Low density lipoprotein from humans supplemented with n-3 fatty acids depresses both LDL receptor activity and LDLr mRNA abundance in HepG2 cells

Saralyn Lindsey, Andrzej Pronczuk, and K. C. Hayes[1]

Foster Biomedical Research Laboratory, Brandeis University, Waltham, MA 02254

Abstract Fish oil supplementation in humans is often associated with an expanded low density lipoprotein (LDL) pool that is not thought to reflect increased production. Since data on clearance of LDL after fish oil supplementation (FO-LDL) are equivocal, normal volunteers (four men and three women) received ten capsules containing 3.6 g eicosapentaenoic acid and 2.9 g docosahexaenoic acid (approximately 2.5% total calories as methyl esters) for 2 weeks. Total plasma cholesterol was unchanged, but triglycerides decreased 30%. Low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were unchanged. Analysis of the LDL particles revealed that increased esterified cholesterol caused the FO-LDL core/surface ratio to be greater than baseline LDL (BL-LDL), resulting in a shift in mean LDL density from 1.060 to 1.056. N-3 fatty acids in FO-LDL were also increased >40% at the expense of n-6 and n-9 fatty acids. Human hepatoma HepG2 cells were used to study the effects of FO-LDL on LDL receptor activity and mRNA abundance for the LDL receptor, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and various apolipoproteins associated with cholesterol metabolism. In this system FO-LDL reduced LDL receptor activity compared to BL-LDL. Scatchard analysis revealed that LDL receptor number (B_{max}) was reduced to one-third normal ($P < 0.001$) whereas particle binding affinity was unchanged. The mRNA abundance for the LDL receptor and apoA-I were also depressed, even by low concentrations (10 μg/ml and 20 μg/ml LDL protein) of FO-LDL as compared to BL-LDL. HepG2 cells incubated with FO-LDL had decreased cellular free cholesterol but increased cholesteryl esters. Thus, moderate supplementation with fish oil n-3 fatty acids in normal humans enriches their LDL particles in cholesteryl esters and n-3 fatty acids. These particles depress both LDL receptor activity and LDL receptor mRNA abundance in HepG2 cells. —**Lindsey, S., A. Pronczuk, and K. C. Hayes.** Low density lipoprotein from humans supplemented with n-3 fatty acids depresses both LDL receptor activity and LDLr mRNA abundance in HepG2 cells. *J. Lipid Res.* 1992. **33:** 647–658.

Supplementary key words cholesterol • HMG-CoA mRNA • eicosapentaenoic acid • docosahexaenoic acid

The hypotriglyceridemic effect of fish oil supplementation both in normal subjects and in many patients with hyperlipemia has been well documented (1–5). At the same time it has been reported that fish oil increases low density lipoprotein (LDL)-cholesterol and apoB levels in some individuals (2, 6–8). This increase presumably does not reflect enhanced LDL production because in humans the n-3 fatty acids in fish oil depress triglyceride synthesis and very low density lipoprotein (VLDL) secretion (2, 7, 9) and appear to inhibit the rate of LDL-apoB production based on kinetic analysis with ^{125}I-labeled LDL (2, 10). In miniature pigs, however, fish oil (18% total calories) increased conversion of VLDL apoB to LDL apoB that was combined with reduced fractional catabolism of LDL apoB, suggesting down-regulation of hepatic LDL receptors had occurred (11). Eicosapentaenoic acid (20:5n-3) incubated directly with HepG2 cells inhibits LDL receptor-mediated uptake, but not the chylomicron remnant receptor, which is less sensitive to down-regulation (12). Further support for down-regulation of LDL receptors has been observed in hepatocyte plasma membranes isolated from rats supplemented with 8% fish oil (13). In vivo, however, the hepatocyte LDL receptor is presumably regulated by cholesterol flux or an oxysterol (14), not fatty acids; and rat hepatic cholesterol metabolism is unlike that in humans. Thus, even though current evidence suggests that the n-3 fatty acids, i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are the active agents in fish oil capable of influencing human lipid metabolism, the specific effect of fish oil supplementation on hepatic cholesterol metabolism remains elusive (15).

Abbreviations: apo, apolipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; C, cholesterol; LDLr mRNA, low density lipoprotein receptor messenger RNA; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ACAT, acyl-CoA:cholesterol acyltransferase; FO, fish oil; BL, baseline

[1]To whom correspondence should be addressed.

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At the molecular level in vitro, 20:5n-3 plus 22:6n-3 (as albumin-bound acids, 1 mM) decreased apoB mRNA in HepG2 cells when compared to 18:1n-9 (12). Whether LDL isolated from individuals consuming fish oil would also depress gene expression of apoB and the LDL receptor has not been reported, but demonstration of this point would provide more convincing evidence of the in vivo condition.

Thus, the present study measured LDL receptor activity in HepG2 cells exposed to LDL isolated from humans before and after dietary fish oil supplementation. The mRNAs encoding the LDL receptor (LDLr mRNA) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, as well as those for certain apolipoproteins involved in cholesterol metabolism, were measured. We found that supplementation of fish oil fatty acids in normal humans produced an LDL particle that depressed both hepatic LDL receptor activity and LDLr mRNA abundance which was correlated with the concentration of n-3 fatty acids in the particle.

TABLE 1. Principal fatty acids in Extra-Strength EPA 1000® capsules

Fatty Acid	(%)
14:0	0.0
16:0	1.0
18:0	2.1
16:1n-9	0.8
18:1n-9	5.5
18:2n-6	1.1
18:3n-3	0.8
18:4n-3	2.1
20:4n-6	4.3
20:5n-3	35.8[a]
22:5n-3	5.6[a]
22:6n-3	28.6[a]
Other	12.3
Saturated fatty acids	3.1
Monounsaturated fatty acids	6.3
Polyunsaturated fatty acids	78.3
n-6	5.4
n-3	72.9

[a]Sum of long chain n-3 fatty acids contributed approximately 2.5% of total dietary calories.

MATERIALS AND METHODS

Subjects and fish oil supplementation

Blood samples were obtained from seven fasting normal human subjects, four men and three women, before (baseline plasma) and after (fish oil plasma) 2 weeks of supplementation with n-3 fatty acid methyl esters (10 g Extra-Strength EPA-cholesterol free, Nature Food Centers, Wilmington, MA) representing 3.6 g of 20:5n-3 (EPA) + 2.9 g of 22:6n-3 (DHA) per day, and <1 mg cholesterol/capsule (**Table 1**). Since the response to supplementation was not gender-related, all data were pooled for analysis. The methyl ester preparation mimics the metabolic aspects of natural fish oil, while allowing for higher concentrations of n-3 fatty acids/capsule and cholesterol and saturated fat-free supplementation when compared to the natural triglycerides (16). Informed consent was obtained from the volunteers in accordance with the policies of the Human Research Committee of Brandeis University.

