in VLDL lipid secretion in LDLr deficient mice fed n-3 PUFA is likely related to decreased levels of triglyceride and cholesteryl esters in the livers of animals fed n-3 PUFA. The marked decrease in hepatic triglyceride levels in LDLr deficient mice fed n-3 PUFA is consistent with a previous report in mice, although the animals in this study were fed a very high level of fish oil (60% of cal) resulting in decreased weight gain (20). Studies in rats have yielded inconsistent results with respect to the effect of n-3 PUFA on hepatic triglyceride levels with most studies showing unchanged (67) or increased (11, 68–71) levels, but others showing decreased levels (72, 73). Studies with liver-derived cells incubated with fatty acids have also yielded variable results. In some studies n-3 PUFA increased cellular triglyceride levels, suggesting a primary effect on apoB secretion or triglyceride recruitment into nascent VLDL (63, 74). In other studies, however, n-3 PUFA decreased cellular triglyceride synthesis rates and levels, suggesting that the primary effect of n-3 PUFA is to limit the amount of triglyceride available for incorporation into VLDL (75). The current studies in mice are more consistent with a mechanism in which n-3 PUFA decreases the amount of hepatic triglyceride available for incorporation into VLDL.

Decreased hepatic triglyceride levels in animals fed n-3 PUFA can be attributed in part to suppression of hepatic fatty acid synthesis. Dietary n-3 PUFA suppressed hepatic fatty acid synthesis and mRNA levels for FAS and ACC by ~40%, whereas dietary n-6 PUFA had no significant effect. More dramatic suppression of FAS and ACC mRNA levels has been reported with extreme levels of fish oil intake (20) or when PUFA is added to fat-free diets (18, 22). Under these conditions, n-6 as well as n-3 PUFA suppress lipogenesis (76) and FAS and ACC mRNA levels (18). Our control diet contained ~5% cal from corn oil to prevent essential fatty acid deficiency and this likely explains the failure of n-6 PUFA to suppress fatty acid synthesis (77). Suppression of hepatic FAS and ACC mRNA levels by n-3 PUFA could be attributed to a marked decrease in nuclear SREBP-1 consistent with previous reports in rats (18), mice (22), and cells (21, 78). Dietary n-3 PUFA modestly decreased SREBP-1 mRNA levels, presumably by accelerating its decay (26). The marked reduction in nuclear SREBP-1 levels in mice fed n-3 PUFA was only partially accounted for by decreased mRNA levels for SREBP-1, consistent with regulation at the mRNA level and at the processing level as described in 293 HEK cells (78).

Dietary n-3 PUFA greatly decreased VLDL cholesteryl ester secretion in LDLr deficient mice, an effect that can be attributed to a marked decrease in hepatic cholesteryl ester levels. Decreased hepatic cholesteryl ester levels accompanied by decreased VLDL cholesterol secretion was also reported in nonhuman primates fed fish oil com-

pared with lard (12). Studies in rat hepatocytes have shown that n-3 PUFA are poor substrates for ACAT and decrease the incorporation of other acyl-CoA substrates into cholesteryl esters (79). This mechanism may have contributed to the marked reduction in hepatic cholesteryl esters found in mice fed n-3 PUFA. However, inhibition of ACAT activity should not increase the rate of hepatic cholesterol synthesis, as was also observed in our studies. Rather, upregulation of hepatic cholesterol synthesis suggests an alteration in whole body sterol balance, such as decreased cholesterol absorption efficiency and/or increased fecal sterol excretion. Cholesterol absorption was modestly decreased in nonhuman primates fed fish oil (17% decrease) or oleic acid-enriched safflower oil (15% decrease) compared with lard (80), but only in animals consuming a high cholesterol diet.

