Dietary modification of high density lipoprotein phospholipid and influence on cellular cholesterol efflux

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Abstract **African green monkeys fed fat-specific diets served as a model to investigate the effect of phospholipid acyl chain modification on high density lipoprotein (HDL)-mediated cellular cholesterol efflux. Diets enriched in saturated, monounsaturated, n–6 polyunsaturated, or n–3 polyunsaturated fats were provided during both low cholesterol and cholesterol-enriched stages; sera and HDL_3 samples were obtained at specific points during the treatment period. Analysis of the HDL phospholipid composition revealed significant acyl chain modification, consistent with the respective fat-specific diet. Cholesterol efflux from mouse L-cell fibroblasts to HDL_3 isolated from the specific diet groups was measured and revealed no differences in the abilities of the particles to accept cellular cholesterol; determination of the bidirectional flux of cholesterol between the cells and HDL_3 species further demonstrated no effect of phospholipid acyl chain modification on this process. The effects of dietary modification of phospholipid acyl chains on cellular cholesterol efflux were directly examined by isolating the HDL phospholipid and combining it with human apolipoprotein A-I to form well-defined reconstituted HDL particles. These complexes did not display any differences with respect to their ability to stimulate cellular cholesterol efflux. Incubations with 5% sera further confirmed that the fat-specific diets do not influence cholesterol efflux. These results suggest that the established influences of specific dietary fats on the progression of atherosclerosis are due to effects on cholesterol metabolism other than the efflux of cellular cholesterol in the first step of reverse cholesterol transport.—**Gillotte, K. L., S. Lund-Katz, M. de la Llera-Moya, J. S. Parks, L. L. Rudel, G. H. Rothblat, and M. C. Phillips. **Dietary modification of high density lipoprotein phospholipid and influence on cellular cholesterol efflux.** ***J. Lipid Res.*** **1998.** 39: **2065–2075.**

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It is well established that high density lipoprotein (HDL) levels in plasma are inversely correlated with the onset of coronary artery disease (1, 2). Much of this protective effect has been attributed to the central role of HDL in the process of reverse cholesterol transport (3, 4), in which excess peripheral cholesterol is transported to sites of metabolism. Structural subclasses of HDL have been shown to exhibit varying abilities to accept cellular cholesterol in the first step of this transport process (5); however, the reasons for these differences remain to be elucidated.

Studies have determined that efficient cholesterol efflux requires the presence of the main protein component of HDL, apolipoprotein (apo) A-I (6), as well as phospholipid (PL). The PL content correlates directly with the capacity of the particle to incorporate cholesterol (7). Experiments utilizing well-characterized reconstituted HDL particles of varying PL content have clearly demonstrated that the degree of saturation of the PL acyl chains strongly influences the ability of the complex to accept cellular cholesterol from mouse fibroblast monolayers (8). As diet influences lipoprotein fatty acid profiles in humans (9, 10), it becomes important to examine further this aspect of HDL-mediated cholesterol efflux. It has been suggested that fatty acid supplementation of the human diet with monounsaturated fat results in enhanced cholesterol efflux to isolated HDL relative to the effects of supplementation with different fatty acids (11). However, no study has clearly established that this effect is a direct result of changes in the PL acyl chain content of the HDL rather than a consequence of other structural modifications of the particle.

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; apo, apolipoprotein; FBS, fetal bovine serum; FC, free (unesterified) cholesterol; GLC, gas–liquid chromatography; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; MEM, minimal essential medium; PAGE, polyacrylamide gradient gel electrophoresis; PBS, phosphate-buffered saline; PL, phospholipid; POPC, 1-palmitoyl,2-oleoyl phosphatidylcholine; RCT, reverse cholesterol transport; rHDL, reconstituted HDL; TLC, thin-layer chromatography.