Preparation and labeling of lipoprotein

Blood was drawn from fasting normolipemic human subjects into EDTA-containing vacuum collection tubes (Vacutainer® Systems, Rutherford, NJ), and plasma was separated by centrifugation at 3,000 rpm at 4°C for 20 min. LDL was isolated from plasma using stock density solutions of d 1.006 g/ml and d 1.38 g/ml containing EDTA, gentamycin sulfate, and sodium azide (17) and benzamidine (18), by sequential ultracentrifugation (19) using a Ti 70.1 rotor in an L6-60 Optima ultracentrifuge (Beckman Instruments, Palo Alto, CA) at 45,000 rpm at 12°C for 24 h. After removal of VLDL and IDL, LDL was isolated from each subject before (at baseline and referred to as BL-LDL) and after fish oil supplementation (referred to as FO-LDL) at d 1.019-d 1.063 g/ml. Each isolated LDL sample was washed once at d 1.063 for 24 h at 45,000 rpm at 12°C and dialyzed at 4°C against normal saline. Protein was assayed by the method of Lowry et al. (20). To obtain VLDL, LDL, and HDL for compositional analysis, lipoproteins were separated by density-gradient ultracentrifugation using a swinging bucket rotor (SW41) with each tube stained with minimal Sudan black to visualize all lipoproteins (21). The visualized fractions were measured, and the LDL density was established from a previously generated standard curve as described by others (22). The VLDL (d < 1.006 g/ml), LDL (d 1.019-1.063 g/ml), and HDL (d 1.063-1.21 g/ml) fractions were then analyzed.

LDL was dialyzed against 1 M glycine (pH 10) for 24 h prior to iodination. LDL was labeled with $Na^{125}I$ using the iodine monochloride technique (23) as modified for lipoproteins (24). Labeled LDL was exhaustively dialyzed against normal saline to remove free iodine. The intramolecular distribution of ^{125}I was determined with a 10 μl aliquot of labeled LDL. The free iodine content was determined on the supernatant after precipitation with trichloroacetic acid (TCA) (25). The supernatant radioactivity was used as a measure of lipid radioactivity and the radioactivity of the TCA pellet gave the protein label. Essentially all (97-99%) of the radioactivity was precipitable by TCA, and 1-3% of the radioactivity was extractable in chloroform-methanol 2:1 (v/v). The final specific activities for both BL-LDL and FO-LDL were between 200 and 500 cpm/ng protein. ^{125}I-labeled LDL was filtered

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(0.45 μm, Millipore) prior to calculation of final specific activity and all LDL was used for metabolic studies within 2 weeks of preparation.

Plasma and lipoprotein analyses

Total cholesterol, HDL-cholesterol and *total triglycerides* in plasma were determined by enzymatic assays (Sigma kit, procedure 352 and 336). HDL-cholesterol was measured after sodium phosphotungstate–Mg^{2+} precipitation (26). Accuracy was calibrated in accord with the Lipid Standardization Program of the U.S. Center for Disease Control.

Alpha-tocopherol levels in plasma were determined using high performance liquid chromatography (HPLC) according to the method of Bieri, Tolliver, and Catignani (27). A 200-μl plasma sample was mixed with 100 μl of an ethanol solution of internal standard of alpha-tocopheryl acetate (Hoffmann-LaRoche Inc., Nutley, NJ) and extracted with 600 μl hexane. The hexane extract was evaporated under nitrogen and redissolved in 100 μl methanol. The methanol solution was injected into a C_{18} reverse phase HPLC column, (Supelcosil LC-18: Supelco, Inc., Bellefonte, PA) and developed with methanol–water 95:5 using 2 ml/min flow rate (Beckman Model 110A pump: Beckman Instruments Inc., Berkeley, CA). Alpha-tocopherol was detected at 290 nm in a Beckman UV detector eluting at 8.5 min.

For *lipoprotein composition*, aliquots of VLDL, LDL, HDL were assayed for protein (20) after adding 25 μl SDS (2.5%). Total cholesterol and triglyceride content of each fraction was determined by enzymatic assays (Sigma) while phospholipid (lecithin) was measured by an enzymatic assay using the Wako phospholipid B kit (Wako Pure Chemical Industries, USA, Ltd., Richmond, VA).

The *fatty acid profile* of both LDL and HepG2 cells was analyzed using the one-step transesterification method of Lepage and Roy (28). Briefly, 100 μl of LDL was heated at 100°C for 1 h in 2 ml of methanol–benzene 4:1 and 0.2 ml acetyl chloride. After neutralization with 6% H_2CO_3, an aliquot of the benzene upper layer containing the fatty acid methyl esters was analyzed on a Hewlett-Packard 5790A gas–liquid chromatograph (GLC) equipped with a flame ionization detector (Hewlett-Packard Co., Avondale, PA). A fatty acid standard mixture (Nu-Check Prep Inc., Elysian, MN) was used to identify and quantitate fatty acids by peak area integration using a Hewlett-Packard #3390A integrator.

Free cholesterol and individual *cholesteryl esters* in both LDL and HepG2 cells were determined by HPLC according to Kim and Chung (29). Each sample was treated with a solution of isopropanol and 0.75 M NaOH (33:17, by volume) followed by an extraction with n-octane after which individual cholesteryl esters were separated using Waters Radial-Pak™ Resolve™ C_{18} column eluted isocratically with acetonitrile–isopropanol (45:55, by volume) at 2.0 ml/min by using Beckman 110B solvent delivery module. The absorbance of the eluate was measured at 210 nm using a Waters (Model 480) LC spectrophotometer. Cholesterol (free and individual esters) concentrations were calculated by comparing the peak areas obtained with the sample with those obtained for the standards (Sigma Chemical Co.).

LDL apolipoprotein concentration present in delipidated LDL (30) were assessed by polyacrylamide gel electrophoresis (31) on 10% sodium dodecyl sulfate (SDS) acrylamide gels stained with Coomassie Brillian Blue. Protein was exclusively apoB-100.

LDL metabolism by cells in culture

HepG2 cells were obtained from American Type Culture Collection (Rockville, MD), and seeded into 25-cm^2 tissue culture flasks for mRNA studies and 2-cm^2 24-well tissue culture clusters (Costar, Cambridge, MA) for binding studies. Cells were cultured for 4 days (25 cm^2) or 2 days (2 cm^2) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.1 mM nonessential amino acids (Gibco). Each determination was examined in triplicate at each concentration of LDL tested.

