Dietary n-3 polyunsaturated fat increases the fractional catabolic rate of medium-sized HDL particles in African green monkeys

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Abstract We have previously described a novel pathway for the metabolism of HDL subfractions in which small [2 apolipoprotein (apoA-I) molecules per particle] HDL particles are converted in a unidirectional manner outside the plasma compartment to medium (3 apoA-I molecules per particle) or large (4 apoA-I molecules per particle) HDL particles, which are subsequently removed from the circulation by the liver (Colvin et al. 1999. *J. Lipid Res.* 40: 1782–1792; Huggins et al. 2000. *J. Lipid Res.* 41: 384–394). The purpose of the present study was to determine whether the reduction in concentration of medium HDL in African green monkeys consuming n-3 polyunsaturated versus saturated fat diets resulted from decreased in vivo production or increased catabolism. Tracer small LpA-I (HDL containing only apoA-I) were isolated, without ultracentrifugation, by gel filtration and immunoaffinity chromatography and radiolabeled. After injection, the specific activity of apoA-I in small, medium, and large HDL was determined, and the kinetic data were analyzed using our previously published multicompartmental model for HDL subfraction metabolism. We found a significant reduction of apoA-I concentration in medium HDL in the animals fed n-3 polyunsaturated fat (31.2 ± 0.7 mg/dl) compared with animals fed saturated fat (85.4 ± 11.9 mg/dl; $P = 0.002$). The production rates of apoA-I in small, medium, and large HDL were similar in both diet groups; however, there was a significant increase in the fractional catabolic rate of apoA-I in medium HDL in the animals fed n-3 polyunsaturated fat (2.188 ± 0.501 pools/day) compared with animals fed saturated fat (0.714 ± 0.191 pools/day; $P = 0.02$).▪ We conclude that n-3 polyunsaturated fat reduces HDL cholesterol concentration by increasing the fractional catabolic rate of medium-sized HDL particles in African green monkeys.—Huggins, K. W., P. L. Colvin, E. R. Burleson, K. Kelley, J. K. Sawyer, P. H. R. Barrett, L. L. Rudel, and J. S. Parks. **Dietary n-3 polyunsaturated fat increases the fractional catabolic rate of medium-sized HDL particles in African green monkeys.** *J. Lipid Res.* 2001. 42: 1457–1466.

Supplementary key words fish oil • LpA-I • apolipoprotein A-I • PUFA • non-human primates • saturated fat • in vivo catabolism

Despite numerous advances in the understanding and treatment of coronary heart disease (CHD), it remains the leading cause of death in Western societies (1). Usually dietary modification is the first step taken to lower the risk for CHD due to the positive relationship between dietary fat and cholesterol consumption and atherosclerosis development (2). The general dietary recommendation includes the replacement of saturated fat in the diet with polyunsaturated fat because, in general, saturated fatty acids increase, whereas polyunsaturated fatty acids decrease, total plasma cholesterol concentrations in humans (3). Humans consuming diets containing n-3 polyunsaturated (n-3 poly) fatty acids found in fish oils have reduced mortality from heart disease (4, 5). Although this benefit is probably due to many mechanisms (6), the influence of n-3 poly fat on lipoprotein metabolism remains unclear (7).

Studies in humans have consistently shown that n-3 poly fat consumption decreases plasma triglyceride concentrations (8). However, the impact of dietary n-3 poly fat on lipoprotein metabolism in humans has been difficult to determine due to differences in study design, such as duration, dose, and source of n-3 poly fat (8). Studies in nonhuman primates have been more consistent in their results. When dietary n-3 poly fat is isocalorically substituted for saturated fat in nonhuman primates, plasma concen-

trations of total cholesterol, LDL, and HDL are decreased (9). In humans, triglyceride lowering typically is inversely associated with HDL elevation, and yet n-3 fatty acid-induced triglyceride reduction has not been associated with changes in HDL (8). In monkeys, where plasma triglycerides are already low, dietary n-3 fatty acids lower HDL. We assume that in humans, the same effect of n-3 fatty acids on HDL metabolism occurs, but it is counterbalanced by the HDL increase that is inversely associated with triglyceride lowering. This appears to be the case where direct effects on HDL metabolism can be studied in the presence of fewer confounding variables. Furthermore, studies on the mechanism by which dietary fish oil modulates HDL metabolism may provide an explanation for this apparent paradox, where lower HDL and reduced atherosclerosis occur together (10, 11).

Along with lowering plasma HDL concentrations, diets rich in fish oil have been shown to alter the subfraction distribution of plasma HDL in human and nonhuman primates. In humans, Sanders et al. (12) demonstrated that men consuming a diet enriched with n-3 poly fat had increased concentration of smaller HDL_3 particles compared with men consuming a diet enriched in saturated fat. In nonhuman primates, we have previously demonstrated that African green monkeys consuming a diet enriched with n-3 poly fat have a decreased concentration of HDL subfractions of intermediate size and density (13). These observations suggest that n-3 poly fat may alter the metabolism of specific subfractions of HDL. However, we know of no previously published studies examining the influence of dietary fat saturation on HDL subfraction metabolism.

Studies of HDL metabolism have been difficult to perform due to the polymorphic nature of plasma HDL. HDL can be separated based on size, density, electrophoretic mobility, and apolipoprotein content. Two major classes of HDL, which can be isolated by immunoaffinity chromatography, have been the subjects of recent investigations. These are HDL that contain both apolipoprotein (apo) A-I and apolipoprotein A-II (LpA-I/A-II) and those that contain only LpA-I (14, 15). Studies have shown that LpA-I concentrations are inversely proportional to the prevalence of CHD in humans and that transgenic mice with elevated concentrations of LpA-I develop less atherosclerosis compared with those with elevated concentrations of LpA-I/A-II (16, 17). LpA-I is also more efficient at removing excess cholesterol from cells in culture than is LpA-I/A-II (18, 19), and it is also more efficient than LpA-I/A-II in the delivery of cholesteryl ester-derived sterol to the bile of rats via the selective cholesteryl ester uptake pathway (20). These results taken together suggest that LpA-I particles are more protective with regard to atherosclerosis development than LpA-I/A-II. Only one study to date has examined the effect of dietary fat on plasma LpA-I and LpA-I/A-II concentration. In humans consuming a diet rich in polyunsaturated fat, total LpA-I concentration was reduced, whereas total LpA-I/A-II concentration was unchanged (21). To our knowledge, there are no studies on the effect of specific dietary fatty acids on LpA-I metabolism.