Fish oil contains a heterogenous mixture of polyunsaturated, monounsaturated, and saturated fatty acids, as well as various sterols. While there is little doubt that the long chain n-3 PUFA, EPA, and DHA are the main biologically active components of fish oil, it is not entirely clear whether EPA, DHA, or a synergistic action of the two is responsible for the effects of fish oil on plasma lipid concentrations. We found that EPA and DHA had similar effects on plasma and liver lipid concentrations in LDLr$^{-/-}$ mice. This is consistent with our previous work in the rat (35) and with recent human trials where EPA and DHA had similar triglyceride-lowering effects (81, 82). As in the human trials, there was a tendency for plasma triglyceride to be lower and HDL-C to be higher in LDLr$^{-/-}$ mice fed DHA compared with EPA. In contrast to the current studies in the mouse and our previous work in the rat (35), several investigators failed to show any effect of dietary DHA on plasma triglyceride concentrations in the rat (67, 83, 84). We currently have no explanation for these apparently contradictory results, but they appear not to be due to differences in the amount or duration of DHA feeding.

In summary, dietary n-3 PUFA markedly decreased the concentration of triglyceride and cholesteryl esters in the liver, and the concentration of apoB-containing lipoproteins in the plasma of LDLr$^{-/-}$ mice. Multiple mechanisms appear to contribute to the lipid-lowering effects of dietary n-3 PUFA in this model including *i)* suppression of SREBP-1 expression and processing leading to decreased hepatic lipogenesis, decreased hepatic triglyceride levels, and decreased VLDL triglyceride secretion, *ii*) a marked decrease in hepatic cholesteryl ester levels and VLDL cholesteryl ester secretion, and *iii*) enhanced hepatic clearance of IDL/LDL through a mechanism that appears to involve apoE but is independent of the LDLr and LRP.

The authors thank Anh Pho and Thomas Van dinter for excellent technical assistance. The authors also thank Melissa Hyatt and Dr. Helen Hobbs for performing immunoblots for LRP, SR-BI, and the VLDL receptor. This work was supported by grants HL-38049, T32-DK07745, and HL-47551 from the National Institutes of Health.

Manuscript received 2 November 2001 and in revised form 15 February 2002.

REFERENCES

1. Kromann, N., and A. Green. 1980. Epidemiological studies in the Upernavik District, Greenland. Incidence of some chronic diseases 1950–1974. *Acta Med. Scand.* **208:** 401–406.
2. Kromhout, D., E. B. Bosschieter, and C. de Leaenne Coulander. 1985. The inverse relation between fish consumption and 20-year mortality from coronary heart disease. *N. Engl. J. Med.* **312:** 1205–1209.
3. Connor, W. E., C. A. DeFrancesco, and S. L. Connor. 1993. N-3 fatty acids from fish oil: Effects on plasma lipoproteins and hypertriglyceridemic patients. *Ann. N. Y. Acad. Sci.* **683:** 16–34.
4. Illingworth, D. R., and E. B. Schmidt. 1993. The influence of dietary n-3 fatty acids on plasma lipids and lipoproteins. *Ann. N. Y. Acad. Sci.* **676:** 60–69.
5. Harris, W. S. 1996. n-3 fatty acids and lipoproteins: comparison of results from human and animal studies. *Lipids.* **31:** 246–252.
6. Harris, W. S., W. E. Connor, N. Alam, and D. R. Illingworth. 1988. Reduction of postprandial triglyceridemia in humans by dietary n-3 fatty acids. *J. Lipid Res.* **29:** 1451–1460.
7. Weintraub, M. S., R. Zechner, A. Brown, S. Eisenberg, and J. L. Breslow. 1988. Dietary polyunsaturated fats of the ω-6 and ω-3 series reduce postprandial lipoprotein levels. *J. Clin. Invest.* **82:** 1844–1893.
8. Worne, H. E., and L. W. Smith. 1959. Effects of certain pure long chain polyunsaturated fatty acid esters on the blood lipids of man. *Am. J. Med. Sci.* **237:** 710–721.
9. Parks, J. S., and L. L. Rudel. 1990. Effect of fish oil on atherosclerosis and lipoprotein metabolism. *Atherosclerosis.* **84:** 83–94.