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In the present study we have directly addressed this question through administration of fat-specific diets to African green monkeys. This non-human primate model has been shown to have striking similarities to the human with respect to the dietary influence on lipoprotein fatty acid profiles and the importance of HDL cholesterol levels in preventing atherosclerosis (12). Prior work has indicated that the administration of a fatty acid-specific diet in this animal model leads to differences in the extent of coronary artery atherosclerosis, depending on the fatty acid enrichment (12, 13). Administration of a fat-specific diet to the African green monkey influences both lipoprotein fatty acid composition and lipoprotein function in atherosclerosis-related processes (12, 14). However, the effect of the fatty acid modifications on the HDL-mediated efflux of cellular cholesterol in the first step of the reverse cholesterol transport process is unknown. Sera and HDL particles were isolated from the monkeys after prolonged consumption of diets enriched with one of four individual fatty acids: saturated (palm oil 16:0), monounsaturated (oleinate-rich safflower oil 18:1), n–6 polyunsaturated (safflower oil 18:2), or n–3 polyunsaturated (fish oil 20:5, 20:6); the dietary approach also consisted of both a low cholesterol and a cholesterol-enriched treatment period. The isolated HDL species were well-characterized with respect to size and composition and were utilized to address the following two functional aspects. *1*) Does dietary modification of the HDL PL acyl chains influence the ability of the lipoprotein to accept cholesterol from mouse L-cell fibroblast monolayers, and *2*) how do well-characterized rHDL comprised of PL isolated from these HDL and human apoA-I compare in their ability to remove cholesterol in this cell culture system. Although marked HDL PL acyl chain modification was achieved by the fatty acid-specific diet, no effects of these changes on cholesterol efflux were detected. The results indicate that the mechanism by which dietary fats exert their effect on the progression of atherosclerosis probably does not involve HDL-mediated cellular cholesterol efflux, the first step of reverse cholesterol transport.

MATERIALS

Cholesterol, cholesteryl methyl ether, and sodium cholate were purchased from Sigma (St. Louis, MO). [1,2-^{3}H]cholesterol (43.5 Ci/mmol) was obtained from DuPont NEN (Boston, MA). Minimal essential medium and phosphate-buffered saline (PBS) were purchased from BioWhittaker (Walkersville, MD). Bovine calf and fetal serum were supplied by Life Technologies, Inc. (Grand Island, NY). All media were supplemented with 50 μg/mL gentamycin (Sigma). All other reagents were analytical grade.

METHODS

Purification of apolipoprotein A-I

Human HDL isolated from the fresh plasma of normolipidemic subjects was delipidated in ethanol/diethyl ether as described by Scanu and Edelstein (15), and purified apoA-I was isolated by anion exchange chromatography on Q-Sepharose and stored in lyophilized form at −70°C (16). Prior to use the purified apolipoprotein was resolubilized in 6 m guanidine HCl and dialyzed extensively against Tris buffer, (10 mm Tris, 150 mm NaCl, 1.0 mm EDTA; pH 8.2). Protein concentration was determined by the Markwell modification of the Lowry protein assay (17).

Dietary modification of monkey HDL

African green monkeys were fed high fat diets containing either saturated (palm oil), monounsaturated (oleic acid-enriched safflower oil), n–6 polyunsaturated (safflower oil), or n–3 polyunsaturated (fish oil, mixed 1:1 with palm oil) fat (Table 1A) in two experimental periods. In the first period, no cholesterol was added to the diets so that all but the fish oil diet were cholesterol free; the processed fish oil contained 2.6 mg cholesterol per g oil which resulted in 0.05 mg cholesterol/kcal diet. These diets are referred to as low cholesterol (≤0.05 mg cholesterol/kcal). In the second experimental period, crystalline cholesterol was added to each diet to result in 0.4 mg cholesterol/kcal and these diets are referred to as cholesterol-enriched. In both experimental periods, the diets consisted of 35% of calories as fat, 48% as carbohydrate, and 17% protein using ingredients similar to those described previously (18). After 21 weeks of diet regimen in each experimental phase, sera and/or plasma was obtained from the animals for HDL isolation, using ultracentrifugation and size-exclusion chromatography (19). The animals were housed at the animal facility at Wake Forest University School of Medicine, which is approved by the American Association for the Accreditation of Laboratory Animal Care and supervised by veterinary staff. All procedures were approved by the Institutional Animal Care and Use Committee.