HDL can be subfractionated by size and apolipoprotein content into small, medium, and large LpA-I particles containing two, three, and four apoA-I molecules per particle, respectively. Recently, we described for the first time a metabolic pathway for HDL subfractions in African green monkeys in which small plasma HDL were converted to medium or large HDL in a unidirectional manner outside of the plasma compartment (22–24). We found no evidence for the conversion of large HDL to smaller HDL particles or for the generation of pre-beta apoA-I during HDL metabolism. Furthermore, we found that large HDL were removed from plasma and catabolized primarily by the liver (25). The purpose of the present study was to examine the effect of dietary n-3 poly fat on the metabolism of plasma HDL subfractions to elucidate the mechanism that accounts for the reduced plasma HDL concentration, altered HDL subfraction distribution, and decreased extent of atherosclerosis in nonhuman primates. We have chosen the vervet monkey for our studies because of its close phylogenetic relationship to humans and because African green (vervet) monkeys that were fed atherogenic diets had lipoprotein concentrations, distributions, and compositions similar to humans at risk for CHD (26).

EXPERIMENTAL PROCEDURES

Animals and diets

The animals used in this study were St. Kitts vervet monkeys (*Cerocopitheus aethiops sabeus*) that were part of a larger atherosclerosis progression study at the time the metabolic studies were performed. Twenty-five adult male vervet monkeys were purchased from a primate importer and quarantined for 10 weeks, during which time they were fed a monkey chow diet. After this equilibration period, the monkeys were challenged for a 4-week period with a diet containing 0.6 mg/kcal of cholesterol and 35% of calories as saturated fat (palm oil) to establish two groups of monkeys with equivalent dietary responsiveness. Plasma total, LDL, and HDL cholesterol measurements were taken at 3 and 4 weeks of dietary challenge, and these values together with body weight were used to establish two groups with similar means ± SD for these endpoints. The groups were then returned to a monkey chow diet for 12 weeks to allow plasma and hepatic cholesterol concentrations to return to baseline. The two groups were then fed one of two experimental diets, saturated (Sat) or n-3 poly, during the atherosclerosis induction phase for ~4 years before the initiation of the present investigations. Both diets contained 0.4 mg/kcal cholesterol and 35% of calories as fat. The diets were prepared in the diet kitchen in the Section of Comparative Medicine (Department of Pathology) at Wake Forest University School of Medicine. The compositions of the different diets are presented in **Table 1**, and the fatty acid analysis has been previously described (27). The animals were maintained on their respective diets during the HDL turnover studies. A subset (n = 5) was selected randomly from each diet group for the HDL turnover studies. All animals were individually housed in an enriched environment in the animal facility at the Wake Forest University School of Medicine, which is approved by the American Association for the Accreditation of Laboratory Animal Care and supervised by a veterinary staff. The Institutional Animal Care and Use Committee approved all experimental procedures.

TABLE 1. Diet composition

| | Experimental Group | | |
Ingredient	All Diets[a]	Sat	n-3 poly
			g/ 100 g
Wheat flour	35.0		
Dextrin	9.6		
Sucrose	10.0		
Casein	9.0		
Lactalbumin	5.0		
Alphacel	7.24		
Hegsted salts	5.0		
Vitamin mixture	2.5		
Fat			
Palm oil		16.4	8.2
Fish oil[b]			8.2
Crystalline cholesterol		0.17	0.15
β-Sitosterol		0.065	0.069
α-Tocopherol		0.0066	
γ-Tocopherol		0.0066	
Tenox 20A[c]		0.0082	

Abbreviations: Sat, saturated; n-3 poly, n-3 polyunsaturated.

[a] All diets contain a calorie distribution of 35% fat, 48% carbohydrate, and 17% protein.

[b] Fish oil contains 2.6 mg cholesterol, 1.0 mg α-tocopherol, 1.0 mg γ-tocopherol, and 1.0 mg Tenox 20A per g of oil.

[c] Tenox 20A is an antioxidant.

Plasma lipid, lipoprotein, and apolipoprotein measurements

Total and HDL cholesterol, triglyceride, and apolipoprotein concentrations were measured in whole plasma, as described previously (26). Whole plasma HDL cholesterol subfraction distribution was measured using proton NMR (28, 29).

Isolation of LpA-I

The small LpA-I tracer used in the kinetic studies was isolated from donor animals on the experimental diets, either Sat or n-3 poly, and administered to study animals on the corresponding diet. Blood was obtained via venipuncture from the femoral vein of two donor animals on each experimental diet on the morning after an overnight fast. The animals were sedated with ketamine HCl (10 mg/kg). Blood was collected into sterile evacuated tubes containing 0.1% EDTA and 0.1% NaN₃. Tubes were immediately placed on ice, and plasma was promptly separated by centrifugation at 2,000 g for 30 min at 4°C. Aprotinin (1 mg/l, final concentration) and phenylmethylsulfonyl fluoride (80 mg/l, final concentration) were then added to the isolated plasma. Gel filtration chromatography was used to separate the plasma lipoprotein classes by applying the plasma to an 8% agarose column (2.5 × 90 cm). Lipoproteins were eluted with 0.15 M NaCl, 0.01% EDTA, 0.01% NaN₃, pH 7.4 (column buffer) at 4°C. The distribution of the lipoproteins eluted from the column and collected in a fraction collector was evaluated by the measurement of total cholesterol in each of the column fractions. The HDL-containing fractions were pooled into three different size ranges from the front (large HDL), middle (medium HDL), and back (small HDL) portions of the HDL peak.