10. Harris, W. S. 1989. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. *J. Lipid Res.* **30:** 785–807.
11. Wong, S. H., P. J. Nestel, R. P. Trimble, G. B. Storer, R. J. Illman, and D. L. Topping. 1984. The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver. *Biochim. Biophys. Acta.* **792:** 103–109.
12. Parks, J. S., M. D. Wilson, F. L. Johnson, and L. L. Rudel. 1989. Fish oil decreases hepatic cholesteryl ester secretion but not apoB secretion in African green monkeys. *J. Lipid Res.* **30:** 1535–1544.
13. Benner, K. C., W. E. Connor, and A. Sasaki. 1985. Omega-3 fatty acid suppression of triglyceride synthesis and secretion by isolated rabbit hepatocytes. *Clin. Res.* **33:** 567A.
14. Nossen, J. O., A. C. Rustan, S. H. Gloppestad, S. Malbakken, and C. A. Drevon. 1986. Eicosapentaenoic acid inhibits synthesis and secretion of triacylglycerols by cultured rat hepatocytes. *Biochim. Biophys. Acta.* **879:** 56–65.
15. Brown, A., J. Castle, A. Hebbachi, and G. F. Gibbons. 1999. Administration of n-3 fatty acids in the diets of rats or directly to hepatocyte cultures results in different effects on hepatocellular apoB metabolism and secretion. *Arterioscler. Thromb. Vasc. Biol.* **19:** 106–114.
16. Iritani, N., K. Inoguchi, M. Endo, E. Fukuda, and M. Morita. 1980. Identification of shellfish fatty acids and their effects on lipogenic enzymes. *Biochim. Biophys. Acta.* **618:** 378–382.
17. Yang, Y-T., and M. A. Williams. 1978. Comparison of C18-, C20- and C22-unsaturated fatty acids in reducing fatty acid synthesis in isolated rat hepatocytes. *Biochim. Biophys. Acta.* **531:** 133–140.
18. Xu, J., M. T. Nakamura, H. P. Cho, and S. D. Clarke. 1999. Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fatty acids. *J. Biol. Chem.* **274:** 23577–23583.
19. Ren, B., A. P. Thelen, J. M. Peters, F. J. Gonzalez, and D. B. Jump. 1997. Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor alpha. *J. Biol. Chem.* **272:** 26827–26832.
20. Kim, H-J., M. Takahashi, and O. Ezaki. 1999. Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse Liver. A Possible mechanism for down-regulation of lipogenic enzyme mRNAs. *J. Biol. Chem.* **274:** 25892–25898.
21. Worgall, T. S., S. L. Sturley, T. Seo, T. F. Osborne, and R. J. Deckelbaum. 1998. Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J. Biol. Chem.* **273:** 25537–25540.

22. Yahagi, N., H. Shimano, A. H. Hasty, M. Amemiya-Kudo, H. Okazaki, Y. Tamura, Y. Iizuka, F. Shionoiri, K. Ohashi, J-I. Osuga, K. Harada, T. Gotoda, R. Nagai, S. Ishibashi, and N. Yamada. 1999. A crucial role of sterol regulatory element-binding protein-1 in the regulation of lipogenic gene expression by polyunsaturated fatty acids. *J. Biol. Chem.* **274:** 35840–35844.
23. Ide, T., H. Kobayashi, L. Ashakumary, I. A. Rouyer, Y. Takahashi, T. Aoyama, T. Hashimoto, and M. Mizugaki. 2000. Comparative effects of perilla and fish oils on the activity and gene expression of fatty acid oxidation enzymes in rat liver. *Biochim. Biophys. Acta.* **1485:** 23–35.
24. Jump, D. B., and S. D. Clarke. 1999. Regulation of gene expression by dietary fat. *Annu. Rev. Nutr.* **19:** 63–90.
25. Ou, J., H. Tu, B. Shan, A. Luk, R. A. DeBose-Boyd, Y. Bashmakov, J. L. Goldstein, and M. S. Brown. 2001. Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. *Proc. Natl. Acad. Sci. USA.* **98:** 6027–6032.
