Regulatory effects of n-3 polyunsaturated fatty acids on hepatic LDL uptake in the hamster and rat

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Abstract These studies were undertaken to investigate the mechanisms involved in the regulation of hepatic low density lipoprotein (LDL) transport by n-3 fatty acids in the hamster and rat. Animals were fed n-3 or n-6 fatty acids with a cholesterol-free, very-low-fat semisynthetic diet, or with a diet enriched with cholesterol and saturated fat. Although the enrichment of liver lipids with dietary n-3 fatty acids was similar in hamsters and rats, the effect of n-3 fatty acids on hepatic LDL transport differed in the two species. In the hamster, n-3 fatty acids had no effect on hepatic receptor-dependent LDL uptake in animals fed a cholesterol-free, very-low-fat diet and suppressed receptor-dependent transport in animals fed a diet enriched with cholesterol and saturated triglyceride. In hamsters fed n-3 fatty acids, changes in receptor-dependent LDL transport were accompanied by parallel changes in LDL receptor mRNA, indicating regulation of the receptor at the pretranslational level. In the rat, on the other hand, dietary n-3 fatty acids enhanced hepatic receptor-dependent LDL uptake by nearly twofold regardless of the background diet; however, hepatic LDL receptor protein and mRNA were unchanged. Dietary n-3 fatty acids did not enhance hepatic chylomicron remnant clearance in the rat. These studies confirm marked species differences in response to n-3 fatty acids and suggest that n-3 fatty acids accelerate hepatic receptor-dependent LDL transport in the rat by altering the distribution or recycling of LDL receptors or via effects on a different receptor pathway.—**Spady, D. K., J. D. Horton, and J. A. Cuthbert.** Regulatory effects of n-3 polyunsaturated fatty acids on hepatic LDL uptake in the hamster and rat. *J. Lipid Res.* 1995. **36:** 1009-1020.

Supplementary key words LDL receptor • LDL receptor mRNA • VLDL • LDL receptor-related protein/α_2-macroglobulin receptor

Epidemiological evidence suggesting a low incidence of coronary heart disease in populations consuming diets rich in marine lipids (1, 2) has created considerable interest in the use of these lipids in the prevention and treatment of atherosclerotic complications (3-7). Marine lipids are unique in their content of long-chain n-3 polyunsaturated fatty acids. These fatty acids have widespread biologic activities including effects on platelet function, inflammation, and plasma lipids. Concerning the effects of n-3 fatty acids on plasma lipids, dietary fish oil reduces plasma triglyceride levels in normal and hypertriglyceridemic individuals whereas vegetable oils containing predominantly n-6 fatty acids have little activity (8-10). The effects of dietary fish oil on plasma cholesterol concentrations are less consistent. Fish oil generally does not lower plasma cholesterol when taken as a supplement to a Western diet and frequently raises plasma cholesterol levels in hypertriglyceridemic individuals (10-13). On the other hand, total and LDL-cholesterol levels do fall when fish oil is substituted for saturated fat in the diet, and it appears that the n-3 fatty acids present in fish oil are at least as effective in this regard (on a g/g basis) as the n-6 fatty acids present in vegetable oils (8, 14, 15).

Response to marine lipids varies remarkably among different animal species. In the rat, dietary fish oil consistently lowers plasma cholesterol and triglyceride concentrations. Although the cholesterol content of all lipoprotein fractions declines, the greatest reductions occur in lipoproteins that contain apolipoprotein B-100 or apolipoprotein E (16). The fall in plasma LDL concentrations in rats fed fish oil is due primarily to an increase in receptor-dependent LDL uptake by the liver (16). How n-3 fatty acids enhance LDL uptake by the liver has not been established. In normal animals, ~90% of LDL uptake by the liver is receptor dependent (17). However, ligand binding studies have shown no effect of dietary fish oil on the binding of LDL to rat liver membranes suggesting an alternative mechanism for enhanced clearance of LDL (18). The response to dietary fish oil is quite variable in other species. Indeed, dietary fish oil has been reported to raise

Abbreviations: LDL, low density lipoprotein(s); VLDL, very low density lipoprotein(s); HDL, high density lipoprotein(s); LRP/α_2M, low density lipoprotein receptor-related protein/α_2-macroglobulin receptor; PUFA, polyunsaturated fatty acid.

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plasma triglyceride and cholesterol concentrations in several species commonly used to study sterol metabolism including the hamster (19, 20) and cynomolgus monkey (21). The present studies were undertaken to characterize the response to n-3 fatty acids in the rat and hamster and to investigate the mechanisms responsible for the n-3 fatty acid-induced changes in receptor-dependent LDL transport in the liver.

METHODS

Animals and diets

Sprague-Dawley rats and Golden Syrian hamsters (Sasco, Inc., Omaha, NE) were housed in colony cages and subjected to light cycling for at least 3 weeks prior to introduction of the experimental diets. The control semisynthetic diet used in most studies contained (wt/wt) 20% soy protein, 60.5% corn starch, 2% corn oil, 0.3% DL-methionine, 8.5% salt mix, 1% vitamin mix, 0.2% choline bitartrate, and 7.5% cellulose. The experimental diets were prepared by isocalorically replacing corn starch in the semisynthetic control diet with safflower or fish oil ethyl esters. The n-3 fatty acid concentrate used in these studies was obtained through the National Institutes of Health Fish Oil Test Materials Program. The concentrate contained 79% n-3 fatty acids as the ethyl esters. In some experiments, the effects of the n-3 and n-6 fatty acids were examined in animals fed a diet enriched with cholesterol and triglyceride. This diet was prepared by isocalorically replacing corn starch in the semisynthetic control diet with coconut oil to provide 24% of calories and including cholesterol at the 0.08% level (~200 mg/1000 kcal). All diets were supplemented with vitamin E (0.05%) and TBHQ (0.02%). Diets containing polyunsaturated fatty acids were stored under nitrogen in the dark at −20°C. The various diets were fed ad lib on a daily basis for 1 month and all studies were carried out during the mid-dark phase of the light cycle.