LpA-I was isolated from the small HDL regions of the 8% agarose column eluate by immunoaffinity chromatography, as previously described (22). The eluted LpA-I particles were dialyzed for 36 h (3 changes of 6 liters of column buffer) to ensure complete removal of NaSCN. The LpA-I particles were then concentrated to 1 ml using an Amicon nitrogen pressure cell and stored at 4°C under an argon atmosphere. After isolation, the LpA-I particles were used within 2 weeks for turnover studies.

LpA-I characterization

LpA-I particle size distribution was determined by 4–30% nondenaturing gradient gel electrophoresis for 1,400 Vxh at 10°C, as previously described (30, 31). SDS polyacrylamide gradient gel (4–30%) electrophoresis was performed as previously described (30). The number of apoA-I molecules per LpA-I particle was determined using dimethyl suberimidate (DMS) crosslinking, as previously described (22). LpA-I particles were assayed for phospholipid (32), protein (33), triglyceride (34), and free and total cholesterol (34) by published procedures.

Radioiodination of LpA-I

Small LpA-I was iodinated with carrier-free ¹²⁵I or ¹³¹I (New England Nuclear, Boston, MA.) according to the method of McFarlane (35) as modified by Bilheimer, Eisenberg, and Levy (36). Radiolabels were alternated throughout the course of the studies to control for potential isotope effects. Sufficient ICl was added so that 0.5 mole of radiolabeled iodine was added for every 28,000 g of LpA-I protein. After iodination, the small LpA-I particles were subjected to a second size exclusion chromatography step over a 10% agarose column (1.6 × 100 cm; Bio-Gel 0.5 m, 200–400 mesh, Bio-Rad) equilibrated with 0.15 M NaCl, 0.01% EDTA, pH 7.4. Individual fractions were run on 4–30% polyacrylamide gradient gels for 1,400 Vxh at 10°C, as previously described (22). Gels were then subjected to autoradiography, and column fractions that contained homogeneous small LpA-I particles were selected for reinjection.

Radioiodinated LpA-I doses were characterized by autoradiography of 4–30% nondenaturing gradient gels for particle size homogeneity. SDS-PAGE and autoradiography were used to assess apolipoprotein content of LpA-I. The number of apoA-I molecules per iodinated LpA-I particle was determined using DMS crosslinking. Aliquots (2 μl) of radioiodinated LpA-I were added to tubes containing 0.5 ml of a 1 mg/ml solution of BSA and 0.5 ml 20% TCA. TCA-precipitation was allowed to proceed for 15 min. After low-speed centrifugation, the supernatant was removed and the pellet was counted for radioactivity. The extent of lipid labeling was determined by adding 2 μl of iodinated LpA-I to 0.5 ml of a 1 mg/ml solution of BSA and subjecting this mixture to Bligh-Dyer extraction, as previously described (37). Specific activities of the small LpA-I doses ranged from 350 to 2,500 cpm/ng protein. The LpA-I preparations consistently had >98% of the radiolabel associated with protein (i.e., TCA-precipitable count) and 1–2% associated with lipid.

In vivo turnover studies

All of the turnover studies were performed in pairs from the two diet groups (n = 5). The animals were given a solution of 0.45% NaCl, 0.05% NaI ad libitum 1 week before injection and were kept on the solution for the duration of the turnover study. The radiolabeled small LpA-I tracer from each diet group was administered in the morning after the animals were fasted overnight. The animals were not fed again until 12 h after injection. Approximately 1.0×10^8 cpm of radiolabeled small LpA-I was injected into the saphenous vein of the animals. Blood samples (1.5 ml) were withdrawn from the femoral vein at 5 and 20 min and 1, 2, 2.25, 3.5, 5, 8, 24, 48, 72, 96, 120, and 168 h after injection. Blood samples were collected into chilled tubes containing 0.1% EDTA and placed on ice. Urine was collected each day throughout the study period. Plasma volume was calculated as 3.5% of body weight.

Plasma was isolated by centrifugation of the timed blood samples as described above. An aliquot of plasma (100 μl) was subjected to radiolabel quantification using a Beckman gamma counter (Beckman Instruments, Fullerton, CA). Another aliquot of plasma (20 μl) was run on 4–30% nondenaturing gradient

gels. After fixing in 10% sulfosalicilic acid, the gels were cut based on the migration of the high molecular protein standard thyroglobulin, which was visible on the fixed gels as a brownish band. From this standard, the gels were sliced into 5-mm slices, and the regions corresponding to large (0–15 mm), medium (15–25 mm), and small (25–40 mm) were quantified for the radioactive tracer. The designated migration distances for large, medium, and small HDL were based on the migration distances of the individual doses on 4–30% nondenaturing gradient gels. These migration distances did not change for any of the particles used in these studies. Regions above thyroglobulin (extra large material) and below albumin (pre-beta material) were also quantified for ^{125}I or ^{131}I radioactivity. These regions did not contain significant amounts of radioactivity at any time during the course of the turnover studies. Recovery of applied radioactivity typically ranged from 80% to 95%.

A duplicate nondenaturing gradient gel of the plasma samples from each recipient animal was run to determine the relative distribution of apoA-I among large, medium, and small particles. LpA-I protein was electrophoretically transferred from 4–30% nondenaturing gradient gels to nitrocellulose membranes (0.45 μm, Schleicher & Schull, Keene, NH) in 0.192 M glycine, 0.025 M Tris, pH 8.3, at 35 V for 3 h. The nitrocellulose blots were then blotted for apoA-I, as previously described (22).

The stained blot was scanned into a computer using a digital scanner, and the areas under the curves corresponding to large, medium, and small apoA-I-containing particles were determined using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). These numbers were used to generate the fractional distribution of apoA-I among the HDL particles. The fractional distribution was multiplied times the apoA-I concentration in the plasma sample, as determined by ELISA (38). The specific radioactivity of each size range of HDL was then calculated as cpm/μg from the cpm and micrograms for apoA-I in each size fraction.