26. Xu, J., M. Teran-Garcia, J. H. Y. Park, M. T. Nakamura, and S. D. Clarke. 2001. Polyunsaturated fatty acids suppress hepatic sterol regulatory element-binding protein-1 expression by accelerating transcript decay. *J. Biol. Chem.* **276:** 9800–9807.
27. Dallongeville, J., E. Bauge, A. Taileux, J. M. Peters, F. J. Gonzalez, J-C. Fruchart, and B. Staels. 2001. Peroxisome proliferator activated receptorα is not rate limiting for the lipoprotein-lowering action of fish oil. *J. Biol. Chem.* **276:** 4634–4639.
28. Harris, W. S., W. E. Connor, D. R. Illingworth, D. W. Rothrock, and D. M. Foster. 1990. Effects of fish oil on VLDL triglyceride kinetics in humans. *J. Lipid Res.* **31:** 1549–1558.
29. Kasim-Karakas, S. E., R. Herrmann, and R. Almario. 1995. Effects of omega-3 fatty acids on intravascular lipolysis of very-low-density lipoproteins in humans. *Metabolism.* **44:** 1223–1230.
30. Hulsmann, W. C., M. C. Oerdemanus, and H. Jansen. 1980. Activity of heparin releasable lipase-dependence on the degree of saturation of the fatty acids in the acylglycerol substrates. *Biochim. Biophys. Acta.* **618:** 364–369.
31. Harris, W. S., B. Hustvedt, E. Hagen, M. H. Green, G. Lu, and C. A. Drevon. 1997. N-3 fatty acids and chylomicron metabolism in the rat. *J. Lipid Res.* **38:** 503–515.
32. Huff, M. W., and D. E. Telford. 1989. Dietary fish oil increases conversion of very low density lipoprotein apoprotein B to low density lipoprotein. *Arteriosclerosis.* **9:** 58–66.
33. Illingworth, D. R., W. S. Harris, and W. E. Connor. 1984. Inhibition of low density lipoprotein synthesis by dietary omega-3 fatty acids in humans. *Arteriosclerosis.* **4:** 270–275.
34. Ventura, M. A., L. A. Woollett, and D. K. Spady. 1989. Dietary fish oil stimulates hepatic low density lipoprotein transport in the rat. *J. Clin. Invest.* **84:** 528–537.
35. Spady, D. K. 1993. Regulatory effects of individual n-3 and n-6 polyunsaturated fatty acids on hepatic LDL receptor activity in the rat. *J. Lipid Res.* **34:** 1337–1346.
36. Spady, D. K., J. D. Horton, and J. A. Cuthbert. 1995. Regulation of hepatic LDL transport by n-3 polyunsaturated fatty acids in the rat and hamster. *J. Lipid Res.* **36:** 1009–1020.
37. Ishibashi, S., M. S. Brown, J. L. Goldstein, R. D. Gerard, R. E. Hammer, and J. Herz. 1993. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* **92:** 883–893.
38. Rohlmann, A., M. Gotthardt, R. E. Hammer, and J. Herz. 1998. Inducible inactivation of hepatic LRP gene by Cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *J. Clin. Invest.* **101:** 689–695.
39. Li, X., F. Catalina, S. M. Grundy, and S. Patel. 1996. Method to measure apolipoprotein B-48 and B-100 secretion rates in an individual mouse: evidence for a very rapid turnover of VLDL and preferential removal of B-48- relative to B-100-containing lipoproteins. *J. Lipid Res.* **37:** 210–220.
40. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
41. Horton, J. D., J. A. Cuthbert, and D. K. Spady. 1993. Dietary fatty acids regulate hepatic low density lipoprotein (LDL) transport by altering LDL receptor protein and mRNA levels. *J. Clin. Invest.* **92:** 743–749.
42. Glass, C. K., R. C. Pittman, G. A. Keller, and D. Steinberg. 1983. Tissue sites of degradation of apoprotein A-I in the rat. *J. Biol. Chem.* **258:** 7161–7167.