LDL receptor activity studies. Twenty four hours prior to experiments the medium was replaced with DMEM + pooled human lipoprotein-deficient serum (LPDS) to maximize LDL receptor activity (32). After 24 h, cells were washed and then incubated with one of several concentrations of ^{125}I-labeled LDL (5–100 μg/ml protein) prepared from LDL obtained before and after fish oil supplementation from each subject. Incubations were run in the presence and absence of 500 μg/ml autologous unlabeled LDL for 4 h at 37°C after which cells were placed on ice. Degradation of LDL was calculated from the trichloroacetic acid-soluble, non-iodide radioactivity in the media at the end of this period. Bound LDL was taken to equal the radioactivity released from extensively washed cells followed by addition of heparin (10 mg/ml heparin in 50 mM NaCl/10 mM HEPES (pH 7.4) for 1 h at 4°C (30). Cells were dissolved in 0.2 N NaOH to assay cell protein content.

Determination of apoB, apoE, apoA-I, LDL receptor, and HMG-CoA reductase mRNA abundance

HepG2 cells were derepressed for 24 h with DMEM + LPDS, after which cells were washed once with DMEM and incubated for 1 h at 37°C with BL-LDL or FO-LDL at both 10 μg/ml and 20 μg/ml LDL protein. After the 1 h incubation, cells were washed twice with PBS, dissociated with 0.05% trypsin in 0.02% EDTA solution, and resuspended in 5 ml 6 M urea/3 M lithium chloride, and homogenized for total RNA isolation as previously described (33).

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TABLE 2. Plasma lipid values of human volunteers before and after supplementation with fish oil fatty acids

Criterion	Baseline	Fish Oil
	mg/dl	
Total cholesterol	174 ± 24	172 ± 24
Total triglycerides	105 ± 81	74 ± 29[a]
Total phospholipid	182 ± 23	177 ± 21
α-Tocopherol	1.04 ± 0.2	0.93 ± 0.1
α-Tocopherol/total lipids (μg/mg lipids (TC + TG + PL))	2.4 ± 0.7	2.2 ± 0.2
LDL cholesterol[b]	103 ± 20	106 ± 23
HDL cholesterol[c]	49 ± 8	51 ± 7

Values are means ± SD; n = 7.
[a]Significantly different by paired *t*-test ($P < 0.05$).
[b]Estimated after adjustment for VLDL-C according to ref. 26.
[c]From precipitation, see Methods.

Isolated RNA from baseline and fish oil phases were analyzed at the same time in increasing RNA concentrations (1.0, 2.0, and 3.0 μg) utilizing slot-blot hybridization with yeast tRNA as a negative control as previously described (33). The nitrocellulose filters were dried overnight, baked for 2 h at 80°C, and prehybridized and hybridized for 1 and 16 h, respectively. All RNA blots were hybridized to ^{32}P-labeled recombinant plasmids containing specific cDNA inserts corresponding to an individual mRNA species. Plasmid DNA was labeled by nick translation (>1 × 10^8 cpm/μg) using a labeling kit (Amersham, Arlington Heights, IL), according to the manufacturer's directions. Each nitrocellulose filter was probed consecutively with human apoA-I cDNA (courtesy J. Breslow, Rockefeller University), human apoB cDNA (courtesy V. Zannis, Boston University), human LDL receptor (clone pLDLR3, American Type Culture Collection, Rockville, MD), human 3-hydroxy-3-methylglutaryl CoA reductase (clone pHRed-102, American Type Culture Collection, Rockville, MD), and human apoE cDNA (clone pE-301, American Type Culture Collection, Rockville, MD), hybridized under 50% formamide conditions at 42°C. The filters were stripped of probe before each subsequent hybridization and exposed to X-ray film (X-OMAT-AR, Kodak Diagnostic Film, Eastman Kodak Co., Rochester, NY) to confirm the removal of the previous hybridization. After each hybridization, the filters were washed with 2 × standard saline citrate (0.3 M sodium citrate, 3 M NaCl, pH 7.0), 0.1% SDS, 1% NaPPi for 1 h, followed by a second 1-h wash with 0.2 × standard saline citrate; 0.1% SDS, 1% NaPPi. The filters were exposed to X-ray film, and the resulting autoradiographs were scanned densitometrically (E-C Apparatus Corporation, St. Petersburg, FL) to determine the relative changes in mRNA abundance with untreated cells serving as the reference control (zero effect).

Statistical analysis

Statistical analysis was performed on a Macintosh Plus computer using StatView 512+ (Brain Power Inc., Calabasas, CA) and Cricket Graph 1.2™ (Cricket Software Inc., Philadelphia, PA). Statistical differences between each phase were assessed by paired *t*-test for binding studies, while mRNA experiments were assessed for dietary treatment and LDL concentrations utilizing two-way analysis of variance (ANOVA) and Fisher's protected least significant difference between groups when deemed appropriate by ANOVA. Regression analysis was used to establish correlation coefficients between specific LDL fatty acids affected by fish oil intake and LDL high affinity binding (B_{max}) obtained from HepG2 cells before and after fish oil intake.

RESULTS

Plasma lipids and α-tocopherol

The main effect of fish oil supplementation was to depress plasma triglycerides by 30%. The plasma HDL-C, LDL-C, total cholesterol, and total phospholipids were

TABLE 3. LDL fatty acid composition before and after supplementation with fish oil fatty acids

Fatty Acids	BL-LDL	FO-LDL
	% of total	
14:0	0.5 ± 0.2	0.5 ± 0.2
16:0	16.1 ± 1	16.7 ± 1
16:1n-9	1.1 ± 0.4	1.1 ± 0.5
18:0	6.7 ± 0.9	6.9 ± 0.9
18:1n-9	16.8 ± 1	16.2 ± 2
18:2n-6	39.0 ± 2	32.0 ± 4[a]
20:3n-9	1.5 ± 0.1	1.1 ± 0.3[a]
20:4n-6	8.7 ± 1	8.3 ± 1
20:5n-3	1.1 ± 0.1	6.5 ± 2[a]
22:6n-3	1.8 ± 0.4	3.7 ± 0.8[a]
Total n-3	2.9 ± 0.5	10.3 ± 2[a]
Total n-6	49.3 ± 2	41.3 ± 4[a]
n-3/n-6	0.06 ± 0.01	0.25 ± 0.06[a]

Values are means ± SD; n = 7.
[a]Significantly different by paired *t*-test ($P < 0.05$).