Kinetic analysis

Analysis of the apoA-I specific activity was completed using the SAAM II program (SAAM Institute, Seattle, WA). We previously published the model used in the kinetic analysis of the turnover data (23). The model is shown in **Fig. 1**. This model is characterized by circulating plasma compartments (C) of small (C5), medium (C8), and large (C1) HDL. The circulating plasma compartments exchange with extravascular compartments (C6), (C9), and (C2), respectively. This model includes additional noncirculating compartments of medium (C7) and large (C3) HDL to account for the delayed appearance of tracer in plasma. All apoA-I in small HDL enters the noncirculating compartment

of medium (C7) or large (C3) HDL and then passes through a delay compartment (C10 and C4) before reentering the plasma compartment as medium or large HDL. U(5) is the production rate of apoA-I in small HDL. U(7) and U(3) are the additional production rates of apoA-I in medium and large HDL to account for the acquisition of apoA-I molecules when small HDL particles containing two apoA-I molecules are converted to medium or large HDL particles containing three or four apoA-I molecules. The body iodine pool (C11) is assumed to turn over at a rate of 2.5 pools/day (39). The model estimated parameters in this report were derived from the simultaneous analysis of the specific activity of apoA-I in small, medium, and large HDL and urine radioactivity data after the injection of small LpA-I tracer. Representative fits of the plasma specific activity data are shown in Fig. 3. Appearance of radioactivity in urine during the tracer studies was also monitored and used in the model (data not shown).

Data analysis

Data are given for individual animals as well as the mean ± standard error of the mean, except where indicated. Statistical analysis was done using the Statview™ 4.5 program. P values were determined by Student's t-test.

<div align="center">RESULTS</div>

Analysis of plasma lipids and LpA-I

Plasma lipid and apolipoprotein concentrations for vervet monkeys used in the metabolic studies are shown in **Table 2**. After 5 years of atherosclerosis progression, animals consuming the n-3 poly diet had significantly lower total plasma cholesterol, triglyceride, and HDL cholesterol concentrations as well as lower apoA-I and apoA-II concentrations compared with the animals consuming the Sat diet. There was no statistically significant diet-induced difference in plasma apoE concentration between the two diet groups.

In a previous study, we demonstrated that African green monkeys fed a fish oil diet had a significant reduction in concentration of HDL subfractions of intermediate size, as determined by gradient gel electrophoresis and density gradient ultracentrifugation (13). We analyzed the HDL subfraction distribution in the animals of this study using proton NMR. The data given in **Table 3** show that the animals fed the n-3 poly diet had significantly less cholesterol in the intermediate sized subfractions, HDL_{3a} and HDL_{3b}, compared with the Sat-fed animals. The n-3 poly group also had significantly more cholesterol in the HDL_{2a} subfraction compared with the Sat group.

The chemical compositions of plasma small, medium, and large LpA-I were examined in two animals from each diet group and are shown in **Table 4**. Large LpA-I had increased phospholipid, decreased protein, and slightly more cholesteryl ester compared with small LpA-I in both diet groups. The composition of medium LpA-I was intermediate between large and small LpA-I. These results are consistent with the size data obtained by nondenaturing gradient gel electrophoresis (**Fig. 2**). There were no apparent compositional differences between the two diet groups with the exception of medium LpA-I in the n-3 poly group, which appeared to have more protein, choles-
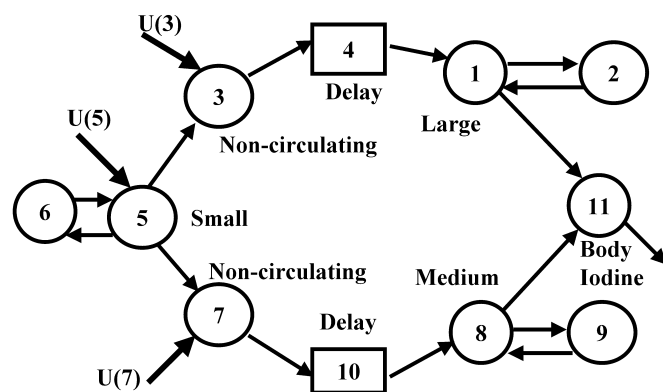

Fig. 1. Model developed by Colvin et al. (23) used in the analysis of HDL apoA-I kinetic data.

TABLE 2. Plasma concentrations of lipids, lipoproteins, and apolipoproteins

Diet, Animal #	TPC	TG	HDL-C	ApoA-I	ApoA-II	ApoE
			mg/dl			
Sat						
794	176	16	70	189	18.3	1.7
803	365	22	56	203	19.6	4.4
817	447	16	55	244	14.6	4.2
834	433	28	74	246	22.3	5.2
837	524	13	42	234	17.7	10.2
Mean ± SEM	389 ± 59	19 ± 3	59 ± 6	229 ± 14	17.2 ± 1.9	5.1 ± 1.4
n-3 Poly						
799	268	6	39	116	8.1	4.7
800	207	6	55	135	7.7	3.7
818	136	6	45	114	7.0	3.6
820	225	12	48	160	11.4	3.3
824	223	13	29	136	9.6	3.3
Mean ± SEM	212 ± 22	9 ± 2	43 ± 4	133 ± 9	8.8 ± 0.8	3.7 ± 0.3
P	0.006	0.003	0.02	0.0003	0.0004	NS

TPC (total plasma cholesterol), HDL-C (HDL cholesterol), and TG (triglyceride) concentrations are means of data from months 3 to 27 of the experimental diet phase (n = 14 measurements). Apolipoprotein E (apoE) concentrations are means of months 4.5–27 (n = 6 measurements). ApoA-I and apoA-II concentrations are the means of data determined from the plasma samples taken during the metabolic studies (n = 13 measurements). NS indicates not significant at $P = 0.05$.

teryl ester, and less phospholipid than in the Sat group. However, too few measurements were made to determine if these differences are statistically significant.

