Role of LDL subfraction heterogeneity in the reduced binding of low density lipoproteins to arterial proteoglycans in cynomolgus monkeys fed a fish oil diet

John S. Parks,[1] Abraham K. Gebre, Iris J. Edwards, and William D. Wagner

Department of Comparative Medicine, Bowman Gray School of Medicine of Wake Forest University, Medical Center Boulevard, Winston-Salem, NC 27157-1040

Abstract Previous studies using cynomolgus monkeys have shown that isocaloric substitution of dietary fish oil for lard reduced the in vitro binding of plasma low density lipoproteins (LDL) to arterial proteoglycans (PG) (Edwards, I. J., A. K. Gebre, W. D. Wagner, and J. S. Parks. 1991. *Arterioscler. Thromb.*, **11:** 1778–1785). The purpose of the present study was to determine whether all LDL subfractions were equally affected by the type of dietary fat with regard to PG binding and to identify compositional changes in LDL subfractions that might relate to the differential in PG binding. Two groups of cynomolgus monkeys (n = 5 each) were fed atherogenic diets (40% calories as fat; 0.26 mg cholesterol/kcal) containing 20% of calories as egg yolk and 20% as either lard or menhaden fish oil. LDL were isolated from plasma by ultracentrifugation and size exclusion chromatography and subfractionated by density gradient centrifugation. Three density ranges of LDL subfractions were collected from the gradients for determination of chemical composition, apoE and apoB content by ELISA, and binding to arterial PG in vitro. The d 1.015–1.025 g/ml subfraction contained 39 ± 8% of the LDL cholesterol in the lard group but only 7 ± 3% for the fish oil group. Values for cholesterol distribution were opposite for the d 1.035–1.045 g/ml subfraction, 8 ± 1% versus 41 ± 8%, respectively. Similar trends were noted for the distribution of apoB. For the lard group, LDL binding to arterial PG increased with decreasing density (i.e., increasing size) of the subfractions. A significant difference ($P = 0.001$) in LDL-PG complex formation was observed for the d 1.015–1.025 g/ml subfraction in which there was a 3- to 4-fold difference between the lard versus fish oil groups (55 ± 6% vs. 15 ± 4% of LDL cholesterol in insoluble PG complex); there was also a 2.5-fold difference in the apoE to apoB molar ratio for this subfraction with the fish oil group having a lower ratio (0.9 ± 0.3 vs. 2.2 ± 0.7; $P = 0.04$) and a significant correlation ($r = 0.64$) was observed between apoE/B molar ratio and LDL-PG complex formation. There was no difference between diet groups for LDL binding to PG or apoE to apoB molar ratio for the d 1.025–1.035 g/ml subfraction. The d 1.035–1.045 g/ml subfraction had a significantly lower apoE/B molar ratio for the fish oil group (0.6 ± 0.2 vs 2.1 ± 0.8; $P = 0.03$) but there was no difference in LDL-PG complex formation between diet groups. In all three subfractions of LDL, chemical compositions were similar between diet groups except for a significant increase in percentage of cholesteryl ester in the d 1.035–1.045 g/ml subfraction of the fish oil group. We conclude that the d 1.015–1.025 g/ml subfraction of LDL binds most avidly to arterial PG. Furthermore, the reduced binding of plasma LDL to PG observed for the fish oil group compared to the lard group may be related to the decreased apoE content of the d 1.015–1.025 g/ml subfraction of LDL in addition to the decreased amount of this subfraction of LDL in plasma. **—Parks, J. S., A. K. Gebre, I. J. Edwards, and W. D. Wagner.** Role of LDL subfraction heterogeneity in the reduced binding of low density lipoproteins to arterial proteoglycans in cynomolgus monkeys fed a fish oil diet. *J. Lipid Res.* 1991. **32:** 2001–2008.

Supplementary key words saturated fat • n-3 fatty acids • apolipoprotein E • apolipoprotein B • lard

Low density lipoproteins (LDL) are the major cholesterol-carrying lipoprotein class in human plasma and the concentration of plasma LDL is correlated with human coronary heart disease (1). LDL are spherical particles composed of a core of cholesteryl esters (CE) and triglycerides surrounded by a monolayer of phospholipid (PL), unesterified cholesterol, and protein. The core CE undergo a thermotropic phase transition near body temperature which is indicative of a reversible liquid crystalline to liquid transition (2). Our previous studies have shown that the composition, concentration, and transition temperature of LDL can be modified by the type of dietary fat fed to nonhuman primates (3–5). Recent studies have shown that the isocaloric substitution of fish oil for saturated fat (lard) in the diet of African green monkeys was associated with a decrease in LDL concentration, size, CE content, and CE transition temperature as well as a

Abbreviations: PG, proteoglycans; LDL, low density lipoproteins; apo, apolipoprotein; ELISA, enzyme-linked immunosorbent assay; CE, cholesteryl esters; PL, phospholipids; VLDL, very low density lipoproteins; HDL, high density lipoproteins; TG, triglyceride; TPC, total plasma cholesterol.

[1]To whom correspondence should be addressed.

decrease in atherosclerosis development (3, 6, 7). Based on these studies we have hypothesized that the changes in the physical and chemical properties of plasma LDL are related to atherosclerosis development in these experimental animals. One potential mechanism by which the physical and chemical properties of LDL could alter atherosclerosis outcome involves the binding of LDL to arterial proteoglycans with subsequent trapping of LDL in the arterial wall. Another mechanism may relate to the ability of LDL to interact with receptors on cells in the artery wall.