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TABLE 4. Composition of lipoproteins before and after supplementation with fish oil fatty acids

Criterion	BL-LDL	FO-LDL
	% distribution	
VLDL		
Cholesterol	11.3 ± 2	14.8 ± 6
Triglyceride	45.3 ± 5	46.2 ± 5
Phospholipid	23.4 ± 2	21.5 ± 11
Protein	20.6 ± 2	17.4 ± 9
LDL		
Cholesterol	38.1 ± 2	42.4 ± 3[a]
Free cholesterol	10.2 ± 1	9.8 ± 1
Esterified cholesterol	27.9 ± 1	32.6 ± 4[a]
Triglyceride	7.6 ± 1	7.8 ± 1
Phospholipid	23.1 ± 3	22.5 ± 0.4
Protein	31.2 ± 4	27.3 ± 2[a]
HDL		
Cholesterol	13.5 ± 1	15.8 ± 1[a]
Triglyceride	3.0 ± 1	2.6 ± 1[a]
Phospholipid	30.5 ± 1	28.8 ± 2
Protein	53.1 ± 2	52.8 ± 3

Values are means ± SD; n = 7.
[a]Significantly different by paired *t*-test ($P < 0.05$).

unchanged. Because the highly polyunsaturated fatty acids in fish oil could potentially stress the vitamin E reserves, this parameter was measured and found unchanged (**Table 2**). To show that both dietary phases resulted in equal antioxidant protection, plasma α-tocopherol/total lipid components (i.e., total cholesterol + total triglyceride + total phospholipid) was compared and found unchanged.

LDL fatty acid profile

Not surprisingly, a significant enhancement in the LDL n-3 fatty acid profile was associated with fish oil intake (**Table 3**) attributable to 20:5n-3 and 22:6n-3 in conjunction with a decline in 18:2n-6. These changes were reflected in a significant increase in the n-3/n-6 ratio after fish oil supplementation.

Lipoprotein composition

Fish oil supplementation altered LDL composition (**Table 4** and **Table 5**) by significantly increasing the percentage of esterified cholesterol, while the percent free cholesterol decreased slightly. By contrast, the percentage of LDL protein was significantly decreased, while the percent cholesterol was significantly increased in FO-HDL, and HDL triglyceride decreased slightly.

The significant increase in esterified cholesterol reflected an increase in 20:5n-3 cholesteryl esters coupled with a concomitant decrease in 18:2n-6 cholesteryl esters (Table 5). Minor changes were observed in the cholesteryl palmitate pool as well as 18:1n-9 and 20:4n-6 esters. When the core (cholesteryl esters and triglycerides)-to-surface (free cholesterol, phospholipids and protein) ratio was assessed as an indicator of LDL particle size (34), FO-LDL was significantly larger ($P < 0.01$) than BL-LDL. In support of this observation, the density-gradient lipoprotein profile before and after fish oil supplementation revealed that the FO-LDL were less dense and more buoyant than BL-LDL, i.e., the BL-LDL density of 1.060 ± 0.002 g/ml shifted to 1.056 ± 0.001 g/ml ($P < 0.02$) with LDL from all individuals revealing the shift, determined from the generated standard curve. LDL α-tocopherol/LDL cholesterol ratio was assessed (35) to demonstrate that the FO-LDL particle contained essentially the same LDL tocopherol per molecule of LDL cholesterol as the BL-LDL particle.

LDL receptor binding and metabolism by HepG2 cells

As revealed by the dose-response curves, the overall catabolism of LDL by HepG2 cells was significantly de-

TABLE 5. LDL cholesterol and protein distribution before and after supplementation with fish oil fatty acids

Criterion	BL-LDL	FO-LDL
LDL esterified cholesterol (mg/mg LDL protein)	0.91 ± 0.1	1.21 ± 0.2[a]
LDL cholesteryl esters (% of total)		
16:0	10.1 ± 1	11.4 ± 1[a]
18:1n-9	15.3 ± 1	14.6 ± 2
18:2n-6	58.3 ± 2	44.6 ± 4[a]
20:4n-6	13.6 ± 1	12.6 ± 1
20:5n-3 (+22:6n-3)	2.7 ± 1	16.8 ± 3[a]
Total cholesteryl esters	73.3 ± 2	76.9 ± 3
LDL free cholesterol (%)	26.6 ± 2	23.2 ± 3
LDL core:surface ratio[b]	0.55 ± 0.05	0.68 ± 0.08[a]
LDL α-tocopherol/LDL cholesterol (molar ratio)[c]	1:106 ± 25	1:115 ± 13

Values are means ± SD.
[a]Significantly different by paired *t*-test ($P < 0.05$).
[b]Core/surface ratio assuming all triglycerides and cholesteryl ester are in the core and all protein, phospholipid, and free cholesterol are on the surface of the particles (34).
[c]Ref. 35.

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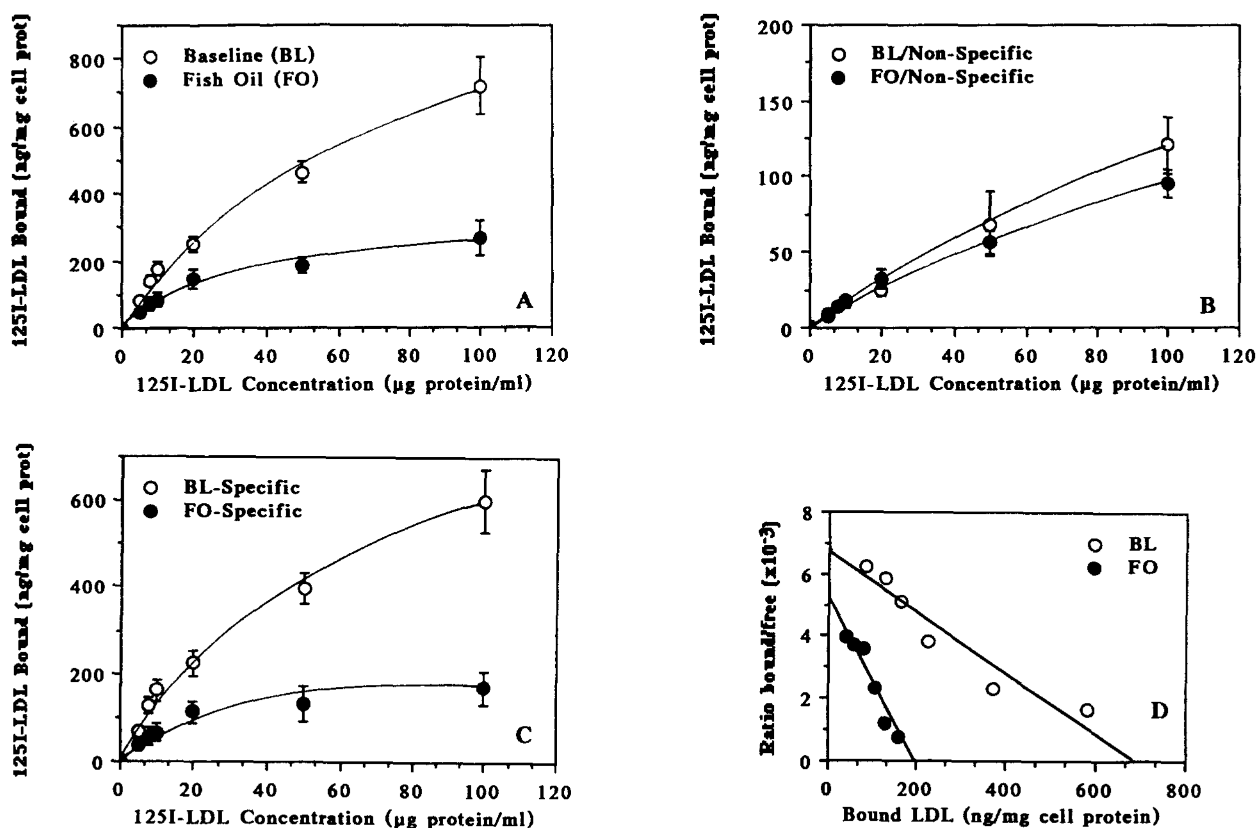