The effect of dietary fat type on the distribution of apoA-I in large, medium, and small plasma HDL, as determined by Western blot analysis, is shown in **Table 5**. There was a significant reduction in the concentration of apoA-I in medium HDL in the n-3 poly animals compared with the Sat animals (31.2 ± 0.7 vs. 85.4 ± 11.9; $P = 0.01$). This resulted in a decrease in the percent distribution of apoA-I in medium HDL (24% vs. 38%) in the animals fed n-3 poly fat. There was also a significant reduction in the concentration of apoA-I in small HDL in the n-3 poly animals compared with the Sat animals (57.5 ± 3.8 vs. 78.8 ± 6.9

mg/dl; $P < 0.001$). However, the percent distribution of plasma apoA-I in small HDL in the animals fed the n-3 poly diet was increased compared with the Sat-fed animals (44% vs. 35%). There were no differences in the apoA-I concentration in large HDL between the two groups. The percent distribution of apoA-I in large HDL was slightly increased in the n-3 poly group (33% vs. 26%).

Kinetic studies

The goal of the present study was to determine the effect of dietary fat type on the metabolism of HDL subfractions as a function of size and apoA-I content. This was achieved by kinetic studies using small (two apoA-I molecules per particle) LpA-I tracer isolated from donor ani-

TABLE 3. NMR analysis of HDL subfraction cholesterol distribution in whole plasma of African green monkeys

Diet, Animal #	HDL Subfraction				
	2b	2a	3a	3b	3c
			mg/dl		
Sat					
794	20.1	12.2	29.7	6.7	4.0
803	15.1	8.2	24.8	8.3	8.0
817	12.2	5.1	14.2	8.4	19.9
834	17.0	10.2	25.0	9.7	11.2
837	7.0	1.8	11.1	7.9	26.0
Mean ± SEM	14.3 ± 2.2	7.5 ± 1.8	21.0 ± 3.5	8.2 ± 0.5	13.8 ± 4.0
n-3 Poly					
799	9.3	8.2	2.7	1.8	20.7
800	17.0	21.5	0.2	3.7	10.5
818	8.2	18.8	1.2	3.6	11.0
820	11.1	19.9	2.1	3.4	12.2
824	13.9	17.1	1.0	3.7	13.8
Mean ± SEM	11.9 ± 1.6	17.1 ± 2.3	1.4 ± 0.4	3.2 ± 0.4	13.2 ± 1.9
P	NS	0.003	<0.0001	0.005	NS

Cholesterol concentrations determined for HDL subfractions by NMR analysis are means of data from months 3–27 of the experimental diet phase (n = 14 measurements). HDL 2b is the largest and HDL 3c is the smallest subfraction. NS indicates not significant at $P = 0.05$.

TABLE 4. Chemical compositions of LpA-I subfractions

Diet	Size	Pro	PL	FC	CE	TG
				%		
Sat	Large	50.7	31.7	3.3	12.8	1.5
	Medium	60.1	27.5	0.9	10.4	1.2
	Small	71.2	17.4	2.1	9.0	0.4
n-3 Poly	Large	52.8	29.5	3.6	12.9	1.5
	Medium	67.8	16.5	0.4	15.3	ND
	Small	74.1	17.0	0.9	6.1	1.9

Values represent the mean of two individual determinations from two animals except for the medium n-3 poly value, which was from a single animal, and the small Sat and n-3 poly values, which were determined from a pooled (n = 2) sample. Pro, protein; PL, phospholipid; FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; ND, none detected.

mals and injected into recipient animals on the same experimental diet. The small LpA-I tracer preparations were isolated by a combination of gel filtration and immunoaffinity chromatography to avoid the alteration of HDL subfraction distribution caused by ultracentrifugation. Fig. 2 shows autoradiographs from a representative isolation of small iodinated LpA-I from donor animals fed the Sat diet that were used for in vivo kinetic studies in recipient animals in the Sat diet group. The nondenaturing gradient gel (right) demonstrates that the small LpA-I is relatively homogeneous in size and migrates with a Stokes' diameter between 7.1 and 8.2 nm. Large LpA-I that migrates with a Stokes' diameter between 10.4 and 12.2 nm is also shown in the figure for reference. Medium particles isolated from a Sat-fed animal were intermediate in size (8.2–10.4 nm) between large and small LpA-I (data not shown). LpA-I particles isolated from n-3 poly animals gave similar results (data not shown). The tracer LpA-I contained only apoA-I at ~28 kD with no apoE and no apoA-II, as determined by SDS-PAGE and autoradiography (left). Crosslinking with DMS showed that LpA-I contained four (large), three (medium), and two (small) apoA-I molecules per particle (data not shown).
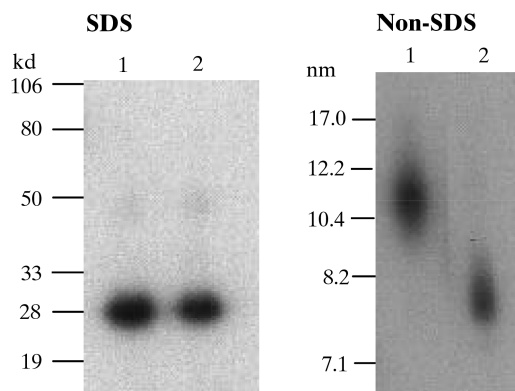
The radiolabeled tracers used for the kinetic studies

Fig. 2. Autoradiagram of an SDS (left) and non-SDS (right) 4–30% gradient gels of representative Sat large and small [125]I- labeled LpA-I (lanes 1 and 2) isolated by apoA-I gel filtration and immunoaffinity chromatography. Molecular weight markers are indicated on the left for the SDS gel, and Stokes' diameters are indicated on the left for the non-SDS gel.