We have recently shown, using cynomolgus monkeys (*Macaca fascicularis*), that isocaloric substitution of dietary fish oil for saturated fat decreased the binding of plasma LDL to arterial PG (8). A crossover study was performed so that each animal served as its own control and eight of ten animals demonstrated a decreased binding of LDL to PG while consuming the fish oil diet. Although the two diets did not alter plasma total or LDL cholesterol concentrations, LDL were smaller in size, were enriched in n-3 fatty acids, had lower CE melting temperatures, and were depleted of phosphatidylcholine and enriched in sphingomyelin and lysophosphatidylcholine when the animals were consuming the fish oil diet (4). These data suggested that one or more of the changes in the physical and chemical properties of plasma LDL was affecting the binding properties of LDL to PG in vitro.

The purpose of the present study was to determine whether all subfractions of LDL were equally affected by the type of dietary fat (lard vs. fish oil) with regard to proteoglycan binding and to identify compositional changes in LDL subfractions that might relate to PG binding. Our results suggest that the largest, lightest subfractions (d 1.015–1.025 g/ml) of LDL bind most avidly to arterial PG in vitro. In addition, the reduced binding of plasma LDL to PG observed during fish oil feeding may be due to the decreased apoE content of the d 1.015–1.025 g/ml subfraction of LDL in addition to the decreased amount of this subfraction of LDL in plasma.

METHODS

Animals and diets

Ten adult male cynomolgus monkeys (*Macaca fascicularis*) were provided for the study through an NHLBI-sponsored nonhuman primate models program at the Bowman Gray School of Medicine. These same animals were previously used in a crossover study on the effects of dietary fish oil versus lard on the physical and chemical properties of LDL (4) and on LDL-PG binding (8). In the present study, five animals were fed a lard-containing diet and five were fed a fish oil diet. The diets contained 40% of calories as fat with 0.26 mg cholesterol/kcal. Half of the fat calories were derived from lard or menhaden oil; the other half were from egg yolk or egg yolk replacement, a low cholesterol mixture that resembles egg yolk in composition. Diets similar to these have been used in studies of African green monkeys and detailed diet compositions have been published (9). In the present study the amount of egg yolk was reduced to give 0.26 mg cholesterol/kcal of diet because cynomolgus monkeys are more responsive to dietary cholesterol than are African green monkeys (10). Processed menhaden oil was obtained from the Southeast Fisheries Center (Charleston, SC) through the NIH Nutrition Committee Fish Oil Test Materials Program. Alpha-tocopherol, Tenox GT-1 (mixture of tocopherols; Eastman Chemical Products, Kingsport, TN) and Tenox 20A (tertiary butyl-hydroquinone, Eastman Chemicals) were added to the lard diet to a final concentration of 16.4 mg, 13.2 mg, and 11 mg/100 g diet, respectively, to balance the amounts contained in the fish oil diet (antioxidants were added to processed fish oil). All other dietary constituents were similar to those given in our previous publication (9). Diets were made in 10-kg batches and were stored frozen until needed. Prior to feeding, the diets were allowed to thaw overnight at 4°C and the animals were fed 30-min meals (15 g diet/kg body wt per meal) twice daily. The fish oil group consumes, on average, 7.4 g n-3 fatty acids/day (7.9 g n-3 fatty acids/1000 calories).

LDL isolation and subfraction

Blood samples were taken from animals after an overnight (18 h) fast. Ketamine hydrochloride (10 mg/kg) was used to restrain each animal while blood was taken from the femoral vein into chilled tubes (4°C) containing 0.1% EDTA and 0.02% NaN_3 (final concentration) at pH 7.4. The animals had consumed their respective experimental diets for at least 6 months before blood samples for LDL subfractionation were taken. LDL was isolated from plasma by ultracentrifugation and high performance liquid chromatography using Superose 6B (Pharmacia, Piscataway, NJ) as described previously (4). Isolated LDL was characterized chemically (3) and used for proteoglycan binding studies or in some cases was subfractionated further by density gradient centrifugation. Briefly, 4 mg of LDL protein was subfractionated in a VTi 50 rotor by the procedure of Marzetta and Rudel (10) with slight modification. The LDL sample was adjusted to d 1.019 g/ml using a saturated KBr solution and 7.3 ml of the LDL solution containing 4 mg protein was layered between 3.85 ml of a d 1.006 g/ml and 3.85 ml of a d 1.050 g/ml solution in a 15-ml centrifuge tube. The samples were ultracentrifuged for 6 h at 50,000 rpm at 20°C. The tubes were drained as described (10) and the density of individual fractions was determined by refractometry using a standard curve that related density to refractive index of KBr solutions. Individual tubes from each gradient run were pooled to give three density fractions: d 1.015–1.025 g/ml, d 1.025–1.035 g/ml, and d 1.035–1.045 g/ml. These LDL

subfractions were then dialyzed against 0.9% NaCl, 0.01% EDTA, 0.01% NaN_3, pH 7.4, and concentrated to >100 μg cholesterol/ml for PG binding studies, chemical composition determinations (3), and ELISA assays for apoB and apoE (11, 12). All LDL samples were stored under N_2 at 4°C.

LDL-PG binding studies

PG was isolated from normal aorta of cynomolgus monkeys (8) and consisted predominantly of chondroitin sulfate PG containing 187 μg/ml protein, 60 μg/ml hexosamine, and 104 μg/ml hexuronic acid. LDL binding to isolated arterial PG was performed for LDL and LDL subfractions as described previously (8). Briefly, 100 μg of LDL cholesterol was incubated with 1 μg PG (measured as hexuronic acid) in 1.1 ml of buffer containing 5 mM Tris, 6 mM KCl, 15 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.2, at room temperature for 30 min. The resulting LDL-PG complexes were precipitated by low speed centrifugation, the supernatant was removed and the pellet was resuspended in 10 μl of 1.5 M NaCl and diluted to 100 μl with deionized water. Cholesterol in the resuspended pellet was measured by an enzymatic cholesterol assay (13) and percentage of LDL cholesterol complexed with PG was calcualted as cholesterol in the pellet divided by LDL cholesterol in the incubation × 100.