Determination of hepatic LDL uptake rates in vivo

Plasma was obtained from normocholesterolemic human donors and from hamsters and rats fed standard rodent diet (Teklab). The LDL was isolated from plasma by preparative ultracentrifugation in the density range of 1.020 to 1.050 g/ml and labeled with [^{125}I]- or [^{131}I]tyramine cellobiose as previously described (22). In previous studies, LDL from control and fish oil-fed rats was found to be transported at similar rates in control animals (16). The human LDL was also reductively methylated to eliminate completely its recognition by the LDL receptor (23). Rates of hepatic LDL uptake were measured using primed-infusions of [^{125}I]tyramine cellobiose-labeled LDL (16, 17). The infusions of [^{125}I]tyramine cellobiose-labeled LDL were continued for 4 h at which time each animal was administered a bolus of [^{131}I]tyramine cellobiose-labeled LDL as a volume marker and killed 10 min later by exsanguination through the abdominal aorta. Tissue samples and aliquots of plasma were assayed for radioactivity in a gamma 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 4 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 cholesterol or LDL protein with time represents the rate of LDL uptake in micrograms of LDL cholesterol or LDL protein taken up per h per g of tissue. As previously described, changes in the level of receptor-dependent LDL transport (defined as the rate of receptor-dependent LDL uptake in an experimental animal relative to the rate of receptor-dependent LDL uptake seen in control animals at the same plasma LDL concentration) were determined by relating the rates of LDL transport in experimental animals to the kinetic curves describing the relationship between hepatic LDL uptake and plasma LDL concentrations in normal animals (16, 17).

Determination of hepatic chylomicron remnant clearance rates

Chylomicrons were labeled in vivo with [^{3}H]retinol and were collected from the mesenteric lymphatic duct of rats as previously described (24). Chylomicron remnants were prepared by injecting chylomicrons into functionally hepatectomized, eviscerated rats (24). The radiolabeled chylomicrons were allowed to circulate for 2 h, at which time the rats were exsanguinated through the abdominal aorta. The chylomicron remnants were isolated from plasma by ultracentrifugation at 22,000 rpm in a SW 25.1 swinging bucket rotor. Rates of hepatic chylomicron remnant clearance were measured using primed-infusions of [^{3}H]retinol-labeled chylomicron remnants. Groups of animals were killed by exsanguination through the abdominal aorta at 5 min or 4 h. Samples of liver and plasma were saponified, neutralized, solubilized, and assayed for radioactivity (with quench correction) as described (25). The tissue space achieved by the labeled chylomicron remnants at 5 min and at 4 h was calculated by dividing the radioactivity in 1 g of liver by the steady-state concentration of radioactivity in the plasma. The increase in the tissue space of labeled chylomicron remnants during the 4 h experimental period equals the rate of chylomicron remnant movement into the liver and is expressed as the μl of plasma cleared of chylomicron remnants per h per g of liver or per whole liver.

Determination of LDL receptor protein levels

Liver membrane proteins were solubilized essentially as described by Schneider et al. (26). Solubilized liver membrane proteins were separated by polyacrylamide gel electrophoresis and transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA) as previously described (27). After electrophoretic transfer, membranes were blocked with 5% nonfat dried milk and incubated with a 1:200 dilution of LDL receptor antiserum. Polyclonal, monospecific antiserum was prepared in New Zealand White rabbits against a synthetic peptide corresponding to the C-terminal 13 amino acids of the rat LDL receptor. After incubation with the primary antibody, the PVDF membrane was incubated with 0.5 μCi/ml ^{125}I-labeled donkey anti-rabbit antibody (Amersham, Arlington Heights, IL). The radioactivity in each band, as well as background radioactivity, was quantified using an isotopic imaging system (Ambis, Inc., San Diego, CA).

Determination of mRNA levels

Hepatic LDL receptor, low density lipoprotein receptor-related protein/α_2-macroglobulin (LRP/α_2M) receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an invariant control) mRNA levels were determined by nuclease protection as previously described (27, 28). A cDNA probe was not available for the rat LRP/α_2M receptor. Therefore, after reverse transcriptase synthesis of the complementary DNA, the polymerase chain reaction (PCR) was used to amplify a fragment of the LRP/α_2M receptor cDNA from rat liver RNA. Oligonucleotide primers used to amplify a 241 nt LRP cDNA fragment (5'-AAAGGATCCCTACTTCTGC GAAGGCATTTGG-3', and 5'-AAAGAATTCCCAGCT TCAAACATCACTCGGTA-3') were selected from areas of 100% homology in the published mouse and human LRP/α_2M receptor sequences (29). The PCR was carried out sequentially for 5 min at 55°C, 2 min at 72°C, and 45 sec at 95°C for 30 cycles in a programmable thermal controller (MJ Research, Inc., Watertown, MA). The oligonucleotide primers were synthesized with restriction sites (*Bam*HI and *Eco*RI) to allow direct subcloning of the amplified DNA into the plasmid pGEM (Promega Corp., Madison, WI) for sequencing and into the bacteriophage M13 (Pharmacia LKB Biotech, Piscataway, NJ) for the preparation of ^{32}P-labeled single-stranded probes. The cDNA probes for rat LDL receptor (186 nt), rat GAPDH (447 nt), hamster LDL receptor (402 nt), and hamster GAPDH (204 nt) were previously described (27, 28, 30). Probes were synthesized as previously described (27) using 0.5 μM [^{32}P]dCTP and 1 μM (LDL receptor) or 300 μM (LRP/α_2M receptor and GAPDH) unlabeled dCTP.

Samples of liver were homogenized in guanidinium thiocyanate and the RNA was isolated by the method of Chomczynski and Sacchi (31). Total RNA (40 μg) was hybridized with the ^{32}P-labeled cDNA probes simultaneously at 48°C overnight. Unhybridized probe, present in excess relative to the amount of specific mRNA, was then digested with 40 units of mung bean nuclease (GIBCO BRL/Life Technologies, Gaithersburg, MD). The mRNA-protected ^{32}P-labeled probes were separated on 7 M urea, 6% 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 an isotopic imaging system (Ambis). The level of GAPDH mRNA did not vary among the various experimental groups and was used to correct for any procedural losses.

Determination of liver and plasma cholesterol distribution

Liver cholesterol was quantified by capillary gas–liquid chromatography. The cholesterol distribution in plasma was determined by simultaneously centrifuging plasma at densities of 1.020, 1.055 and 1.095 g/ml. The cholesterol content of the top one-third of each tube was determined by gas chromatography. Total plasma cholesterol and triglyceride were assayed using enzymatic kits (Boehringer Diagnostics, Indianapolis, IN).

RESULTS

Preliminary dose–response experiments were carried out to determine the effect of the n-3 fatty acid concentrate on plasma triglyceride and cholesterol concentrations in the hamster and rat. Animals were fed a cholesterol-free, very-low-fat semisynthetic diet or the same diet in which the n-3 fatty acid concentrate was substituted for corn starch (on a cal/cal basis) to provide 3%, 12%, or 24% of total calories. As shown in **Fig. 1** (top panel), n-3 fatty acids had no significant effect on plasma triglyceride levels in the hamster. In rats, on the other hand, n-3 fatty acids reduced plasma triglyceride concentrations by 60% and 67% in animals ingesting the n-3 concentrate at the 12% and 24% levels, respectively. As shown in the bottom panel, n-3 fatty acids lowered plasma cholesterol concentrations by 15% and 20% in hamsters fed the n-3 fatty acid concentrate at the 12% and 24%, respectively. The cholesterol-lowering effect of n-3 fatty acids was greater in the rat with reductions in plasma cholesterol of 44% and 53% in animals fed the n-3 fatty acid concentrate at the 12% and 24% levels, respectively. Weight gain did not differ significantly among the various groups and all further studies were performed using diets in which the n-3 concentrate provided 12% of total energy. For comparative purposes, animals were also fed diets containing 12% of total energy as safflower oil ethyl esters (predominantly n-6 fatty acids).