TABLE 5. Plasma apoA-I distribution in large, medium, and small HDL subfractions

Diet, Animal #	ApoA-I in Large HDL	ApoA-I in Medium HDL	ApoA-I in Small HDL
		mg/dl	
Sat			
794	60.0	62.1	66.5
803	69.4	56.5	77.1
817	60.3	120.8	62.6
834	68.9	88.9	87.9
837	35.7	98.5	100.0
Mean ± SEM	58.9 ± 6.1 (26%)	85.4 ± 11.9 (38%)	78.8 ± 6.9 (35%)
n-3 Poly			
799	24.9	28.9	62.0
800	48.3	31.4	55.1
818	23.9	30.9	59.0
820	62.1	31.1	67.0
824	57.5	33.5	44.5
Mean ± SEM	43.3 ± 8.1 (33%)	31.2 ± 0.7 (24%)	57.5 ± 3.8 (44%)
P	NS	0.01	<0.001

Total plasma apoA-I concentrations, as determined by ELISA, are means of data from the individual plasma samples (n = 13) obtained during the course of the turnover studies. This value was multiplied by the percent distribution of apoA-I, as determined by Western blot analysis (see Experimental Procedures) to calculate the concentration of apoA-I in large, medium, and small HDL. P value was determined by Student's t-test. NS indicates not significant at P = 0.05.

were small LpA-I. The small LpA-I tracer preparation typically contained 85% small LpA-I and 15% medium LpA-I as analyzed by gradient gel electrophoresis before injection.

Figure 3 shows the plasma die-away of large, medium, and small HDL specific activity after the injection of radiolabeled small LpA-I tracer into representative monkeys fed the Sat and n-3 poly diets. The radioactivity in small HDL decreased rapidly after injection and, after a delay, increased in medium and large HDL particles. This outcome was seen in all animals studied, regardless of diet; however, the amount of radioactivity appearing in medium and large particles varied from animal to animal. This result suggests that there was a precursor-product relationship between small and medium (or large) HDL in plasma and is consistent with our earlier reported observations (23). The HDL apoA-I kinetic model derived values (represented by the lines) are consistent with the plasma experimental data (represented by the data points) as seen in Fig. 3. Data from a representative animal from the Sat group are shown in Fig. 3A. After injection of the radiolabeled small LpA-I tracer, the specific activity in small HDL rapidly decreased, and, after a delay, a peak in specific activity of apoA-I in medium HDL was observed. A peak in specific activity of large HDL was reached at the same time as the peak in medium HDL. In the representative animal from the n-3 poly group, the peak in both medium and large HDL was delayed until after the initial rapid decline in the small HDL biphasic curve (Fig. 3B).

As in our previous study, all injected tracer radioactivity was accounted for in the model. There was no evidence that the delayed peak was the result of an artifact in the
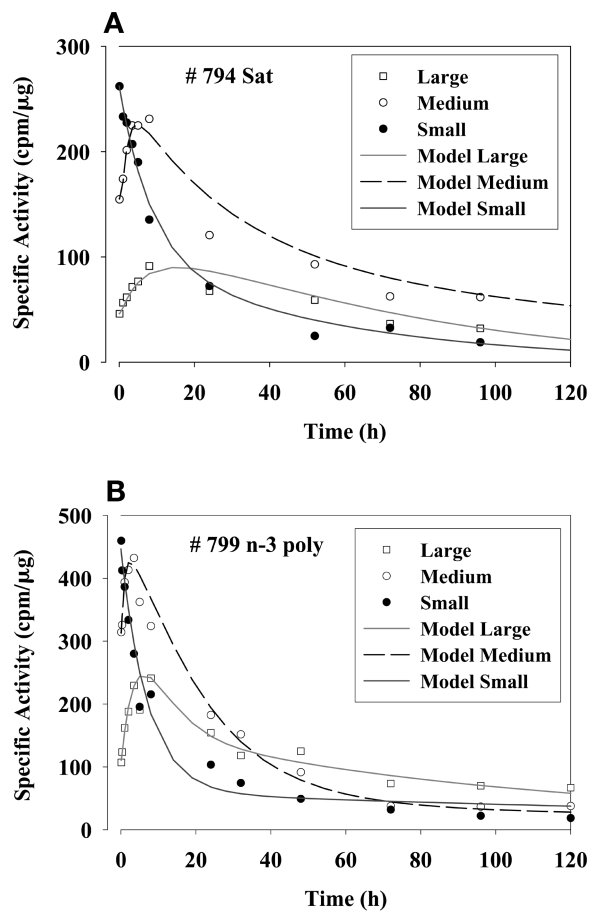
Fig. 3. Specific activity for apoA-I in large, medium, and small HDL after injection of small LpA-I in representative animals fed the Sat (A) or n-3 poly (B) diets. Lines are the computer-derived values. Symbols are the experimental data expressed as cpm/μg apoA-I.

experimental method. The experimental kinetic data provide clear evidence of the transfer of tracer radioactivity from small HDL to medium or large HDL in all monkeys studied. There was no evidence of significant transfer of tracer radioactivity from large HDL to medium, to small, nor to pre-beta HDL and no evidence of transfer from medium to large HDL.

The simultaneous analysis of the plasma specific activity in small, medium, and large HDL and urine radioactivity after the injection of small LpA-I tracer was used in the kinetic model to characterize the interconversion among the subpopulations of HDL and to calculate the production rate and fractional catabolic rate for each. The model used for the analysis of the plasma specific activity and urine data is shown in Fig. 1. The model-derived production rate of apoA-I in small, medium, and large HDL after injection of radiolabeled small LpA-I is shown in **Table 6**. There was no difference in the total apoA-I production rate nor in the production rate of apoA-I in the HDL subfractions between the two diet groups. In contrast, as shown in **Table 7**, animals fed n-3 poly fat had a threefold increase in the fractional catabolic rate of apoA-I in medium HDL compared with the animals fed the Sat diet (2.188 ± 0.501 vs. 0.714 ± 0.191 pools/day; $P = 0.02$).