Data analysis

Values are presented as mean ± SEM. The Student's *t*-test was used to test for statistically significant differences. In some cases a natural ln transformation of the data was performed to normalize the data before statistical analysis.

RESULTS

The results of an in vitro binding assay of LDL with isolated arterial PG are shown in **Fig. 1.** The LDL isolated from animals fed the fish oil diet formed significantly less insoluble complex with PG compared to those fed the lard diet (8.1 ± 0.9 vs 18.2 ± 3.1% LDL cholesterol in insoluble PG complex; $P = 0.014$). These results are similar to the previously published results of a crossover dietary study in which LDL was tested for PG complex formation during a lard and fish oil dietary phase for each animal (8). The results in Fig. 1 were generated using a different PG preparation and after the animals had been consuming the experimental diets for a longer period of time (~1 yr vs. 15 wk in ref. 8). However, the results show that LDL from monkeys fed the fish oil diet are less than half as effective at forming insoluble complexes with PG (Fig. 1, ref. 8) as those LDL isolated from animals fed the lard diet. Plasma lipid values for these animals have been reported previously (4) and at the time the data in Fig. 1

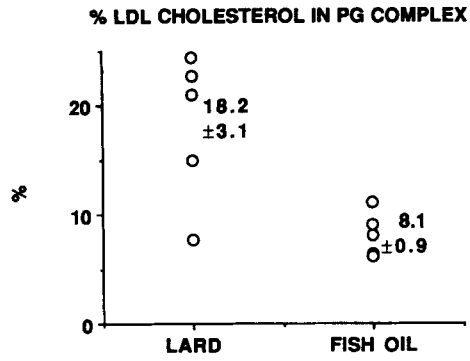

Fig. 1. Binding of plasma LDL, isolated from cynomolgus monkeys consuming two types of dietary fat, to arterial chondroitin sulfate proteoglycans. LDL (100 μg as cholesterol) was incubated with PG (1 μg as hexuronic acid) for 30 min at room temperature in buffer containing 5 mM Tris, 6 mM KCl, 15 mM $CaCl_2$, 1.5 mM $MgSO_4$, pH 7.2. The formation of PG:LDL complexes after 30-min incubations was measured as μg cholesterol in a 1500 *g* pellet and was represented as a percentage of the total cholesterol in the incubation tube. Values shown are for individual animals and the mean ± SEM is given for each diet group.

were generated the TPC and TG concentrations were similar for both diet groups (TPC: 367 ± 17 [lard] vs. 370 ± 42 mg/dl [fish oil]; TG: 9 ± 3 [lard] vs. 10 ± 2 mg/dl [fish oil]). However, HDL cholesterol concentrations were significantly lower for animals fed the fish oil diet (23 ± 2 vs. 58 ± 8 mg/dl; $P < 0.01$) similar to previously reported results (4).

In order to determine whether all LDL subfractions from animals fed the fish oil diet demonstrated diminished binding to LDL or whether only selected subfractions were affected, isolated plasma LDL were subfractionated by density gradient centrifugation and characterized for each diet group. Density gradient profiles for two animals in each diet group are shown in **Fig. 2.** Striking differences in the density distribution of LDL subfractions were apparent; the LDL from animals fed the lard diet were less dense and distributed predominantly in the upper half of the gradient while those from animals fed the fish oil diet were denser and were distributed predominantly in the lower half of the gradient. Three density cuts (indicated by the brackets) from each gradient run were taken for further analyses.

The LDL subfraction cholesterol and apoB distribution for the three density cuts taken from the gradients are shown in **Fig. 3.** The d 1.015–1.025 g/ml subfraction contained 38.7 ± 7.6% versus 6.8 ± 2.7% ($P = 0.01$) of the total LDL cholesterol for the lard versus fish oil groups, respectively. The d 1.025–1.035 g/ml subfractions contained nearly equivalent amounts of LDL cholesterol for both diet groups (53.0 ± 6.8% vs 52.1 ± 7.0% for lard vs. fish oil, respectively). The third subfraction (d 1.035–1.045 g/ml) contained significantly ($P = 0.02$) more of the LDL cholesterol for animals fed the fish oil diet (41.2 ± 7.9%) compared to those fed the lard diet (8.2 ± 1.0%). Similar

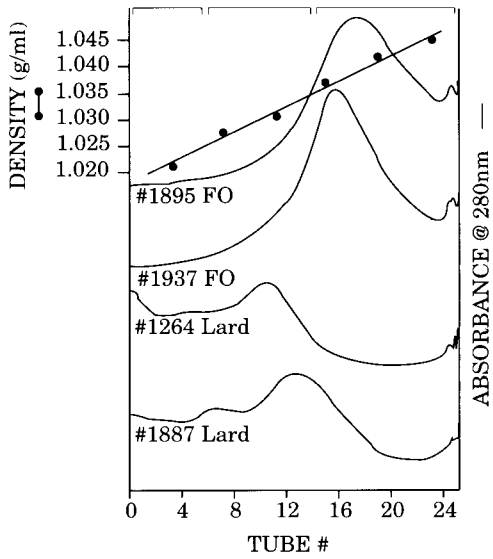

Fig. 2. Density gradient profiles of LDL, isolated from plasma by ultracentrifugation and column chromatography, from cynomolgus monkeys consuming diets containing lard or fish oil. Details of the density gradient ultracentrifugation procedure are given in the Methods section. Brackets indicate where subfractions were pooled for subsequent analyses.

results were found for apoB distribution among density gradient fractions. The distribution of apoB from the lightest to heaviest LDL subfractions for the lard versus fish oil groups was 30.3 ± 8.3 versus 8.3 ± 1.8% (P = 0.033), 55.3 ± 6.0 versus 57.1 ± 7.6% and 14.3 ± 2.5 versus 34.6 ± 8.6% (P = 0.05).