Fig. 1. HepG2 cells were incubated for 24 h in 10% LPDS. Cells were then incubated for 4 h at 37°C with the indicated concentration of ^{125}I-labeled BL-LDL or ^{125}I-labeled-FO-LDL. Specific activity was 200-500 cpm/ng protein. The total binding (A) was measured as described in Materials and Methods. Nonspecific binding (B) was determined by parallel incubations in the presence of 500 µg/ml of unlabeled LDL. Specific binding (C) was obtained by subtracting the nonspecific binding from the total binding, and Scatchard analysis was determined for high affinity binding by regressing the ratio of bound/free ^{125}I-labeled LDL. The intercept of the regression with the x-axis represents the number of receptors on the cell surface, and the slope of the curve reflects the affinity (K_D) of the receptor to BL-LDL or FO-LDL (D). Values are means ± SD from duplicates for each subject before and after fish oil supplementation.

creased after fish oil consumption (**Fig. 1**, panel A). To determine whether this reflected defective binding to receptors or a reduced number of receptors associated with metabolism of FO-LDL, the separate effect of fish oil on specific (high affinity) versus nonspecific (low affinity) LDL binding was determined. Only receptor-mediated, high affinity LDL binding (defined as the difference between total and nonspecific LDL binding) was depressed by fish oil supplementation (Fig. 1, panel C) while nonspecific binding remained unchanged (Fig. 1, panel B). In addition, the actual receptor number (B_{max}) was separated from binding affinity (K_D) by Scatchard plot analysis. Using this technique we determined that FO-LDL decreased the maximum binding capacity (B_{max}) from 698 ± 337 to 214 ± 91 ng of ^{125}I-labeled LDL apoprotein/mg of cell protein ($P < 0.001$) (Fig. 1, panel D) while the binding affinity (K_D) tended to decrease (11.7 ± 9 vs 5.1 ± 3 µg/ml for BL-LDL and FO-LDL, respectively) but the difference was not significant.

HepG2 cell fatty acid and cholesterol content

When the fatty acid composition of HepG2 cells was examined after incubating cells for 4 h with BL-LDL or FO-LDL (at 20 µg/ml), the profile from cells incubated with FO-LDL was enriched with n-3 fatty acids (**Table 6**). Specifically, although a minor component, the total 20:5n-3 and 22:6n-3 increased 173% after exposure to FO-LDL, while the relative concentrations of 18:2n-6 and 20:4n-6 were significantly reduced.

When the cell suspension was assayed for cholesterol, the total cellular cholesterol content (per mg cell protein) was reduced by FO-LDL. Although the mass of cellular free cholesterol was decreased 33% (n.s.), the esterified cholesterol doubled ($P < 0.05$). When expressed as a percent of total cholesterol, both the decrease in cellular free cholesterol and the increase in esterified cholesterol were significant (**Table 7**).

mRNA abundance in HepG2 cells

The mRNA data indicate that derepressed HepG2 cells responded to both BL-LDL and FO-LDL during a 1-h incubation (**Fig. 2**). Abundance of the various mRNAs depended on the concentration of LDL added, and the responses to the two LDL sources were often divergent and substantially different from untreated cells. The abbreviated incubation period (1 h) and the two low concen-

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TABLE 6. HepG2 cell fatty acid profile after incubation with LDL isolated from human plasma before and after supplementation with fish oil fatty acids

Fatty Acids	BL-LDL	FO-LDL
	percent	
14:0	0.9 ± 0.4	0.7 ± 0.4
16:0	19.7 ± 0.4	20.0 ± 3
16:1n-7	5.4 ± 0.9	5.6 ± 1
18:0	10.4 ± 0.9	11.4 ± 3
18:1n-9	53.7 ± 10	54.0 ± 12
18:2n-6	3.2 ± 0.3	1.9 ± 0.8[a]
20:4n-6	5.1 ± 1	3.8 ± 0.7[a]
20:5n-3	0.1 ± 0.1	0.9 ± 0.5
22:6n-3	1.4 ± 0.1	1.7 ± 0.1
Total n-3	1.5 ± 0.3	2.6 ± 0.8

Values are means ± SD.
[a]Significantly different from baseline by paired *t*-test ($P < 0.05$).

trations of LDL added (10 μg, 20 μg) were designed to evaluate concentrations of LDL leading to saturation of the LDL receptor, as opposed to the high concentration (≥50 μg/ml) and longer incubation (4 h) known to saturate the receptor and lead to bulk phase endocytosis (36).

Whereas the abundance of LDLr mRNA was considerably depressed by FO-LDL relative to either concentration of BL-LDL, the mRNA abundance for HMG-CoA reductase, a membrane-bound enzyme whose activity is inversely related to cholesterol concentration (37), was strikingly elevated by FO-LDL at 10 μg, but depressed by BL-LDL at this concentration. However, both LDL preparations caused equal depression in the reductase message at 20 μg (Fig. 2).