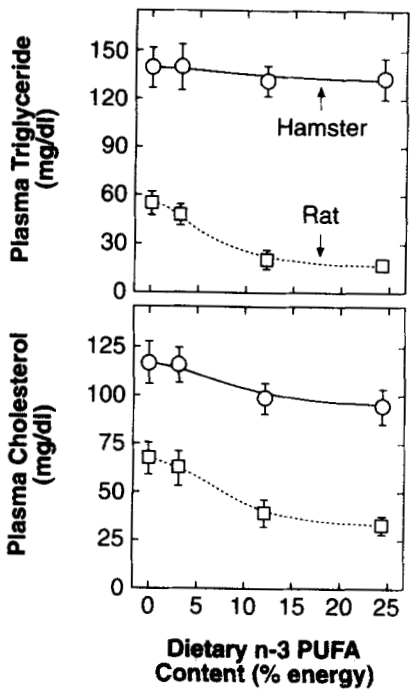

Fig. 1. Dose-response relationship between plasma triglyceride and cholesterol concentrations and the content of n-3 PUFAs in the diet. Animals were fed a cholesterol-free, very-low-fat semisynthetic diet or the same diet in which an n-3 PUFA concentrate replaced carbohydrate on a cal/cal basis. Each value represents the mean ± 1 SD for data obtained in six animals.

The fatty acid distribution of liver lipids is shown in **Table 1.** In both species the fatty acid distribution in liver lipids shifted in the direction of the fed fatty acids. In control hamsters, n-3 fatty acids accounted for 8.6% of the fatty acids present in liver lipids; this value increased to 31% in hamsters fed the n-3 fatty acid concentrate. Similarly, the content of n-3 fatty acids in liver lipids increased from 6% in control rats to 34% in rats fed the n-3 fatty acid concentrate.

The effect of n-3 fatty acids on the distribution of cholesterol in plasma in animals fed a cholesterol-free semisynthetic diet is shown in **Table 2.** In the hamster, n-3 fatty acids reduced total plasma cholesterol levels by 29%; however, this reduction in total plasma cholesterol was confined entirely to the d 1.055–1.095 g/ml and d > 1.095 g/ml fractions. Hamster plasma contains relatively little apoE-containing HDL_1 so that the fall in HDL cholesterol occurred largely in apoA-I-containing HDL_2 and HDL_3. In the rat, on the other hand, the n-3 concentrate reduced total plasma cholesterol by 45% with the greatest relative reductions occurring in the d < 1.020 g/ml (83% reduction), d 1.020–1.055 g/ml (50% reduction) and d 1.055–1.095 g/ml (62% reduction) fractions. In the rat, much of the cholesterol carried in the d 1.055–1.095 g/ml fraction is present in apoE-containing HDL_1 particles. Thus, although n-3 PUFAs reduced the cholesterol content of all density fractions, the greatest relative reductions were in lipoproteins containing apoB-100 or apoE. In contrast to n-3 fatty acids, n-6 fatty acids had little effect on total plasma cholesterol concentrations or on the distribution of cholesterol in plasma when fed at the 12% level in either the hamster or the rat.

To examine the effect of n-3 fatty acids under dietary conditions more relevant to Western humans, experiments were carried out using diets enriched with cholesterol and saturated triglyceride. Western diets typically contain 100–300 mg cholesterol/1000 kcal and provide ~40% of calories as triglyceride. For these studies, animals were fed a control semisynthetic diet containing 200 mg/1000 kcal cholesterol, 24% of total energy as saturated triglyceride (coconut oil) and 5% of total energy as unsaturated triglyceride (corn oil). The experimental diets were prepared by substituting n-3 or n-6 fatty acids for starch on a cal/cal basis to provide 12% of total energy. The effect of n-3 PUFAs on plasma cholesterol levels in animals fed a high fat diet is shown in **Table 3.** In hamsters fed a high-fat diet, n-3 fatty acids significantly increased cholesterol carried in the d < 1.020 and d 1.020–1.055 g/ml fractions and greatly reduced cholesterol in the d 1.055–1.095 and d > 1.095 g/ml density fractions. In the rat, n-3 PUFAs reduced total plasma cholesterol

TABLE 1. Effect of n-3 and n-6 fatty acids on fatty acid profile of liver lipids

	Fatty Acid							
Diet	16:0	18:0	18:1	18:2	20:4	20:5 n-3	22:5 n-3	22:6 n-3
	wt %							
Hamster								
Control	20 ± 1	20 ± 1	14 ± 2	20 ± 3	13 ± 1	0.3 ± 0.1	0.3 ± 0.1	8 ± 1
n-6 PUFA	19 ± 1	20 ± 2	12 ± 3	23 ± 3	13 ± 2	0.2 ± 0.1	0.3 ± 0.1	9 ± 1
n-3 PUFA	19 ± 1	22 ± 1	6 ± 1	11 ± 2	6 ± 1	7 ± 1	2.9 ± 0.5	21 ± 1
Rat								
Control	20 ± 1	23 ± 1	12 ± 0.8	12 ± 1	23 ± 1	0.2 ± 0.1	0.2 ± 0.1	6 ± 1
n-6 PUFA	15 ± 1	21 ± 2	7 ± 0.7	22 ± 3	25 ± 3	0.2 ± 0.1	0.2 ± 0.1	6 ± 1
n-3 PUFA	15 ± 1	18 ± 1	7 ± 0.4	13 ± 1	9 ± 1	12 ± 0.5	2.0 ± 0.1	20 ± 1

Each value represents the mean ± 1 SD for data obtained in six animals.