The percentage chemical composition of the LDL subfractions for four of five animals in each diet group is given in **Table 1.** As expected, the lighter subfractions contained relatively more lipid and less protein than did the more dense subfractions. The experimental diets had little effect on the composition of LDL subfractions between diet groups with the exception of the d 1.035–1.045 g/ml subfraction. In this subfraction the LDL from animals fed fish oil were relatively enriched in CE (P = 0.01) and poor in protein and free cholesterol compared to their lard counterparts.

The individual LDL subfractions (100 μg cholesterol) were incubated with isolated arterial PG to determine the ability of each subfraction to form insoluble complexes with PG. The results are shown in **Fig. 4.** A significant difference in LDL-PG complex formation was seen for the lightest LDL subfraction (i.e., d 1.015–1.025 g/ml) in which there was a 3.7-fold difference (P = 0.001) between the lard versus fish oil groups (55 ± 6% vs. 15 ± 4% of LDL cholesterol in insoluble PG complex). There was no difference in LDL-PG complex formation between the lard versus fish oil groups, respectively, for the d 1.025–1.035 g/ml (37 ± 10% vs. 36 ± 7%) or the d 1.035–1.045 g/ml subfraction (23 ± 5% vs. 23 ± 4%). Note that for the lard diet group there was a decrease in LDL-PG complex formation with increasing density of the LDL subfractions; the d 1.025–1.035 g/ml and 1.035–1.045 g/ml subfractions of the fish oil group also followed the same trend.

The results of the PG binding studies suggested that the lightest LDL subfractions were most active in binding with PG, at least for the lard diet group. We previously have documented that the LDL of the animals fed the lard diet have larger average sizes, measured as LDL molecular weight, compared to those fed the fish oil diet and that plasma apoE concentrations was significantly correlated with LDL size in cynomolgus monkeys (4, 14). In addition, the increased plasma apoE in hypercholesterolemic cynomolgus monkeys was associated with LDL particles with little change in concentration of apoE in the HDL fraction compared to those animals that are not hypercholesterolemic. Since apoE has two binding sites for the

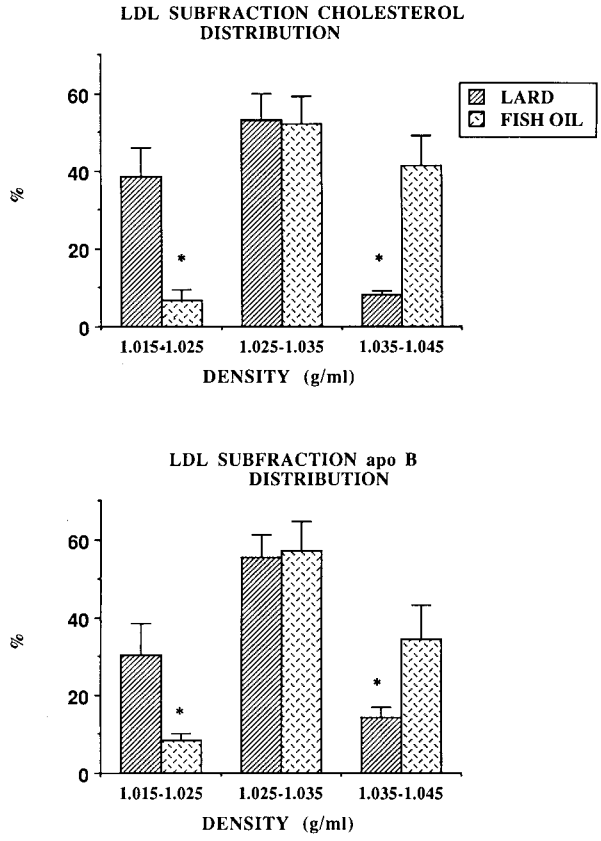

Fig. 3. Percentage distribution of plasma LDL subfraction cholesterol (top panel) and apoB (bottom panel) from cynomolgus monkeys consuming lard- or fish oil-containing diets. LDL were isolated from plasma by ultracentrifugation and size exclusion chromatography and subfractionated by density gradient ultracentrifugation. Pooled LDL subfractions were assayed for cholesterol and apoB by enzymatic and ELISA procedures, respectively. Values represent the mean ± SEM (n=5) for each diet group, respectively. Asterisk denotes statistically significant difference between diet groups (P < 0.05).

TABLE 1. Chemical composition of LDL subfractions of cynomolgus monkeys fed diets containing lard or fish oil

Density	Diet	% Composition				
		Pro	PL	TG	FC	CE
1.015–1.025	Lard	18.8 ± 2.4[a]	18.9 ± 0.9	0.3 ± 0.2	5.9 ± 0.5	56.1 ± 2.2
	Fish oil	17.1 ± 2.4	18.1 ± 1.3	0.7 ± 0.4	8.3 ± 0.9	55.8 ± 3.3
1.025–1.035	Lard	20.1 ± 0.9	18.5 ± 0.7	0.1 ± 0.1	3.4 ± 1.4	57.9 ± 1.4
	Fish oil	20.7 ± 0.9	19.8 ± 0.8	0.4 ± 0.3	2.3 ± 0.9	56.7 ± 1.1
1.035–1.045	Lard	28.1 ± 1.9	18.9 ± 1.9	N.D.[b]	9.9 ± 1.8	43.1 ± 1.7
	Fish oil	24.6 ± 0.9	17.8 ± 1.2	0.6 ± 0.3	4.7 ± 1.4	52.3 ± 2.2[c]