43. Mahley, R. W., K. H. Weisgraber, G. W. Melchior, T. L. Innerarity, and K. S. Holcombe. 1980. Inhibition of receptor-mediated clearance of lysine and arginine-modified lipoproteins from the plasma of rats and monkeys. *Proc. Natl. Acad. Sci. USA.* **77:** 225–229.
44. Spady, D. K., S. D. Turley, and J. M. Dietschy. 1985. Receptor-independent low density lipoprotein transport in the rat in vivo. Quantitation, characterization, and metabolic consequences. *J. Clin. Invest.* **76:** 1113–1122.
45. Spady, D. K., M. Huettinger, D. W. Bilheimer, and J. M. Dietschy. 1987. Role of receptor-independent low density lipoprotein transport in the maintenance of tissue cholesterol balance in the normal and WHHL rabbit. *J. Lipid Res.* **28:** 32–41.
46. Spady, D. K., J. A. Cuthbert, M. N. Willard, and R. S. Meidell. 1998. Overexpression of cholesterol 7α-hydroxylase (CYP7A) in mice lacking the low density lipoprotein (LDL) receptor gene: LDL transport and plasma LDL concentrations are reduced. *J. Biol. Chem.* **273:** 126–132.
47. Stein, O., G. Halperin, and Y. Stein. 1980. Biological labeling of very low density lipoproteins with cholesteryl linoleyl ether and its fate in the intact rat. *Biochim. Biophys. Acta.* **620:** 247–260.
48. Halperin, G., O. Stein, and Y. Stein. 1986. Synthesis of ether analogs of lipoprotein lipids and their biological applications. *Methods Enzymol.* **129:** 816–848.
49. Glass, C., R. C. Pittman, D. B. Weinstein, and D. Steinberg. 1983. Dissociation of tissue uptake of cholesterol ester from that of apoprotein A-I of rat plasma high density lipoprotein: selective delivery of cholesterol ester to liver, adrenal and gonad. *Proc. Natl. Acad. Sci. USA.* **80:** 5435–5439.
50. Pittman, R. C., T. P. Knecht, M. S. Rosenbaum, and C. A. Taylor, Jr. 1987. A nonendocytotic mechanism for the selective uptake of high density lipoprotein-associated cholesterol esters. *J. Biol. Chem.* **262:** 2443–2450.
51. Hough, J. L., and D. B. Zilversmit. 1984. Comparison of various methods for in vitro cholesteryl ester labeling of lipoproteins from hypercholesterolemic rabbits. *Biochim. Biophys. Acta.* **792:** 338–347.
52. Spady, D. K., L. A. Woollett, R. S. Meidell, and H. H. Hobbs. 1998. Kinetic characteristics and regulation of HDL cholesteryl ester and apolipoprotein transport in the apoA-I$^{-/-}$ mouse. *J. Lipid Res.* **39:** 1483–1492.
53. Spady, D. K., and J. A. Cuthbert. 1992. Regulation of hepatic sterol metabolism in the rat. Parallel regulation of activity and mRNA for 7α-hydroxylase but not 3-hydroxy-3-methylglutaryl-coenzyme A reductase or low density lipoprotein receptor. *J. Biol. Chem.* **267:** 5584–5591.
54. Landschulz, K. T., R. K. Pathak, A. Rigotti, M. Krieger, and H. H. Hobbs. 1996. Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. *J. Clin. Invest.* **98:** 984–995.
55. Stangl, H., G. Cao, K. L. Wyne, and H. H. Hobbs. 1998. Scavenger Receptor, class B, type I-dependent stimulation of cholesterol esterification by high density lipoproteins, low density lipoproteins, and nonlipoprotein cholesterol. *J. Biol. Chem.* **273:** 31002–31008.
56. Frykman, P. K., M. S. Brown, T. Yamamoto, J. L. Goldstein, and J. Herz. 1995. Normal plasma lipoproteins and fertility in gene-targeted mice homozygous for a disruption in the gene encoding very low density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA.* **92:** 8453–8457.