ApoA-I mRNA abundance reflected LDLr mRNA in that FO-LDL at both 10 μg and 20 μg depressed the apoA-I message, whereas 10 μg of BL-LDL caused a substantial increase in message abundance that was eventually depressed at 20 μg (Fig. 2).

The response in apoB mRNA was affected more by the concentration of LDL than by fish oil supplementation. Both LDL preparations increased apoB mRNA abundance at 10 μg, with FO-LDL causing the greatest increase, but both LDL decreased the message abundance at 20μg (Fig. 2).

ApoE mRNA was notable for its nonresponsiveness (Fig. 2).

Correlations

In an attempt to identify the LDL factor(s) regulating LDL receptor high affinity binding on HepG2 cells, the maximum binding capacity (B_{max}) representing the number of LDL receptors was correlated with the descriptors of BL-LDL or FO-LDL composition. In keeping with the above results, the number of LDL receptors decreased as the density of LDL particles decreased (**Fig. 3A**) and as the n-3/n-6 fatty acid ratio of LDL particles increased (Fig. 3B), clearly linking the degree of n-3 fatty acid modification of the LDL particle with the reduced number of LDL receptors on HepG2 cells.

DISCUSSION

Our objective was to determine whether the beneficial hypotriglyceridemic response commonly observed in humans fed fish oil would be associated with the potentially adverse and commonly encountered increase in the circulating LDL pool and, if so, whether the LDL expansion could be linked to decreased LDL receptor activity. In our study a modest daily increment of n-3 fatty acids, representing about 2.5% of total energy, decreased the plasma triglyceride concentration without changing the size of either the total or LDL circulating pools of cholesterol. However, this intake of fish oil fatty acids did cause the FO-LDL to reduce receptor-dependent LDL uptake by HepG2 cells without affecting receptor-independent uptake. The observed down-regulation in LDL receptor activity by FO-LDL particles suggests that the LDL particle itself (as opposed to a related serum factor or a non-fatty acid component in fish oil) modulates this activity.

Data from rats fed fish oil are equivocal concerning the effect on LDL receptor activity. When 20% fish oil was added to rat chow, LDL receptor activity was reportedly increased (38) whereas 8% fish oil mixed with chow de-

TABLE 7. HepG2 cell free and esterified cholesterol content after 4 h incubation with LDL isolated from human plasma before and after supplementation with fish oil fatty acids

	BL-LDL	FO-LDL
	μg/mg HepG2 cell protein[a]	
Total cellular cholesterol	185 ± 59	157 ± 37
Free	160 ± 64 (85 ± 9)[c]	107 ± 37 (67 ± 7)[bc]
Esterified	25 ± 11 (15 ± 9)[c]	50 ± 4[b] (33 ± 7)[bc]

Values are means ± SD.
[a]Values calculated per mg HepG2 cell protein after 4 h incubation with 20 μg/ml LDL protein.
[b]Significantly different from baseline by paired *t*-test ($P < 0.05$).
[c]Values in parentheses represent % distribution.

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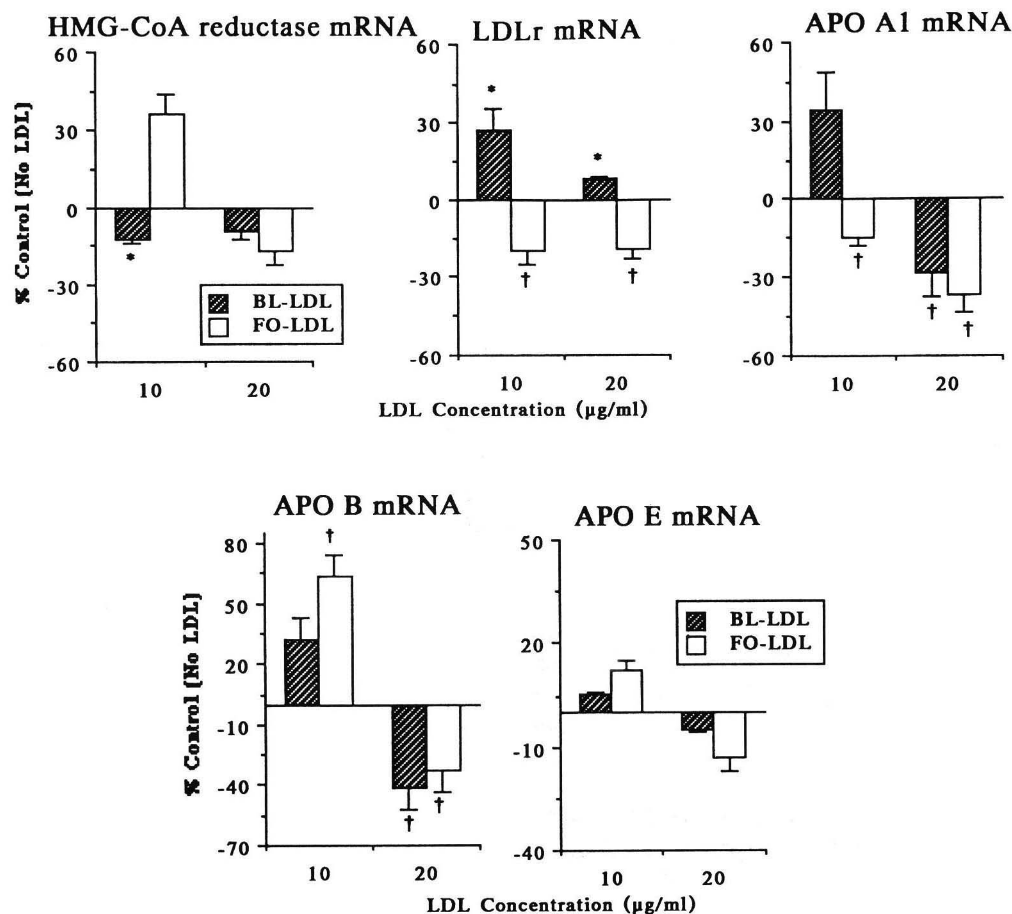

Fig. 2. HepG2 cells were incubated for 24 h in 10% LPDS and then received either 10 µg/ml or 20 µg/ml BL-LDL or FO-LDL for 1 h at 37°C. mRNA was isolated and probed as indicated in Materials and Methods. Values are means of triplicate flasks ± SD. Significant differences between BL-LDL and FO-LDL (*) or between either LDL and cells without LDL added (†) are indicated.

creased hepatic LDL receptor activity as measured by colloidal gold-labeled LDL binding to hepatic membranes (13). Even though both rat diets lowered plasma cholesterol and triglycerides, it seems likely that an extraordinary intake of fish oil (e.g., 40% total energy) might elicit a different response than a more modest fish oil consumption.