[a]Mean ± SEM (n = 4).
[b]N.D., not detectable.
[c]P = 0.019, Student's t-test (lard vs. fish oil).

glycosaminoglycan heparin (15), we investigated whether the larger, lighter LDL from animals fed the fish oil diet were poor in apoE, which might account for the reduced ability to bind to PG. We first looked at the association between LDL size (i.e., LDL molecular weight) and plasma apoE concentrations for the animals of this study. There was a very strong positive correlation between these two variables (r = 0.91). For the lard and fish oil groups the mean ± SEM for LDL molecular weight was 4.94 ± 0.21 and 4.37 ± 0.14 g/μmole (P = 0.05), respectively, while values for plasma apoE were 9.5 ± 2.6 and 6.1 ± 1.2 mg/dl, respectively.

The distribution of apoE and apoB among LDL subfractions was measured by ELISA and the results were converted to an apoE to apoB molar ratio (**Table 2**). For the group fed fish oil there was a significantly lower apoE/apoB molar ratio for the d 1.015–1.025 g/ml subfraction and the d 1.035–1.045 g/ml subfraction compared to the group fed the lard diet. This trend was similar to that observed for the PG binding results in Fig. 4 for the d 1.015–1.025 g/ml subfraction. The apoE/B molar ratio was not statistically different between diet groups for the d 1.025–1.035 g/ml.

A plot of the LDL subfraction apoE/B molar ratio versus the percentage of LDL cholesterol in the insoluble PG complex for individual animals is shown in **Fig. 5.** Several points can be emphasized from these data. First, only for the d 1.015–1.025 g/ml subfraction was there a statistically significant association (r = 0.64) between apoE/B molar ratio and LDL-PG complex formation. In addition, even though the mean values for the apoE/B molar ratio in the d 1.015–1.025 and 1.035–1.045 g/ml subfractions were similar for the lard group (2.19 vs. 2.07, respectively; Table 2), this was due to one animal having a high apoE/B molar ratio (i.e., 5.2) in the d 1.035–1.045 g/ml subfraction (Fig. 5). Except for that animal, the apoE/B molar ratios in the d 1.035–1.045 g/ml subfraction were close to or below 1.

The apoE/B molar ratio of the d 1.035–1.045 g/ml subfraction on average was greater than that of the d 1.025–1.035 g/ml subfraction (Table 2). To determine whether this was the result of an accumulation of apoE that had dissociated from the less dense LDL subfractions during density gradient centrifugation, the d 1.035–1.045 g/ml subfraction was subjected to size exclusion chromatography on Superose 6B (HPLC) to separate unbound apoE from the LDL subfraction. These studies were performed on a subset of six animals (four from fish oil group and

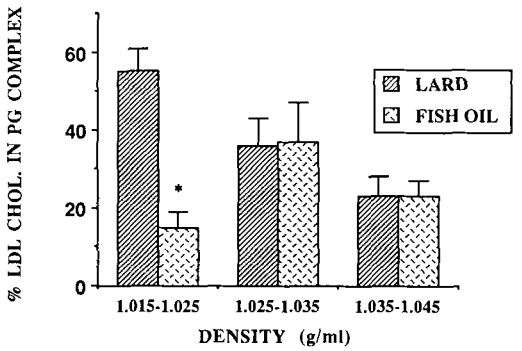

Fig. 4. Binding of subfractions of plasma LDL to arterial chondroitin sulfate PG. LDL subfractions isolated by density gradient ultracentrifugation as described in Methods and Fig. 2 were incubated with PG as described in the legend to Fig. 1. Values represent the mean ± SEM (n = 5 for each diet group); asterisk denotes statistically significant difference between diet groups (P < 0.05).

TABLE 2. LDL subfraction apoE/B molar ratio

Diet Group	LDL Subfraction (g/ml)		
	d 1.015–1.025	d 1.025–1.035	d 1.035–1.045
Lard (n = 5)	2.19 ± 0.65[a]	0.71 ± 0.24	2.07 ± 0.79
Fish oil (n = 5)	0.85 ± 0.25	0.32 ± 0.10	0.62 ± 0.20
P Value[b]	0.036	NS	0.026

[a]Values are mean ± SEM. ApoE and B were measured on individual LDL subfractions by ELISA and converted to a molar ratio using 35,000 and 512,000 as molecular weights for apoE and apoB, respectively.
[b]P value determined by Student's t-test after a natural logarithm transformation of the data. NS, not significant at P = 0.05.

two from lard group) using the d 1.035–1.045 g/ml subfraction of plasma LDL isolated from a separate bleeding. The rechromatographed d 1.035–1.045 g/ml subfractions were then analyzed for apoE and apoB by ELISA and assayed for ability to bind to PG. Analysis of the results by a paired *t*-test showed no significant difference in the apoE to B molar ratio (1.6 ± 0.3 vs 1.3 ± 0.6) or on LDL-PG complex formation (9 ± 1 vs. 18 ± 6% LDL cholesterol in PG complex) for the d 1.035–1.045 g/ml subfraction before versus after reisolation on the Superose 6B column to separate unbound apoE.