TABLE 6. Model-derived production rates (PR) of HDL apoA-I after injection of radiolabeled small LpA-I

Diet, Animal #	Small HDL PR	Medium HDL PR	Large HDL PR
		mg/h	
Sat			
794	17.5	12.6	18.4
803	26.2	33.9	7.2
817	24.4	26.7	13.0
834	17.1	11.4	19.0
837	26.0	31.8	9.6
Mean ± SEM	22.2 ± 2.0	23.3 ± 3.2	13.4 ± 2.4
n-3 Poly			
799	30.2	42.0	4.6
800	25.4	23.4	19.6
818	16.2	10.8	18.0
820	30.0	34.8	13.8
824	14.8	17.1	6.8
Mean ± SEM	23.3 ± 3.3	25.6 ± 5.7	12.6 ± 3.0
P	NS	NS	NS

Data of the model-derived PR of apoA-I for African green monkeys consuming a Sat or n-3 poly diet. Values were calculated from the model to explain the kinetic data of injected radiolabeled small LpA-I shown in Fig. 1. *P* value was determined by Student's *t*-test. NS indicates not significant.

DISCUSSION

We have previously shown that diets enriched in n-3 poly fat result in lower plasma HDL cholesterol and apoA-I concentrations than do diets enriched in saturated fat (9, 10). Although the n-3 poly-fed animals had a lower average concentration of all plasma HDL subfractions, the decreased concentration of medium HDL subfractions was particularly striking (13). Animals fed the n-3 poly diets also developed significantly less coronary and aortic atherosclerosis in spite of the paradoxical reduction in plasma HDL concentrations (10). Although several studies

TABLE 7. Model-derived fractional catabolic rates (FCR) of HDL apoA-I after injection of radiolabeled small LpA-I

Diet, Animal #	Small HDL FCR	Medium HDL FCR	Large HDL FCR
		pools/d	
Sat			
794	0.629	0.533	0.737
803	0.815	1.420	0.247
817	0.936	0.535	0.516
834	0.466	0.307	0.660
837	0.626	0.775	0.638
Mean ± SEM	0.694 ± 0.082	0.714 ± 0.191	0.560 ± 0.086
n-3 Poly			
799	1.170	3.480	0.434
800	1.104	2.760	1.060
818	0.658	0.828	1.810
820	1.075	2.660	0.538
824	0.776	1.214	0.283
Mean ± SEM	0.957 ± 0.101	2.188 ± 0.501	0.825 ± 0.279
P	NS	0.02	NS

Data are from the model-derived FCR of apoA-I for African green monkeys consuming a Sat or n-3 poly diet. Values were calculated from the model to explain the kinetic data of injected radiolabeled small LpA-I shown in Fig. 1. *P* value was determined by Student's *t*-test. NS indicates not significant.

have investigated the effect of dietary n-6 poly fat on HDL metabolism (40–43), there have been no published reports on the effect of dietary n-3 poly fat on HDL metabolism. In addition, previous HDL metabolic studies were performed with unfractionated plasma HDL, and it is not known how dietary fat saturation affects the metabolism of specific HDL subfractions. The purpose of the present study was to determine the effect of dietary n-3 poly relative to Sat fat on the metabolism of HDL subfractions of defined size and containing two, three, or four molecules of apoA-I per particle. Using our previously described model of HDL subfraction metabolism (22–24), we found that the total production rate of apoA-I and the production rate of apoA-I in small, medium, and large HDL was not affected by dietary fat composition. In contrast, the fractional catabolic rate of apoA-I in medium HDL was markedly increased in animals fed n-3 poly fat. Although the kinetic data in this study are limited to the turnover of apoA-I, this outcome suggests that dietary n-3 poly fat does not increase the production of small HDL nor does dietary n-3 fat increase the conversion of small HDL to medium or large HDL. However, dietary n-3 poly fat increases the catabolism of HDL, in particular medium HDL, accounting for the observed decrease in the plasma concentration of medium HDL. The experimental observations support the assumption that HDL particles are cleared from plasma by the liver as an intact entity. Although the movement of cholesterol through HDL was not measured during these experiments, we hypothesize that dietary n-3 poly fat does not change the reverse cholesterol transport pathway, because HDL production was unchanged by diet (Table 6). Thus, the decrease in plasma HDL concentration in monkeys consuming a diet enriched with n-3 poly fat is paradoxical because the decrease in plasma HDL concentration may not signify a decrease in reverse cholesterol transport and does not result in an increase in the extent of coronary artery atherosclerosis. Given these results, the anti-atherogenic effect of dietary n-3 poly fat in African green monkeys is likely mediated through effects on plasma LDL or some aspect of HDL that was not examined during this study (10).

We recently developed the model used to analyze our kinetic data with homogeneous immunoaffinity-isolated LpA-I particles that contained either two or four molecules of apoA-I per particle and were not exposed to ultracentrifugation (22–24). There are several novel aspects to this kinetic model that should be emphasized. First, the conversion of small HDL to medium or large HDL particles was unidirectional (i.e., no evidence for recycling of apoA-I from medium or large HDL to small HDL) and occurred primarily outside the plasma compartment. Small HDL particles rapidly disappeared from plasma and, after a time delay of 2–4 h, reappeared in plasma as medium or large HDL. Evidence supporting a similar metabolic scheme in humans has been reported by Fisher et al. (44) using infused radiolabeled leucine tracer. Although they did not study the kinetics of HDL subfractions, Fisher et al. found that apoA-I was rapidly cleared from plasma, and a portion reappeared in plasma after a delay of less than half a day. This kinetic behavior of apoA-I is consistent with the concept that during reverse cholesterol transport, HDL particles must leave the plasma compartment to pick up additional cholesterol, phospholipid, and apoA-I in the interstitial fluid before reentering plasma as larger, lipid-enriched HDL particles. The delivery of the cholesterol from peripheral tissues to the liver by the hepatic uptake of larger, mature HDL particles with subsequent secretion of cholesterol into bile completes the reverse cholesterol pathway.