TABLE 2. Effect of n-3 and n-6 fatty acids on liver and plasma cholesterol concentrations in animals fed a cholesterol-free semisynthetic diet

Diet	Liver Cholesterol	Plasma Cholesterol Concentration				
			Density Fraction (g/ml)			
		Total	<1.020	1.020–1.055	1.055–1.095	>1.095
	mg/g		*mg/dl*			
Hamster						
Control	2.6 ± 0.3	112 ± 13	10 ± 1	31 ± 3	53 ± 6	23 ± 2
n-6 PUFA	3.0 ± 0.2	109 ± 11	10 ± 1	27 ± 3	55 ± 8	22 ± 3
n-3 PUFA	2.7 ± 0.3	79 ± 10[a]	9 ± 1	32 ± 2	21 ± 3[a]	17 ± 2[a]
Rat						
Control	2.2 ± 0.2	64 ± 6	6 ± 1	10 ± 1	13 ± 2	35 ± 5
n-6 PUFA	2.5 ± 0.2	61 ± 7	5 ± 1	10 ± 1	12 ± 2	34 ± 5
n-3 PUFA	2.3 ± 0.3	35 ± 4[a]	1 ± 1[a]	5 ± 1[a]	5 ± 1[a]	24 ± 3[a]

Each value represents the mean ± 1 SD for data obtained in six animals.
[a]Significantly different from the corresponding control group at $P < 0.05$.

levels (77 to 48 mg/dl) and, again, the greatest relative reductions were seen in the d < 1.095 g/ml density fractions. In animals fed the high-fat diet, as in animals fed the low-fat diet, n-6 fatty acids had little effect on the concentration or distribution of cholesterol in plasma.

Studies were next undertaken to determine the effect of n-3 PUFAs on LDL transport in the hamster and rat. Rates of total and receptor-independent LDL uptake were measured in vivo using homologous and methylated human LDL, respectively. Receptor-dependent LDL uptake was taken as the difference between total and receptor-independent uptake. As receptor-dependent LDL transport is saturable, and as plasma LDL concentrations varied greatly among the different experimental groups, changes in absolute rates of LDL uptake could not be equated directly with changes in receptor activity. To quantify changes in receptor activity in vivo, absolute rates of LDL uptake in the experimental animals were related to standard kinetic curves describing the relationship between LDL uptake and circulating LDL concentrations in control hamsters and rats.

Figure 2 shows the kinetic curves for hepatic LDL transport in control hamsters. The shaded areas represent the relationship between total (stippled) and receptor independent (hatched) LDL uptake and plasma LDL concentrations over the range of LDL levels observed in these experiments. These standard kinetic curves were previously established by quantifying rates of total and receptor-independent LDL uptake in control animals under conditions where plasma LDL concentrations were acutely raised and maintained at various levels by infusions of unlabeled LDL (17). The mean values for total

TABLE 3. Effect of dietary n-3 and n-6 fatty acids on liver and plasma cholesterol concentrations in animals fed a diet enriched with cholesterol and saturated triglyceride

Diet	Liver Cholesterol	Plasma Cholesterol Concentration				
			Density Fraction (g/ml)			
		Total	<1.020	1.020–1.055	1.055–1.095	>1.095
	mg/g		*mg/dl*			
Hamster						
Control	5.3 ± 0.4	232 ± 28	56 ± 9	88 ± 10	61 ± 9	36 ± 5
n-6 PUFA	7.2 ± 0.5[a]	221 ± 18	51 ± 11	83 ± 8	63 ± 8	34 ± 4
n-3 PUFA	5.5 ± 0.4	304 ± 41[a]	98 ± 14[a]	159 ± 22[a]	32 ± 4[a]	26 ± 3[a]
Rat						
Control	2.5 ± 0.3	77 ± 8	5 ± 1	17 ± 3	19 ± 3	27 ± 4
n-6 PUFA	2.7 ± 0.2	75 ± 9	5 ± 1	16 ± 1	20 ± 3	24 ± 4
n-3 PUFA	2.4 ± 0.3	48 ± 5[a]	2 ± 1[a]	9 ± 2[a]	11 ± 2[a]	15 ± 3[a]

Each value represents the mean ± 1 SD for data obtained in six animals.
[a]Significantly different from the corresponding control group at $P < 0.05$.

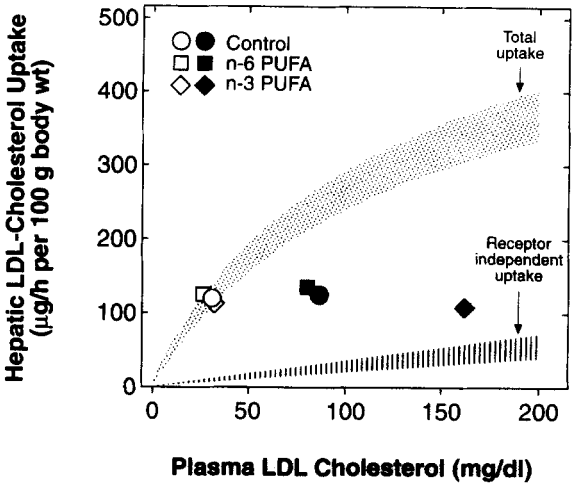

Fig. 2. Effect of dietary n-3 PUFAs on LDL uptake in the hamster. Control hamsters were fed a cholesterol-free, very-low-fat semisynthetic diet (open symbols) or a semisynthetic diet enriched with cholesterol and saturated triglyceride (solid symbols). Experimental diets were prepared by replacing carbohydrate in the control diets with n-3 or n-6 PUFAs (on a cal/cal basis) to provide 12% of total energy. The shaded areas represent the kinetic curves for total (stippled) and receptor-independent (hatched) LDL-cholesterol uptake determined in control hamsters as described in Methods. Superimposed on these normal kinetic curves are the absolute rates of total LDL-cholesterol uptake in the experimental animals plotted as a function of the plasma LDL-cholesterol concentration in the same animals.

hepatic LDL uptake in hamsters fed n-6 and n-3 PUFAs are superimposed on these normal kinetic curves. Rates of receptor-independent LDL transport were normal in all experimental groups and are not shown. In animals fed the cholesterol-free, very-low-fat control diet (open circle), rates of total (117 μg/h) and receptor-independent (8 μg/h) LDL uptake fell on the kinetic curves for LDL transport in normal hamsters, and receptor-dependent transport in these animals was assigned a value of 100%. Under these conditions, rates of total and receptor-independent LDL uptake in the liver were not significantly displaced from the standard kinetic curves by n-3 (open diamond) or n-6 (open square) PUFAs indicating no change in the receptor-dependent or -independent transport processes. In hamsters fed a diet enriched with cholesterol and saturated triglyceride (solid circle), rates of total hepatic LDL uptake equalled 124 μg/h at a plasma LDL-cholesterol concentration of 88 mg/dl whereas normal animals would transport ~243 μg/h at this plasma LDL concentration. Because receptor-independent LDL uptake in these animals was normal, the decrease in total LDL uptake could be attributed entirely to suppression of receptor-dependent LDL transport. Moreover, in hamsters fed the high fat diet, n-3 fatty acids further reduced total LDL uptake by the liver (relative to normal animals with the same plasma LDL concentration) to a level only modestly exceeding the rate of receptor-independent transport (solid diamond) whereas n-6 fatty acids (solid square) had little effect.