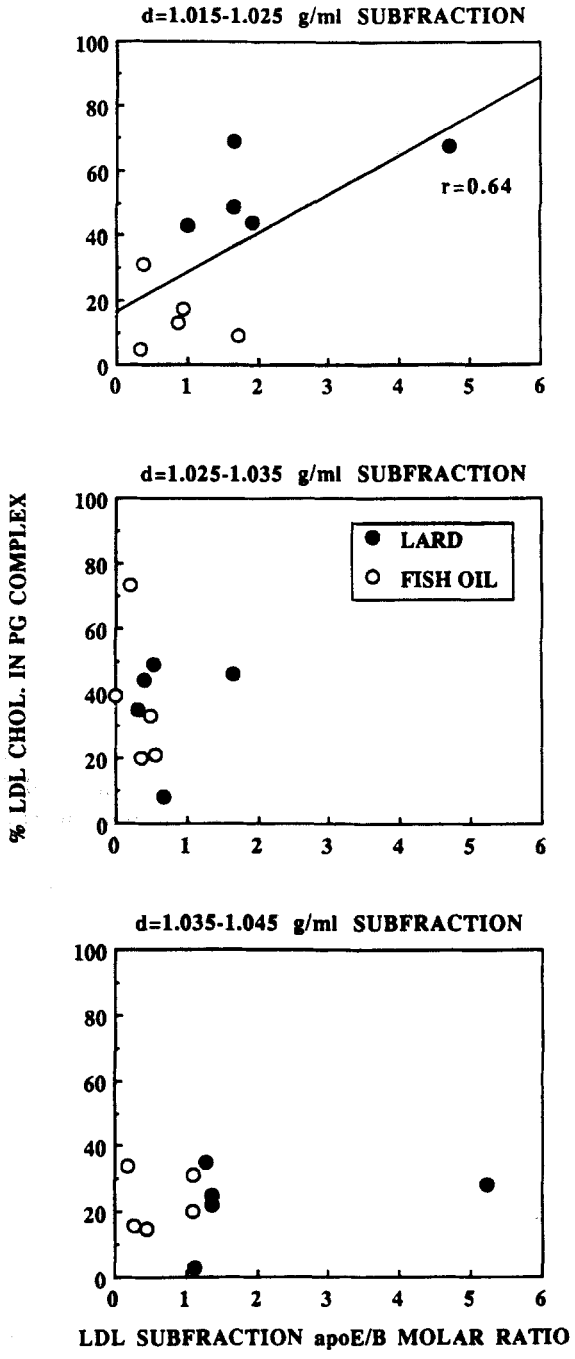

Fig. 5. Plots of LDL subfraction apoE/B molar ratio versus percentage of LDL cholesterol in the insoluble PG complex. Each point represents data from a single animal. In the d 1.015–1.025 g/ml subfraction the line of best fit and correlation coefficient determined by linear regression analysis are shown.

DISCUSSION

Our studies have demonstrated that isocaloric substitution of fish oil for lard in the diet of cynomolgus monkeys alters properties of LDL important in the binding to arterial PG (Fig. 1; ref. 8). LDL from animals fed the fish oil-containing diet had reduced binding to arterial PG compared to those LDL from animals fed lard. The results from the present study show that not all LDL subfractions are equally effective at forming insoluble complexes with arterial PG. For the animals fed lard, LDL complex formation with PG decreased with increasing density of the LDL subfraction (Fig. 4). A 4-fold difference in LDL-PG complex formation between diet groups was noted for the least dense LDL subfraction (d 1.015–1.025 g/ml) and this difference in complex formation appeared to be related to the apoE to B molar ratio for that subfraction of LDL, with lower LDL-PG complex formation occurring when the apoE to B molar ratio was low (Fig. 5). In addition, the LDL of animals fed the fish oil diet had fewer particles in the d 1.015–1.025 g/ml fraction (i.e., less apoB) relative to those fed the lard diet (Fig. 3). Taken together, these data suggest that the reduced capacity of plasma LDL from animals fed fish oil to form insoluble complexes with arterial PG is due, in part, to the decreased amount of larger, less dense LDL and perhaps to reduced apoE to B molar ratio of the LDL particles relative to those from the lard diet group. Since the binding of LDL to arterial proteoglycans is thought to be an important step in the trapping of LDL in the arterial wall and in the development of atherosclerosis, the alteration of properties of LDL particles by diets containing fish oil may be partly responsible for the decreased atherosclerosis observed in animals fed diets enriched with fish oil (6, 16, 17).

Our results suggest that the apoE content of LDL may be an important determinant of PG binding. ApoE has been shown to have two heparin binding sites, one close to the LDL receptor binding domain and one at the carboxyterminus of the protein (15). However, when apoE is bound to lipid, only the heparin binding site in the receptor binding domain is active (15). Because the glycosaminoglycan chains are thought to be important in the binding of PG to LDL, an increased content of apoE on LDL could provide additional binding sites for the glycosaminoglycan chains of PG. However, it is clear that apoE content alone is not responsible for the diet-induced differences in LDL binding to PG since an apoE to apoB ratio difference was observed in both the d 1.015–1.025

g/ml and 1.035–1.045 g/ml subfractions (Table 2), but the LDL-PG complex formation difference was only observed in the least dense LDL subfraction (Fig. 4). There may be several possible explanations for these results. ApoE may need to be in an appropriate conformation to bind to PG and that conformation can only occur on the large CE-enriched LDL such as those in the d 1.015–1.025 g/ml subfraction isolated from the density gradient. Several studies have suggested that apoE must be in the correct conformation to bind to the LDL receptor (18, 19) or the LDL receptor-related protein (20). In addition, conformational changes in apoE may be responsible for the masking of the heparin binding domain in the carboxyterminus of apoE when it is bound to phospholipid (15). Alternatively, the increased content of apoE in the d 1.015–1.025 g/ml subfraction of LDL may simply reflect a change in some other surface property of LDL important in PG binding that also affects the affinity of apoE for the surface of LDL. Although plasma LDL cholesterol concentrations were similar between diet groups, the LDL from the animals fed the fish oil diet were different from their lard counterparts in several respects. The fish oil group had plasma LDL particles that were smaller, enriched in n-3 fatty acids, with lower CE transition temperatures, and with changes in surface phospholipid distribution including an increase in sphingomyelin and lysophosphatidylcholine and a decrease in phosphatidylcholine (4). One or more of these changes induced by dietary fish oil may be responsible for the decreased binding of LDL to PG directly or indirectly by altering the conformation of apoE at the LDL particle surface. Additional studies will be necessary to distinguish among these possibilities.