The second point to emphasize about our kinetic model is that there was no evidence for the creation of pre-beta HDL or pre-beta A-I during the catabolism of small, medium, or large HDL. This outcome is not consistent with results from in vitro studies, which suggest that cholesteryl ester transfer protein and hepatic lipase remodel large HDL particles into smaller HDL particles, with the generation of pre-beta A-I (45). Our studies in the non-human primate suggest that remodeling of medium and large HDL in vivo is insufficient to result in the generation of pre-beta A-I, despite high plasma cholesteryl ester transfer protein concentrations and activity (46) and documented hepatic lipase activity (47) in this animal model. A more likely explanation for the different outcomes of the in vivo versus in vitro studies is that the routine use of ultracentrifugation to isolate HDL results in metastable particles that have some molecules of apoA-I that are destabilized on the particle surface, resulting in the generation of pre-beta A-I. Indeed, Cheung and Wolf (48) demonstrated that ultracentrifugation resulted in the selective loss of larger HDL subfractions, equivalent to large particles in our study, and the generation of particles in the size range of lipid-free or lipid-poor HDL. This observation suggests that HDL tracers used in previous kinetic studies that were isolated by ultracentrifugation may not have included large LpA-I.

The final novel aspect of our kinetic model is that medium and large HDL subfractions were directly catabolized from plasma, primarily by the liver (25). Although other studies have suggested that the kidney is a major contributor to the catabolism of plasma apoA-I (49), our results in primates do not support this view. Again, this difference seems to be due to the destabilizing effects of ultracentrifugation on HDL (50). We have considerable confidence in the kinetic data and this new model of HDL subfraction metabolism because we have now completed two studies using different groups of African green monkeys, different experimental designs (high vs. low HDL animals in the first study and Sat vs. n-3 poly in the current study), and different laboratory personnel. The data from both studies have fit the kinetic model well, supporting the general applicability of this model of HDL subfraction metabolism.

To understand how dietary fat type influences HDL metabolism and the conversion of small HDL to medium or large HDL, we isolated small LpA-I tracers from donor animals and injected these into recipient animals fed diets enriched with a saturated or n-3 poly fat. The kinetic analysis, using the model diagrammed in Fig. 1, did not show any difference in the production rate of total apoA-I or

the production rate of apoA-I in small, medium, and large HDL (Table 6). We conclude that dietary fat type does not change the production or conversion of small HDL to medium or large HDL. In an earlier study, we found that African green monkeys with the highest HDL cholesterol concentrations within a group had a threefold higher apoA-I production rate compared with animals in the same atherogenic diet group with the lowest HDL concentrations (23). This outcome was due to genetic differences in the study groups, because animals were selected from the extremes of the distribution of HDL cholesterol concentrations. The lack of difference in apoA-I production rate in the present study likely was due to the careful matching for responsiveness of animals to an atherogenic challenge diet at the start of the induction phase of the study (see Experimental Procedures). Certain subpopulations of HDL that were not radiolabeled or included in the tracer dose could be influenced by dietary fat. The model-derived apoA-I production rates for apoA-I in medium and large HDL includes both apoA-I derived from the conversion of small HDL plus an additional source of apoA-I production not derived from the subpopulation of small HDL traced in these turnover studies. Newly secreted lipid-free or lipid-poor apoA-I would be one source of apoA-I not contained in our tracer dose that could combine with small HDL outside of plasma to result in medium or large HDL (24). Another subpopulation of HDL apoA-I not included in the tracer dose is the apoA-I on LpA-I/A-II particles. The metabolism of apoA-I on LpA-I/A-II particles was not addressed in this study.

In the present study, in which factors that determine individual responsiveness to an atherogenic diet were carefully controlled, dietary fat affected the fractional catabolic rate (FCR) of apoA-I. In animals fed the n-3 poly diet, the apoA-I FCR for medium HDL was increased approximately threefold compared with the Sat group. This increased FCR explains the reduced plasma concentration of medium HDL particles in the n-3 poly group that was observed despite the similar production rate in the two diet groups. The n-3 poly animals also had increased FCR values for large and small HDL compared with the Sat animals; however, this increase was not statistically significant due to the heterogeneity of the FCR values in the groups. Taken together, these observations suggest that the decrease in HDL concentration induced by n-3 poly fat did not result in a decrease in reverse cholesterol transport, because the production of apoA-I and the conversion of small HDL to medium and large HDL was unchanged in a metabolic setting where the catabolism of medium HDL was increased. Spady, Kearney, and Hobbs (51) have described an up-regulation of scavenger receptor BI in hamsters fed an n-6 poly diet relative to a saturated fat. In agreement with our study, they found that HDL concentrations were reduced and HDL cholesteryl ester uptake by the liver was stimulated, but there was no evidence for increased delivery of cholesterol from peripheral tissues to the liver, suggesting that reverse cholesterol transport was not stimulated by the n-6 poly diet.

The increased catabolism of medium HDL is likely explained by a unique conformation of apoA-I on the surface of the HDL particles or to an increase in the fluidity of the phospholipid surface. A recent study demonstrated that large HDL compared with small HDL were bound to scavenger receptor BI on the surface of CHO cells with higher affinity, and the authors concluded that HDL size and/or apoA-I conformation influences the binding of HDL subfractions to scavenger receptor BI (52). We have previously shown that recombinant HDL made with phosphatidylcholine containing n-3 fatty acids (docosahexanoic and eicosapentaenoic acid) in the sn-2 position have apoA-I that is in a different conformation and has a decreased stability compared with particles containing phosphatidylcholine with oleic acid in the sn-2 position (53). It is unlikely that a difference in lipid fluidity, per se, will manifest itself exclusively in the medium HDL subfractions, resulting in a selective increase in catabolism. On the other hand, the phospholipid surface environment may affect the conformation of apoA-I on medium particles, resulting in a more efficient uptake by the liver or other organs involved in HDL clearance. Alternatively, the enrichment of membrane phospholipids with n-3 fatty acid may alter the interaction of medium HDL particles with scavenger receptor BI or other as yet unidentified receptors involved in whole particle uptake of HDL by the liver.

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