Figure 3 shows absolute rates of LDL transport in rats fed n-3 PUFAs. In animals fed the cholesterol-free, very-low-fat control diet, rates of total and receptor-independent LDL transport were not significantly displaced from the normal kinetic curves by n-6 PUFAs (open square). In animals fed n-3 PUFAs, the rate of total LDL uptake (open diamond) equalled 34 μg/h at a plasma LDL-cholesterol concentration of 5 mg/dl whereas normal animals transport ~18 μg/h per 100 g at this plasma LDL concentration. Receptor-independent LDL uptake was normal in these animals indicating up-regulation of the receptor-dependent pathway. In contrast to hamsters, rats fed the high-fat diet (solid circle) manifested rates of total and receptor-independent LDL transport that fell on the normal kinetic curves for hepatic LDL uptake indicating no effect of this diet on receptor-dependent transport. When superimposed on the high-fat diet, n-3 PUFAs significantly increased total LDL transport relative to control rats at the same plasma LDL-cholesterol concentration (solid diamond) whereas n-6 PUFAs (solid square) had no significant effect. Thus, in the rat n-3 PUFAs produced similar changes in hepatic LDL transport whether provided in the context of a cholesterol-free, very-low-fat diet or a diet enriched with cholesterol and saturated triglyceride.

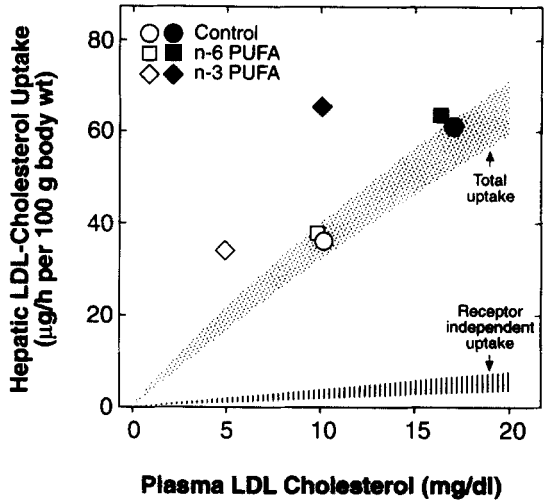

Fig. 3. Effect of dietary n-3 PUFAs on LDL uptake in the rat. Control rats were fed a cholesterol-free, very-low-fat semisynthetic diet (open symbols) or a semisynthetic diet enriched with cholesterol and saturated triglyceride (solid symbols). Experimental diets were prepared by replacing carbohydrate in the control diets with n-3 or n-6 PUFAs (on a cal/cal basis) to provide 12% of total energy. The shaded areas represent the kinetic curves for total (stippled) and receptor-independent (hatched) LDL-cholesterol uptake determined in control rats as described in Methods. Superimposed on these normal kinetic curves are the absolute rates of total LDL-cholesterol uptake in the experimental animals plotted as a function of the plasma LDL-cholesterol concentration in the same animals.

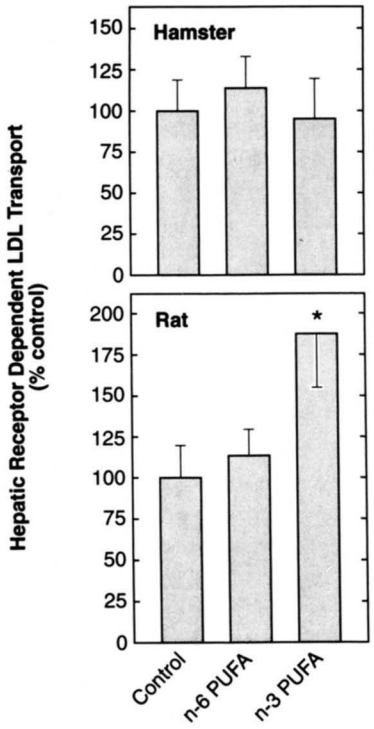

Fig. 4. Effect of dietary n-3 PUFAs on hepatic receptor dependent LDL transport in animals fed a low-fat diet. Hamsters and rats were fed a cholesterol-free, very-low-fat semisynthetic diet or the same diet in which n-3 or n-6 PUFAs replaced carbohydrate on a cal/cal basis. Changes in whole liver receptor-dependent LDL transport were determined as illustrated in Figs. 3 and 4. Each value represents the mean ± 1 SD for data obtained in 12 animals. *Significantly differs from the corresponding control group, $P < 0.05$.

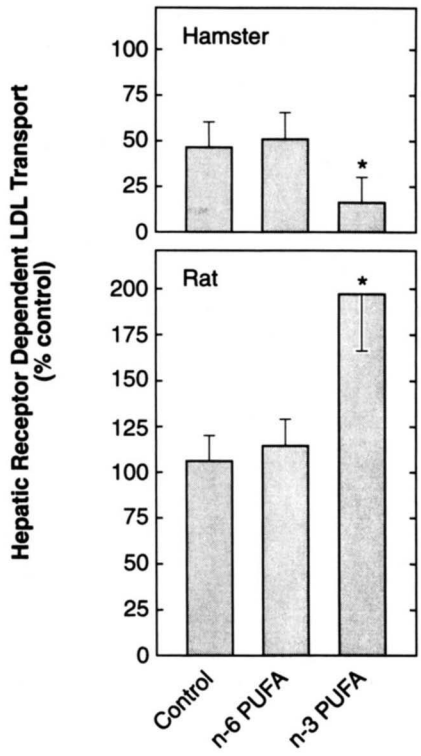

Fig. 5. Effect of dietary n-3 PUFAs on hepatic receptor-dependent LDL transport in animals fed a high-fat diet. Hamsters and rats were fed a semisynthetic diet enriched with cholesterol and saturated triglyceride or the same diet in which n-3 or n-6 PUFAs replaced carbohydrate on a cal/cal basis. Changes in whole liver receptor-dependent LDL transport were determined as illustrated in Figs. 3 and 4. Each value represents the mean ± 1 SD for data obtained in 12 animals. *Significantly differs from the corresponding control group, $P < 0.05$.

From the type of analysis shown in Figs. 2 and 3, changes in the level of receptor-dependent LDL transport (the rate of receptor-dependent LDL uptake in experimental animals relative to the rate of receptor-dependent LDL uptake in control animals at the same plasma LDL-cholesterol concentration) were calculated for each of the experimental groups and these values are summarized in Figs. 4 and 5. **Figure 4** shows the effect on hepatic receptor-dependent LDL transport of dietary PUFAs when fed in the context of a cholesterol-free, very-low-fat diet. As seen in the top panel, n-3 fatty acids had no effect on receptor-dependent LDL transport in the hamster. In contrast, n-3 fatty acids increased hepatic receptor-dependent LDL uptake in the rat by 87% (bottom panel). As also shown in Fig. 4, n-6 fatty acids had no significant effect on hepatic receptor-dependent LDL uptake in either the hamster or rat when fed at the 12% energy level. Thus, in animals fed a cholesterol-free, very-low-fat diet, n-3 fatty acids accelerated receptor-dependent LDL uptake by the liver and markedly lowered the plasma concentration of LDL (and other lipoproteins containing apoB and/or apoE) in the rat but had no effect on these processes in the hamster.