The importance of apoB in the binding of LDL to PG is supported by several observations. ApoB has seven known heparin binding sites that may be involved in binding with the glycosaminoglycan chains of PG (21, 22). Tryptic peptides of LDL apoB that are enriched in positively charged lysine and arginine residues have been found to be important in the binding of LDL to the negatively charged PG (23). Chemical modifications of apoB that result in a decrease in net positive charge also decrease the binding of LDL to glycosaminoglycans (24). These results suggest an important role for apoB in the binding of LDL to PG.

The dietary fish oil-induced modifications of LDL may influence apoB conformation through surface-core interactions. Studies with human subjects have shown that LDL that are highly reactive with PG had core CE in an ordered state, while less reactive LDL have disordered or melted CE cores (25). At body temperature the CE core of LDL from animals fed fish oil is disordered while the LDL core of animals fed the lard diet is predominantly ordered (3, 4). The disordered core may influence the conformation of surface apoB since studies using monoclonal antibodies have suggested that the conformation of apoB changes with LDL particle size (26) and is influenced by the CE core of the particle (27). Taken together, these data suggest another hypothetical mechanism by which dietary fish oil might decrease the interaction of LDL with PG through changes in the conformation of apoB, but further studies will be necessary to support this hypothesis.

The decreased interaction of LDL from animals fed fish oil with PG may be related to the differences in surface phospholipid distribution between diet groups. Phospholipids are also known to participate in the binding of PG to LDL, presumably through the binding of phospholipid phosphate groups via Ca^{2+} ions to PG (28). The changes in phospholipid distribution accompanying fish oil feeding (i.e., decreased phosphatidylcholine, increased sphingomyelin and lysophosphatidylcholine) as well as fatty acid composition may directly affect this type of interaction or may indirectly affect the affinity of LDL for PG by altering the conformation of apoB or by decreasing the binding of apoE to the LDL particle surface.

The striking difference in LDL subfraction distribution between diet groups with little change in the chemical composition of LDL subfractions may result for several reasons. Our previous studies in African green monkeys have demonstrated that there is a strong correlation between hepatic cholesteryl ester content and plasma LDL size as well as hepatic VLDL cholesterol secretion during recirculating liver perfusion (29). Therefore, the contribution of hepatic CE to plasma LDL, via acyl CoA-acyltransferase, may be diminished with fish oil feeding. In addition, the plasma phospholipids of monkeys fed fish oil are poor substrates for lecithin:cholesterol acyltransferase, the enzyme responsible for the generation of CE in plasma, compared to those plasma phospholipids from animals fed the lard diet (30). These observations taken together suggest that, when monkeys are fed fish oil-containing diets, the plasma and intracellular generation of CE is diminished leading to smaller, denser LDL subfractions in plasma that are poor in CE compared to their lard counterparts. Alternatively, selective catabolism of LDL subfractions through increased activity of the LDL receptor (31) may result in a different distribution of LDL subfractions between diet groups. However, this alternative seems less likely since dietary fish oil does not influence plasma apoB concentrations relative to lard in either the cynomolgus (4) or African green monkey (7, 29), and the cynomolgus monkeys fed fish oil actually have a greater number of LDL particles in plasma that are smaller in size compared to their lard counterparts (4).