57. Shimano, H., I. Shimomura, R. E. Hammer, J. Herz, J. L. Goldstein, M. S. Brown, and J. D. Horton. 1997. Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for a targeted disruption of the SREBP-1 gene. *J. Clin. Invest.* **100:** 2115–2124.
58. Asset, G., E. Bauge, J-C. Fruchart, and J. Dallongeville. 2001. Lack of triglyceride-lowering properties of fish oil in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **21:** 401–406.
59. El-Sohemy, A., and M. C. Archer. 1999. Regulation of mevalonate synthesis in low density lipoprotein receptor knockout mice fed n-3 or n-6 polyunsaturated fatty acids. *Lipids.* **34:** 1037–1043.
60. Veniant, M. M., C. H. Zlot, R. L. Walzem, V. Pierotti, R. Driscoll, D. Dichek, J. Herz, and S. G. Young. 1998. Lipoprotein clearance mechanisms in LDL receptor-deficient "Apo-B48-only" and "Apo-B100-only" mice. *J. Clin. Invest.* **102:** 1559–1568.
61. Mahley, R. W., and Z-S. Ji. 1999. Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. *J. Lipid Res.* **40:** 1–16.
62. Manning, J., I. Edwards, W. Wagner, L. Rudel, and J. Parks. 1992. Polyunsaturated dietary fat reduces interaction of low density lipoproteins with arterial proteoglycans. *Circ.* **86 (Suppl. I):** I747.

63. Lang, C. A., and R. A. Davis. 1990. Fish oil fatty acids impair VLDL assembly and/or secretion by cultured rat hepatocytes. *J. Lipid Res.* **31:** 2079–2086.
64. Wang, H., X. Chen, and E. A. Fisher. 1993. N-3 fatty acids stimulate intracellular degradation of apoprotein B in rat hepatocytes. *J. Clin. Invest.* **91:** 1380–1389.
65. Kendrick, J. S., and J. A. Higgins. 1999. Dietary fish oils inhibit early events in the assembly of low density lipoproteins and target apoB for degradation within the rough endoplasmic reticulum of hamster hepatocytes. *J. Lipid Res.* **40:** 504–514.
66. Nozaki, S., A. Garg, G. L. Vega, and S. M. Grundy. 1991. Postheparin lipolytic activity and plasma lipoprotein response to ω-3 polyunsaturated fatty acids in patients with primary hypertriglyceridemia. *Am. J. Clin. Nutr.* **53:** 638–642.
67. Froyland, L., H. Vaagenes, D. K. Asiedu, A. Garras, O. Lie, and K. E. Berge. 1996. Chronic administration of eicosapentaenoic acid and docosahexaenoic acid as ethyl esters reduced plasma cholesterol and changed the fatty acid composition in rat blood and organs. *Lipids.* **31:** 169–178.
68. Brown, A., P. W. Baker, and G. F. Gibbons. 1997. Changes in fatty acid metabolism in rat hepatocytes in response to dietary n-3 fatty acids are associated with changes in the intracellular metabolism and secretion of apolipoprotein B-48. *J. Lipid Res.* **38:** 469–481.
69. Otto, D. A., C. E. Tsai, J. K. Baltzell, and J. T. Wooten. 1991. Apparent inhibition of hepatic triacylglycerol secretion, independent of synthesis, in high-fat fish oil-fed rats: role for insulin. *Biochim. Biophys. Acta.* **1082:** 37–48.
70. Yaqoob, P., E. J. Sherrington, N. M. Jeffery, P. Sanderson, D. J. Harvey, E. A. Newsholme, and P. C. Calder. 1995. Comparison of the effects of a range of dietary lipids upon serum and tissue lipid composition in the rat. *Int. J. Biochem. Cell Biol.* **27:** 297–310.