Modulation of LDL receptor activity

The decrease in LDL catabolism by FO-LDL could result from intracellular events that down-regulated receptors or from poor particle binding. If the latter prevailed, reduced binding affinity (K_D) would occur, but it did not. Rather a decreased number of receptors was measured by Scatchard analysis. Since larger LDL (e.g., FO-LDL) tend to metabolize faster than small LDL (e.g., BL-LDL) and several pieces of evidence (HepG2 total lipid and fatty acid profiles, mRNA abundance fluctuations, LDL receptor number) indicate FO-LDL particles were, indeed, metabolized by HepG2, we would argue that minimal metabolism of FO-LDL was sufficient to induce down-regulation of LDL receptors.

The assumption that the LDL receptor was down-regulated from within as a consequence of FO-LDL metabolism is difficult to explain in traditional terms because, despite the lower intracellular free cholesterol content following FO-LDL, the number of high-affinity LDL receptors was reduced. Furthermore, the relative increase in cholesteryl esters in HepG2 cells suggests that FO-LDL either delivered more cholesteryl esters, enhanced cholesterol esterification, or depressed cholesteryl ester removal from the cells. Although FO-LDL were modestly enriched with cholesteryl esters, the total mass of cholesterol in HepG2 cells was reduced after incubation with FO-LDL. This might reflect increased ACAT activity, which has been documented in isolated liver microsomes from rats and rabbits fed fish oil (14% or 10% by weight, respectively) (39, 40). Enhanced ACAT activity was related to fatty acid unsaturation because dietary n−3 fatty acids increased ACAT activity when compared to

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cocoa butter, while in another study dietary n-6 fatty acids increased ACAT activity when compared to coconut oil (41). This suggests that the polyunsaturation of LDL, and not specifically fish oil fatty acids, was the stimulant for ACAT. However, in our case the total percentages (but not necessarily the mass) of unsaturated fatty acids in BL-LDL and FO-LDL were essentially equal.

Livers from hamsters fed a 10% fish oil diet contained the same concentrations of free and esterified cholesterol as hamsters fed corn oil or olive oil, even though higher cholesterol esterification rates were associated with fish oil (42). In contrast to our findings with human LDL, the LDL from perfused livers of African green monkeys fed fish oil (as 20% total energy plus 0.76 mg chol/kcal) for 2.5-3 years were smaller than LDL from lard-fed monkeys, attributed to a decreased rate of cholesteryl ester secretion by livers from fish oil-fed monkeys. Under those conditions it was hypothesized that cholesterol esterification was less efficient with n-3 fatty acids (43). By contrast, our human FO-LDL appeared to be enlarged. These divergent results fail to clarify the impact of fish oil on hepatic cholesterol metabolism. Differences in fatty acid metabolism between species, the amount of n-3 fatty acids fed, the concentration of dietary cholesterol, or the duration of study may all be important variables affecting the inconsistent response.

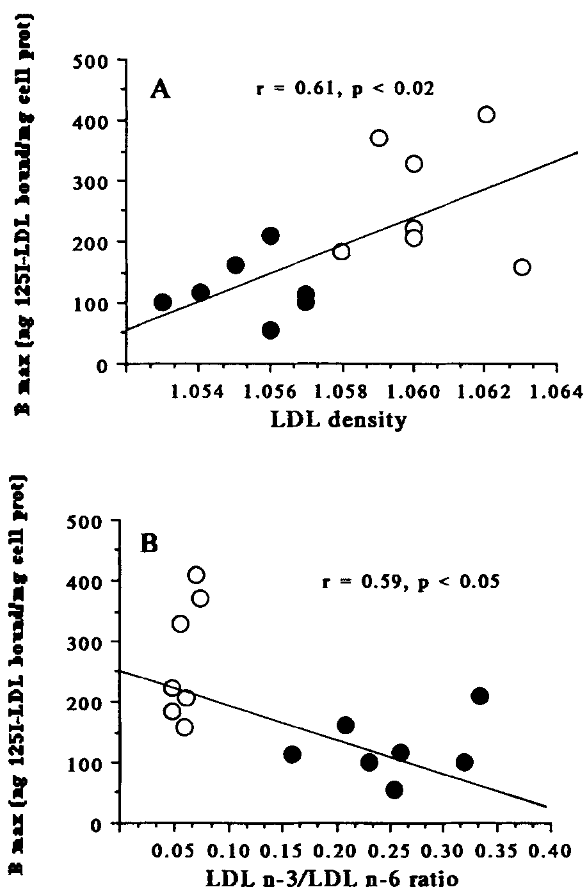

Fig. 3. The high affinity binding on HepG2 cells (B_{max}) was directly correlated with the LDL density (A) and inversely with the n-3/n-6 fatty acid content of each LDL (B): before (open circle) after (closed circle) fish oil supplementation.

Dietary modification of the fatty acid composition of biological membranes has been postulated to alter receptor activity by modulating the biophysical characteristics of membranes (44). In support of this concept, human mononuclear cell membranes were more fluid following incubation of these cells with linoleate or oleate, than with stearate. Linoleate and oleate also increased the rate of LDL uptake, leading the authors to suggest that LDL clearance is enhanced as unsaturated fatty acids are incorporated into membrane phospholipids (45). On this basis, our observation that fish oil decreased LDL receptor activity would suggest that fish oil fatty acids decreased HepG2 cell membrane fluidity, which is opposite to the effect actually observed when 22:6n-3 is incorporated in membranes (46). The possibility that specific decreases in 18:2n-6 or 20:4n-6 reduced the number of LDL receptors cannot be excluded by our results, but such an explanation is not consistent with any known model of receptor regulation.

LDL particle composition

Fish oil supplementation altered the composition of LDL, increasing the cholesteryl esters by 17% without changing the triglyceride content while decreasing the protein to lipid ratio. When we compared ratios for all core (TG, CE) to surface (FC, protein, PL) components, the FO-LDL particles had relatively more core lipid ($P < 0.01$) than BL-LDL particles.