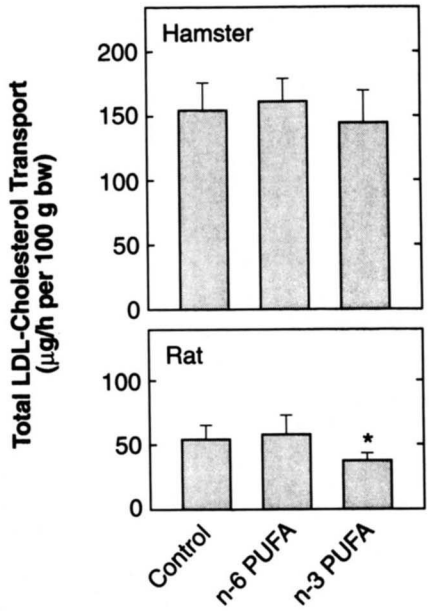

Fig. 6. Effect of dietary n-3 PUFAs on total LDL-cholesterol formation (transport) in animals fed a low-fat diet. Hamsters and rats were fed a cholesterol-free, very-low-fat semisynthetic diet or the same diet in which n-3 or n-6 PUFAs replaced carbohydrate on a cal/cal basis. Each value represents the mean ± 1 SD for data obtained in 12 animals. *Significantly differs from the corresponding control group, $P < 0.05$.

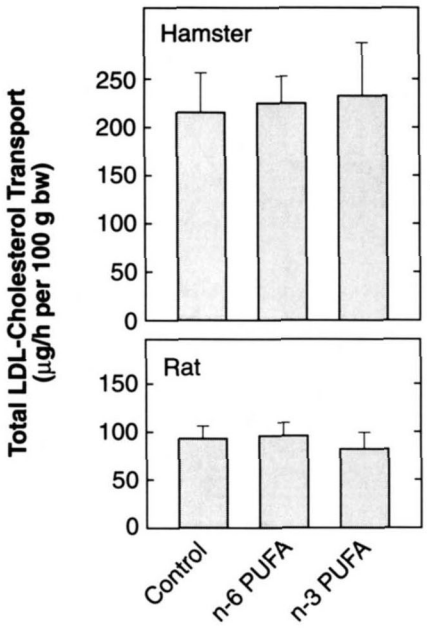

Fig. 7. Effect of dietary n-3 PUFAs on total LDL-cholesterol transport in animals fed a high-fat diet. Hamsters and rats were fed a semisynthetic diet enriched with cholesterol and saturated triglyceride or the same diet in which n-3 or n-6 PUFAs replaced carbohydrate on a cal/cal basis. Each value represents the mean ± 1 SD for data obtained in 12 animals.

Figure 5 shows the effects of n-3 fatty acids, when fed with a diet enriched with cholesterol and saturated triglyceride, on hepatic receptor-dependent LDL transport in the hamster and rat. As shown in the top panel, rates of hepatic receptor-dependent LDL transport were significantly suppressed in hamsters fed a diet enriched with cholesterol and saturated triglyceride and under these conditions, n-3 fatty acids further suppressed the rate of receptor-dependent LDL uptake. In contrast, a diet enriched with cholesterol and saturated triglyceride did not suppress hepatic receptor-dependent LDL transport in the rat (Fig. 5, bottom panel). Moreover, in the rat, n-3 fatty acids increased the rate of hepatic receptor-dependent LDL uptake as effectively in animals fed a diet enriched with cholesterol and saturated triglyceride as in animals consuming a cholesterol-free, very-low-fat diet. Thus, whereas dietary n-3 fatty acids increased the rate of hepatic receptor-dependent LDL uptake in the rat regardless of the background diet, dietary n-3 fatty acids had no effect on hepatic receptor-dependent LDL uptake in hamsters fed a cholesterol-free, very-low-fat diet and significantly suppressed receptor-dependent uptake in this species when superimposed on a diet containing cholesterol and saturated triglyceride.

The concentration of LDL in plasma is determined by the rate at which LDL is formed in plasma relative to the rate at which LDL is cleared from plasma, primarily by