71. Hebbachi, A., M. C. L. Seelaender, P. W. Baker, and G. F. Gibbons. 1997. Decreased secretion of very-low-density lipoprotein triacylglycerol and apolipoprotein B is associated with decreased intracellular triacylglycerol lipolysis in hepatocytes derived from rats fed orotic acid or n-3 fatty acids. *Biochem. J.* **325:** 711–719.
72. Iritani, N., E. Fukuda, K. Inoguchi, M. Tsuboska, and S. Tashiro. 1980. Reduction of lipogenic enzymes by shellfish triglycerides in rat liver. *J. Nutr.* **110:** 1664–1670.
73. Topping, D. L., R. P. Trimble, and G. B. Storer. 1987. Failure of insulin to stimulate lipogenesis and triacylglycerol secretion in perfused liver from rats adapted to dietary fish oil. *Biochim. Biophys. Acta.* **927:** 423–428.
74. Wong, S., E. A. Fisher, and J. B. Marsh. 1989. Effects of eicosapentaenoic and docosahexaenoic acids on apoprotein B mRNA and secretion of very low density lipoprotein in HepG2 cells. *Arterioscle-rosis.* **9:** 836–841.
75. Rustan, A. C., J. O. Nossen, E. N. Christiansen, and C. A. Drevon. 1988. Eicosapentaenoic acid reduces hepatic synthesis and secretion of triacylglycerol by decreasing the activity of acyl-coenzyme A:1,2-diacylglycerol acyltransferase. *J. Lipid Res.* **29:** 1417–1426.
76. Flick, P. K., J. Chen, and P. R. Vagelos. 1977. Effect of dietary linoleate on synthesis and degradation of fatty acid synthetase from rat liver. *J. Biol. Chem.* **252:** 4242–4249.
77. Clarke, S. D., D. R. Romsos, and G. A. Leveille. 1976. Specific inhibition of hepatic fatty acid synthesis exerted by dietary linoleate and linolenate in essential fatty acid adequate rats. *Lipids.* **11:** 485–490.
78. Hannah, V. C., J. Ou, A. Luong, J. L. Goldstein, and M. S. Brown. 2000. Unsaturated fatty acids down-regulate SREBP isoforms 1a and 1c by two mechanisms in HEK-293 cells. *J. Biol. Chem.* **276:** 4365–4372.
79. Rustan, A. C., J. O. Nossen, H. Osmundsen, and C. A. Drevon. 1988. Eicosapentaenoic acid inhibits cholesterol esterification in cultured parenchymal cells and isolated microsomes from rat liver. *J. Biol. Chem.* **263:** 8126–8132.
80. Parks, J. S., and J. R. Crouse. 1992. Reduction of cholesterol absorption by dietary oleinate and fish oil in African green monkeys. *J. Lipid Res.* **33:** 559–568.
81. Grimsgaard, S., K. H. Bonaa, J. Hansen, and A. Nordoy. 1997. Highly purified eicosapentaenoic acid and docosahexaenoic acid in humans have similar triacylglycerol-lowering effects but divergent effects on serum fatty acids. *Am. J. Clin. Nutr.* **66:** 649–659.
82. Mori, T. A., V. Burke, I. B. Puddey, G. F. Watts, D. N. O'Neal, J. D. Best, and L. J. Beilin. 2000. Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoprotein, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. *Am. J. Clin. Nutr.* **71:** 1085–1094.
83. Kobatake, Y., K. Kuroda, H. Jinnouchi, E. Nishide, and S. Innami. 1984. Differential effects of dietary eicosapentaenoic and docosahexaenoic fatty acids on lowering of triglyceride and cholesterol levels in the serum of rats on hypercholesterolemic diet. *J. Nutr. Sci. Vitaminol.* **30:** 357–372.
84. Ikeda, I., K. Wakamatsu, A. Inayoshi, K. Imaizumi, M. Sugano, and K. Yazaw. 1994. α-Linolenic, eicosapentaenoic and docosahexaenoic acids affect lipid metabolism differently in rats. *J. Nutr.* **124:** 1898–1906.