LDL particles from rhesus monkeys fed a high level of dietary cholesterol also were less dense and down-regulated the LDL receptors on smooth muscle cells in culture (47). An increased cholesteryl ester content of those LDL also increased particle size. However, the rhesus plasma cholesterol increased 4- to 5-fold, unlike the present study where plasma total cholesterol was unchanged, and cholesteryl esters represented a much greater percentage (53%) of the rhesus LDL (vs 33% herein) while protein was decreased 20%. Our data reveal a much smaller shift in LDL composition (17% increase in esterified cholesterol and 13% decrease in protein due to fish oil) without a change in apoE (data not shown). To determine whether differences in LDL cholesterol mass or uptake of specific fatty acids incorporated in LDL lipids could explain the FO-LDL effect on HepG2 cells, mRNA abundance for several key proteins was assessed at two low concentrations of LDL.

mRNA abundance studies

If reduced LDL catabolism associated with FO-LDL was due to defective binding and uptake, metabolism of

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the HepG2 cells should be minimally perturbed at progressively lower concentrations of FO-LDL. To test this and/or the possible sequence of regulatory events contributing to decreased LDL receptor activity by FO-LDL at 50 μg/ml LDL protein, we monitored changes in mRNA abundance during an abbreviated incubation (1 h) of HepG2 cells with two low concentrations of LDL (10 μg/ml and 20 μg/ml LDL protein). This manipulation modulated mRNA abundance for the LDL receptor, HMG-CoA reductase, and two of three apolipoproteins involved in hepatic control of cholesterol metabolism. Furthermore, the impact of FO-LDL was in contrast to BL-LDL. Whereas BL-LDL actually increased the mRNA abundance for the LDL receptor at 10 μg LDL, both low concentrations of FO-LDL depressed this message about 20% in concert with a 60% depression in LDL receptor activity. As such, these data are original in their demonstration that LDL isolated from humans supplemented with n-3 fatty acids exerts the coordinated depression of both LDLr mRNA abundance and receptor activity. We would further argue that this concurs with a model of down-regulation of LDL receptors by internal events as opposed to a failed processing of defective (poor ligand binding) FO-LDL particles.

To our knowledge the FO-LDL data also describe the first inverse association between the mRNA abundance for HMG-CoA reductase and LDLr mRNA, which are generally thought to be regulated in the same direction if not to the same degree (48). In the current study we chose a 1-h incubation with low LDL concentrations to measure the dynamics of message induction, a situation not previously evaluated. Moreover, BL-LDL and FO-LDL induced opposite mRNA abundance patterns for these two messages. Thus, while HMG-CoA reductase mRNA abundance was elevated and LDLr abundance was depressed by FO-LDL at 10 μg/ml, this level of BL-LDL depressed HMG-CoA reductase message while elevating LDLr mRNA. These contrasting profiles clearly demonstrate that even minimal processing of FO-LDL (10 μg/ml) decreased LDLr mRNA and receptor activity more effectively than twice the mass (20 μg/ml) of BL-LDL. At the same time, the HMG-CoA reductase message was increased, putatively attempting to maintain cellular cholesterol via synthesis.

The suppressive impact of minimal FO-LDL suggests that the fatty acid composition of LDL lipids, rather than the lipid mass per se (e.g., mass cholesterol delivered), was the more important regulatory factor. In fact, three observations suggest that the regulation of the LDL receptor by n-3 fatty acids was more potent than that exerted by the free cholesterol pool. First, EPA and DHA added directly to HepG2 cells also down-regulated the LDL receptor (12). Second, the decrease in free cholesterol in HepG2 cells exposed to FO-LDL would be expected to increase synthesis and up-regulate LDL receptors (36). Synthesis appeared to increase (HMG-CoA mRNA) but LDLr mRNA abundance and receptor activity were depressed, which suggests that the decrease in cellular free cholesterol was unable to override the negative impact of n-3 fatty acids on LDL receptor number. If a regulatory pool of free cholesterol within the cell was modulating receptor activity, it should have increased receptor message and protein activity along with the observed increase in HMG-CoA message to restore the free cholesterol pool depleted by n-3 fatty acids. The inhibition by n-3 fatty acids would appear to be at the transcriptional level because receptor message abundance was depressed. In summary, the minimal mass of FO-LDL needed to regulate the LDL receptor suggests that n-3 fatty acids enhanced ACAT activity and cholesterol synthesis, even as the n-3 fatty acids present were reducing LDLr mRNA abundance and receptor number.

The mRNA abundance for the LDLr and apoA-I were modulated in concert and opposite to the HMG-CoA reductase message. Similar to LDLr mRNA, apoA-I mRNA abundance was reduced at both concentrations of FO-LDL, but only at 20 μg/ml of BL-LDL. The coordinated response between LDLr mRNA and apoA-I mRNA abundance has been noted on several occasions in hamsters (33, 49, 50). This relationship seems more than circumstantial and suggests that hepatic synthesis of apoA-I (HDL) and the LDL receptor may be linked metabolically. For instance, a high intake of cholesterol in several animal species, e.g., rabbit (51), cynomolgus monkey (52), hamster (53) increases the circulating LDL pool (while down-regulating the LDL receptor), a situation not unlike obesity in humans (54). In both metabolic circumstances the plasma HDL pool is decreased, supporting the above argument. If this association were real, an agent that increases LDL receptor activity might be expected to increase HDL production and the circulating mass of HDL. Both estrogen (55) and cholestyramine (56) therapy are noteworthy examples that support this scenario. In rats given ethinyl estradiol for 5 days, an increase of hepatic LDL receptor number was associated with an increase in both mRNA abundance and synthesis (57). On the other hand, fish oil-fed hamsters experience marked depression in circulating HDL in conjunction with lower hepatic mRNA abundance for both apoA-I and the LDLr (49).

Whereas LDLr mRNA abundance was decreased by both concentrations of FO-LDL, the mRNA abundance for apoB was selectively decreased only at 20 μg/ml FO-LDL and BL-LDL. At 10 μg/ml concentration, apoB mRNA abundance was significantly increased by both LDL preparations. In another study with HepG2 cells (58), apoB mRNA abundance was similar when either EPA (20:5n-3) or DHA (22:6n-3) was supplied at concentrations 30-fold higher than those contributed by our FO-LDL. Since a low concentration of LDL (independent of

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fish oil) was able to increase apoB mRNA abundance in derepressed cells (to a greater degree than any other message, in fact), the possibility exists that the apoB message is more sensitive to modulation than previously thought.

Taken together, these results demonstrate that modest supplementation of n-3 fatty acids in fish oil in normal humans produces a more buoyant LDL particle enriched with n-3 fatty acids primarily as cholesteryl esters. These LDL particles depress both hepatic LDLr mRNA abundance and LDL receptor activity in HepG2 cells, apparently related to their n-3 fatty acid content rather than mass lipid transport. Since fish oil consumption in humans depresses secretion of VLDL triglyceride (2, 9) and apoB (2) as well as LDL apoB production (10), our data support the notion that the often observed increase in LDL cholesterol and apoB reflects a concomitant decrease in LDL receptor activity.

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