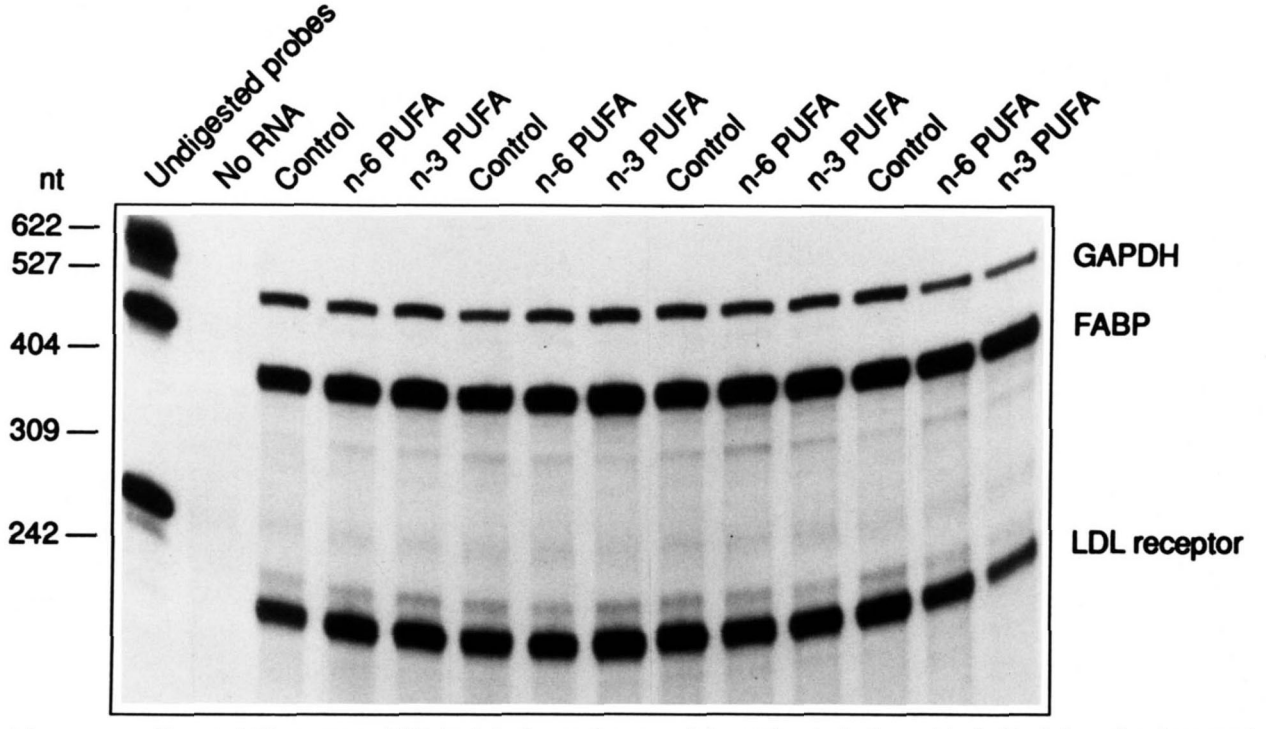

Fig. 8. Measurement of hepatic LDL receptor mRNA levels in the rat. Rats were fed a semisynthetic diet enriched with cholesterol and saturated triglyceride or the same diet in which n-3 or n-6 PUFAs replaced carbohydrate on a cal/cal basis. Total RNA (40 μg) was hybridized with ^{32}P-labeled single-stranded cDNA probes and the protected bands resistant to mung bean nuclease digestion were analyzed by polyacrylamide gel electrophoresis followed by autoradiography. nt, nucleotides; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FABP, fatty acid binding protein.

receptor-dependent uptake into the liver. The effect of n-3 PUFAs on the rate of LDL formation (total LDL-cholesterol transport) in animals fed the low and high fat diets is shown in Figs. 6 and 7, respectively. As shown in **Fig. 6,** n-3 PUFAs modestly reduced the rate of LDL formation in rats (bottom panel) but not in hamsters (top panel) fed the cholesterol-free, very-low-fat diet. As shown in **Fig. 7,** total LDL-cholesterol transport increased in both species when placed on a high-fat diet. In the context of a high-fat diet, n-3 PUFAs had no significant effect on total LDL transport in the hamster (top panel) or rat (bottom panel). Thus, in both species, n-3 PUFA-induced changes in circulating LDL levels were due predominantly to changes in receptor-dependent LDL uptake by the liver.

In the hamster, hepatic LDL receptor activity is modestly suppressed by dietary cholesterol, an effect that was greatly augmented by coconut oil and partially prevented by unsaturated vegetable oils. Under these conditions, changes in hepatic LDL receptor activity are accompanied by parallel changes in LDL receptor mRNA indicating that dietary fatty acids modulate the LDL receptor pathway at the pretranslational level (27). To determine whether a similar mechanism accounts for the changes in hepatic LDL receptor activity induced by n-3 fatty acids, changes in receptor-dependent LDL transport were correlated with changes in LDL receptor mRNA in hamsters and rats fed the same diets as those shown in Fig. 5.

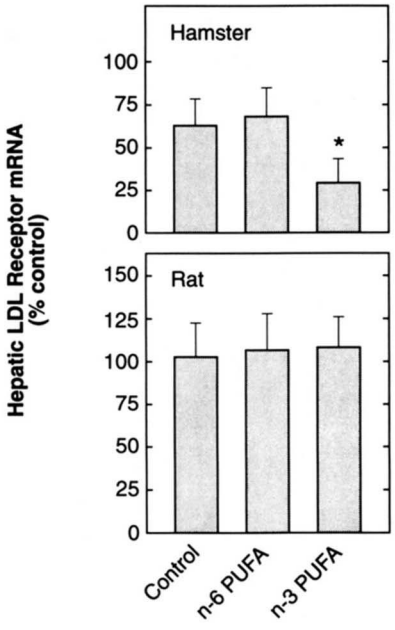

Fig. 9. Effect of dietary n-3 PUFAs on hepatic LDL receptor mRNA in animals fed a high-fat diet. Hamsters and rats were fed a semisynthetic diet enriched with cholesterol and saturated triglyceride or the same diet in which n-3 or n-6 PUFAs replaced carbohydrate on a cal/cal basis. Each value represents the mean ± 1 SD for data obtained in 12 animals. *Significantly differs from the corresponding control group, $P < 0.05$.

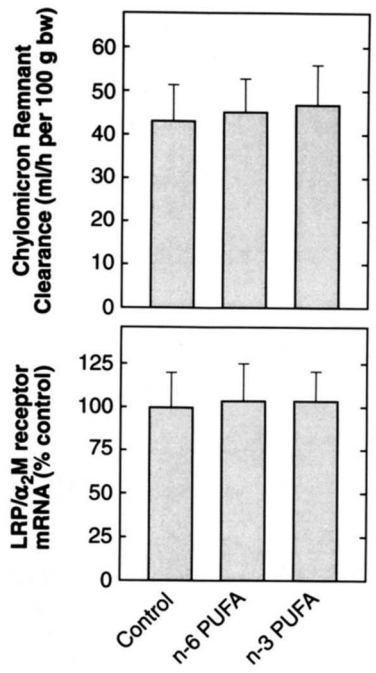

Fig. 10. Effect of dietary n-3 PUFAs on hepatic chylomicron remnant clearance and LRP mRNA. Rats were fed a cholesterol-free, very-low-fat semisynthetic diet or the same diet in which n-3 or n-6 PUFAs replaced carbohydrate on a cal/cal basis. Each value represents the mean ± 1 SD for data obtained in 12 animals. *Significantly differs from the corresponding control group, $P < 0.05$.

Hepatic LDL receptor mRNA levels were quantified by nuclease protection using species-specific cDNA probes. An autoradiogram showing the effect of n-6 and n-3 fatty acids on hepatic LDL receptor mRNA in the rat is shown in **Fig. 8** and the mean changes in LDL receptor mRNA levels are summarized in **Fig. 9.** In the hamster, the n-3 fatty acid-induced suppression of hepatic receptor-dependent LDL transport was accompanied by parallel changes in LDL receptor mRNA levels (top panel). In the rat, on the other hand, hepatic LDL receptor mRNA levels remained unchanged under conditions in which receptor-dependent LDL transport increased by nearly twofold (bottom panel). Hepatic LDL receptor protein was measured in these same animals by immunoblotting and, like LDL receptor mRNA, LDL receptor protein was not altered in rats fed the n-3 fatty acids (data not shown). Administration of 5 mg/kg body wt 17α-ethinylestradiol subcutaneously to rats for 4 days produced the expected marked increase in hepatic LDL transport with parallel increases in hepatic LDL receptor mRNA and protein (data not shown). Thus, in terms of receptor-dependent LDL uptake by the liver, not only was the response to n-3 fatty acids qualitatively different in the hamster and rat but so was the mechanism of this response.