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REFERENCES

1. The Lipid Research Clinics Program. 1984. Lipid Research Clinics Coronary Primary Prevention Trial results. II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering. *J. Am. Med. Assoc.* **251:** 365–374.
2. Deckelbaum, R. J., G. G. Shipley, and D. M. Small. 1977. Structure and interaction of lipids in human plasma low density lipoproteins. *J. Biol. Chem.* **252:** 744–754.
3. Parks, J. S., and B. C. Bullock. 1987. Effect of fish oil versus lard diets on the chemical and physical properties of low density lipoproteins of nonhuman primates. *J. Lipid Res.* **28:** 173–182.
4. Parks, J. S., and A. K. Gebre. 1991. Studies on the effect of dietary fish oil on the physical and chemical properties of low density lipoproteins in cynomolgus monkeys. *J. Lipid Res.* **32:** 305–315.
5. Rudel, L. L., J. K. Sawyer, and J. S. Parks. 1987. Heterogeneity of low density lipoproteins in atherosclerosis: nonhuman primate species and diet composition. *In* Proceedings of the Workshop on Lipoprotein Heterogeneity. NIH Publication #87-2646. 263–278.
6. Parks, J. S., J. Kaduck-Sawyer, B. C. Bullock, and L. L. Rudel. 1990. Effect of dietary fish oil on coronary artery and aortic atherosclerosis in African green monkeys. *Arteriosclerosis.* **10:** 1102–1112.
7. Parks, J. S., and L. L. Rudel. 1990. Effect of fish oil on atherosclerosis and lipoprotein metabolism. *Atherosclerosis.* **84:** 83–94.
8. Edwards, I. J., A. K. Gebre, W. D. Wagner, and J. S. Parks. 1991. Reduced proteoglycan binding of low density lipoproteins from monkeys (*Macaca fascicularis*) fed a fish oil versus lard diet. *Arterioscler. Thromb.* **11:** 1778–1785.
9. Parks, J. S., J. A. Martin, B. L. Sonbert, and B. C. Bullock. 1987. Alteration of high density lipoprotein (HDL) subfractions and plasma lipoprotein concentrations of nonhuman primates fed fish oil diets. Selective lowering of HDL subfractions of intermediate size and density. *Arteriosclerosis.* **7:** 71–79.
10. Marzetta, C. A., and L. L. Rudel. 1986. A species comparison of low density lipoprotein heterogeneity in nonhuman primates fed atherogenic diets. *J. Lipid Res.* **27:** 753–762.
11. Koritnik, D. L., and L. L. Rudel. 1983. Measurement of apolipoprotein A-I concentration in nonhuman primate serum by enzyme-linked immunosorbent assay (ELISA). *J. Lipid Res.* **24:** 1639–1645.
12. Sorci-Thomas, M., M. D. Wilson, F. L. Johnson, D. L. Williams, and L. L. Rudel. 1989. Studies on the expression of genes encoding apolipoproteins B-100 and B-48 and the low density lipoprotein receptor in nonhuman primates. Comparison of dietary fat and cholesterol. *J. Biol. Chem.* **264:** 9039–9045.
13. Auerbach, B. J., J. S. Parks, and D. Applebaum-Bowden. 1990. A rapid and sensitive micro-assay for the enzymatic determination of plasma and lipoprotein cholesterol. *J. Lipid Res.* **31:** 738–742.
14. Stevenson, S. C., J. L. Haines, and L. L. Rudel. 1988. Plasma apolipoprotein E concentration is directly proportional to total and LDL cholesterol concentrations in hypercholesterolemic nonhuman primates. *J. Cell Biol.* **107:** 652a.
15. Weisgraber, K. H., S. C. Rall, Jr., R. W. Mahley, R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1986. Human apolipoprotein E. Determination of the heparin binding sites of apolipoprotein E3. *J. Biol. Chem.* **261:** 2068–2076.
16. Davis, H. R., R. T. Bridenstine, D. Vesselinovitch, and R. W. Wissler. 1987. Fish oil inhibits development of atherosclerosis in rhesus monkeys. *Arteriosclerosis.* **7:** 441–449.
17. Kim, D. N., H. T. Ho, D. A. Lawrence, T. Schmee, and W. A. Thomas. 1989. Modifications of lipoprotein patterns and retardation of atherogenesis by a fish oil supplement to a hyperlipidemic diet for swine. *Atherosclerosis.* **76:** 35–54.
18. Innerarity, T. L., K. H. Weisgraber, K. S. Arnold, S. C. Rall, Jr., and R. W. Mahley. 1984. Normalization of receptor binding of apolipoprotein E2. Evidence for modulation of the binding site conformation. *J. Biol. Chem.* **259:** 7261–7267.
19. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1979. Binding of arginine-rich (E) apoprotein after recombination with phospholipid vesicle to the low density lipoprotein receptors of fibroblasts. *J. Biol. Chem.* **254:** 4186–4190.
20. Kowal, R. D., J. Herz, K. H. Weisgraber, R. W. Mahley, M. S. Brown, and J. L. Goldstein. 1990. Opposing effects of apolipoprotein E and C on lipoprotein binding to low density lipoprotein receptor-related protein. *J. Biol. Chem.* **265:** 10771–10779.
21. Weisgraber, K. H., and S. C. Rall. 1987. Human apolipoprotein B-100 heparin-binding sites. *J. Biol. Chem.* **262:** 11097–11103.
22. Cardin, A. D., and H. J. R. Weintraub. 1989. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis.* **9:** 21–32.
23. Camejo, G., S. Olofsson, F. Lopez, P. Carlsson, and G. Bondjers. 1988. Identification of apoB-100 segments mediating the interaction of low density lipoproteins with arterial proteoglycans. *Arteriosclerosis.* **8:** 368–377.
24. Mahley, R. W., K. H. Weisgraber, and T. L. Innerarity. 1979. Interaction of plasma lipoproteins containing apolipoproteins B and E with heparin and cell surface receptors. *Biochim. Biophys. Acta.* **575:** 81–91.
25. Camejo, G. 1982. The interaction of lipids and lipoproteins with the intercellular matrix of arterial tissue: its possible role in atherogenesis. *Adv. Lipid Res.* **19:** 1–53.
26. Teng, B., A. Sniderman, R. M. Krauss, P. O. Kwiterovich, Jr., R. W. Milne, and Y. L. Marcel. 1985. Modulation of apolipoprotein B antigenic determinants in human low density lipoprotein subclasses. *J. Biol. Chem.* **260:** 5067–5072.
27. Marcel, Y. L., M. Hogue, P. K. Weech, and R. W. Milne. 1984. Characterization of antigenic determinants on human solubilized apolipoprotein B. Conformational requirements for lipids. *J. Biol. Chem.* **259:** 6952–6957.
28. Kim, Y. C., and T. Nishida. 1979. Nature of the interaction of dextran sulfate with high and low density lipoproteins in the presence of Ca^{2+}. *J. Biol. Chem.* **254:** 9621–9626.
29. 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.
30. Parks, J. S., B. C. Bullock, and L. L. Rudel. 1989. The reactivity of plasma phospholipids with lecithin:cholesterol acyltransferase is decreased in fish oil-fed monkeys. *J. Biol. Chem.* **264:** 2545–2551.
31. Spady, D. K., and L. A. Woollett. 1990. Interaction of dietary saturated and polyunsaturated triglycerides in regulating the processes that determine plasma low density lipoprotein concentrations in the rat. *J. Lipid Res.* **31:** 1809–1819.