Because the n-3 fatty acid-induced changes in hepatic receptor-dependent LDL transport in the rat could not be explained by changes in LDL receptor mRNA or protein,

additional studies were carried out in an attempt to understand the mechanisms involved in the regulation of hepatic LDL transport by n-3 PUFAs in this species. An increase in receptor-dependent LDL uptake in the absence of any change in LDL receptor expression suggests the possibility of enhanced LDL uptake via an alternate receptor pathway or an alteration in the cycling or cellular distribution of LDL receptors. To investigate the possibility of enhanced clearance through an alternative lipoprotein receptor pathway, experiments were undertaken to determine whether n-3 fatty acids alter the hepatic clearance of chylomicron remnants. In addition, the effect of n-3 PUFAs on the expression of the hepatic LRP/α_2M receptor was determined as this receptor appears to play an important role in chylomicron remnant uptake into the liver (32). Chylomicron remnant uptake by the liver was determined in rats fed n-3 or n-6 fatty acids using radiolabeled rat chylomicron remnants that were prepared in vivo. In control rats, the liver cleared chylomicron remnants ~40-fold more rapidly than LDL. As shown in **Fig. 10,** top panel, dietary n-3 and n-6 fatty acids had no significant effect on the hepatic clearance of chylomicron remnants under conditions in which receptor-dependent LDL uptake by the liver was increased nearly twofold. Similarly, dietary n-3 fatty acids had no effect on the level of LRP/α_2M receptor mRNA in the liver (Fig. 10, bottom panel).

DISCUSSION

Plasma total and LDL-cholesterol concentrations consistently fall in humans and various animal species when saturated triglyceride is replaced by vegetable oil rich in n-6 polyunsaturated or n-9 monounsaturated fatty acids (33-35). In contrast, the response to dietary n-3 fatty acids in humans and among different animal species is highly variable. In the present studies, dietary n-3 fatty acids were incorporated into liver lipids to a similar extent in the hamster and rat; nevertheless, the effect of dietary n-3 fatty acids on hepatic LDL transport varied remarkably in the two species. The most striking differences were seen when n-3 fatty acids were incorporated into diets also containing cholesterol and saturated fatty acids. Under these conditions n-3 fatty acids suppressed hepatic receptor-dependent LDL uptake in the hamster but had the opposite effect in the rat. In both species, changes in hepatic receptor-dependent LDL uptake were associated with reciprocal changes in the plasma concentration of lipoproteins containing apoB and/or apoE.

As an attempt to understand the differences in response to n-3 fatty acids, the mechanism whereby n-3 fatty acids alter hepatic LDL transport in the hamster and rat was investigated. These studies showed that suppression of hepatic receptor-dependent LDL uptake in hamsters fed n-3 PUFAs was accompanied by parallel changes in LDL receptor protein and mRNA. These findings are consistent with previous studies in the hamster suggesting that dietary fatty acids regulate hepatic LDL receptor activity by altering LDL receptor protein and mRNA (27, 30). In the rat, on the other hand, LDL receptor protein and mRNA levels were not affected by dietary n-3 PUFAs under conditions in which receptor-dependent LDL uptake by the liver was increased by nearly twofold. These observations are consistent with previous ligand binding studies showing no effect of fish oil on LDL binding to hepatic membranes in the rat (18). The finding of enhanced receptor-dependent LDL uptake in the absence of any change in LDL receptor expression raises the possibility of enhanced clearance of LDL via a receptor-dependent mechanism other than the classic LDL receptor pathway.

In addition to the LDL receptor, the liver expresses a second lipoprotein receptor, the chylomicron remnant receptor, that mediates the uptake of chylomicron remnants via a high velocity transport process that recognizes apoE (36, 37). Recently a cell surface receptor, the LRP/α_2M receptor, has been shown to bind and internalize apoE-enriched lipoproteins (38-40). The LRP/α_2M receptor appears to play an important role in the hepatic uptake of chylomicron remnants although the extent of this involvement under various metabolic conditions remains to be established (32, 40). Because uptake through the chylomicron remnant receptor is mediated by apoE, and as it is very difficult to prepare rat LDL completely free of apoE, we investigated the possibility that n-3 fatty acids might alter the activity of the chylomicron remnant receptor and thereby affect LDL clearance. These experiments showed that n-3 fatty acids had no effect on the hepatic clearance of chylomicron remnants under conditions in which the receptor-dependent clearance of LDL was nearly doubled. Similarly, dietary n-3 fatty acids had no effect on hepatic mRNA levels for the LRP/α_2M receptor.

Dietary n-3 fatty acids are incorporated into liver membranes and apparently alter the physical properties of these membranes. By modifying the fatty acid composition of membrane lipids, dietary fatty acids may alter the microenvironment of membrane-associated proteins and thereby influence receptor activity (41). In this way dietary n-3 fatty acids could potentially alter the affinity of the LDL receptor for its ligands or alter the distribution or recycling rate of LDL receptors in the hepatocyte. Indeed, in some studies dietary fatty acid-induced changes in LDL receptor activity have correlated with changes in the composition and physical properties of cell membranes (42). Data in the rat, where n-3 fatty acids increased hepatic LDL receptor activity but not LDL receptor protein or mRNA, would be compatible with such a mechanism. However, liver lipids showed similar enrichment with n-3 fatty acids in the rat and hamster

whereas dietary n-3 fatty acids had the opposite effects on hepatic LDL receptor activity in these two species. Further studies will be necessary to determine whether n-3 fatty acids actually alter the distribution or recycling rate of LDL receptors in rat hepatocytes.

Different animal species also vary greatly in their response to dietary cholesterol. In this respect the rat is highly resistant to the effects of dietary cholesterol whereas the hamster is modestly responsive. The major factors appear to be a much higher basal rate of hepatic bile acid (and cholesterol) synthesis in the rat allowing a much greater inflow of dietary cholesterol to be balanced by suppression of hepatic sterol synthesis (43). In addition, the rat is able to increase the conversion of cholesterol to bile acids in response to excessive amounts of dietary cholesterol (44). The relationship between variability in response to dietary cholesterol and variability in response to n-3 fatty acids is unknown. However, it would appear that species that are highly resistant to dietary cholesterol are more likely to experience a reduction in plasma total and LDL-cholesterol in response to dietary n-3 fatty acids.

Studies in humans have shown extraordinary variability in response to n-3 fatty acids, with effects ranging from marked reductions (15) to significant elevations in plasma total and LDL-cholesterol concentrations (10). In general, adding modest amounts of n-3 fatty acids to a typical Western diet does not lower plasma LDL concentrations and frequently raises LDL levels in hypertriglyceridemic individuals (10). On the other hand, large doses of n-3 fatty acids may markedly lower plasma total and LDL-cholesterol concentrations in some individuals especially when the n-3 fatty acids replace saturated fat in the diet (8, 15). The present studies demonstrate that n-3 fatty acids may exert divergent and, under some circumstances, opposite effects on hepatic LDL transport in the hamster and rat. Elucidating the mechanisms that underlie these species differences may help in understanding the marked variability of responses to n-3 PUFAs in humans.

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