Isolation of PL from isolated monkey HDL

After extensive dialysis against 5 mm ammonium bicarbonate (pH 7.3), the HDL samples were lyophilized overnight to dryness. The lipid species were extracted from the powder by the method of Bligh and Dyer (20). The resulting sample was applied as a streak at a volume of approximately 200 μL to a 1000-μm Silica Gel-G plate (Analtech) and developed using a benzene–ethyl acetate 20:1 (v/v) system for isolation of total phospholipid. These manipulations were all performed in a nitrogen environment. Bands corresponding to PL standards were scraped off the plate and extracted extensively with chloroform–methanol–water 5:5:1 (v/v). The organic phase was concentrated under nitrogen, assayed for phospholipid as inorganic phosphorus by the methods of Sokoloff and Rothblat (21), and stored at 4°C until use.

Phospholipid head group and fatty acid analysis

The PL head group distribution was determined from a pool of HDL (22) from each diet group during the cholesterol-enriched dietary phase (0.4 mg chol/kcal). PL fatty acids were transmethylated by the procedure of Metcalfe and Schmitz (23). The fatty acid methyl esters were separated on a 100 × 0.25 mm Chrompack CPSil 88 column using a Hewlett-Packard model 5890 series II gas–liquid chromatograph equipped with a cool on-column capillary injector, automatic sampler, flame ionization detector, and integrator. During fatty acid separation the column temperature was programmed as follows: 150°C for 0.5 min, 2°C/min to 170°C, 5°C/min to 220°C, and hold at 220°C for 25 min.

Fluorescence polarization measurements

Fluorescence polarization measurements were made with an ISS Model K2 Spectrofluorometer (Champaign, IL) using the

probe TMA-DPH [1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene] (24). The TMA-DPH probe was dissolved in dimethylformamide at a concentration of 10^{-4} m and added to HDL (50 μg/mL PL). The molar ratio of PL:probe was 330:1. The probe was allowed to equilibrate for at least 15 min at the appropriate temperature before measurements were taken. Polarization measurements were taken at 37°C in the L-format (25) with blank subtraction using the following conditions: excitation wavelength = 366 nm, 2 mm slit widths, and KV418 emission filters. The temperature was regulated to ±1°C with a circulating water bath.

Preparation of rHDL comprised of monkey HDL PL and human apoA-I

Particles were prepared using the cholate dispersion/Bio-Bead removal technique as described in detail previously (26). The isolated PL was combined with human apoA-I at a starting mole ratio of 72:1 (PL:protein) to form rHDL as these proportions were determined to result in the most homogeneous complexes. The homogeneity and size of the complexes were assessed by non-denaturing gradient gel electrophoresis using precast 8–25% polyacrylamide gels (Pharmacia Biotech, Inc., Piscataway, NJ). In cases when the electrophoresis indicated that the preparations were heterogeneous, the samples were purified by high performance gel filtration (Superdex 200 HR 10/30 Pharmacia Biotech Inc.). The column was calibrated with standard proteins (26) and the hydrodynamic diameters of the particles were calculated from the elution volumes.

Characterization of rHDL

The particles were analyzed chemically using the Markwell modification of the Lowry protein assay (17) while PL were determined as inorganic phosphorus by the method of Sokoloff and Rothblat (21). The average α-helix content of apoA-I when complexed to PL was determined by circular dichroism spectroscopy using a Jasco J41A spectropolarimeter. Spectra were measured at 25°C in a 0.1-cm path length quartz cuvette as described previously (26); the percent α-helix was determined from the molar ellipticities at 222 nm.

Preparation of cells for measurement of lipid efflux

Mouse L-cell fibroblasts were plated in 22 mm, 12-well plates and grown to confluence in minimal essential medium (MEM) supplemented with 10% fetal calf serum in a 37°C humidified incubator in the presence of 95% air and 5% CO_2 (6). Upon reaching confluence, the cells were labeled with 2 μCi/mL [1,2-^{3}H]cholesterol in bicarbonate-buffered MEM with 1% fetal calf serum for 48 h; the final ethanol concentration was always less than 1% (v/v). This labeling period was followed by an 18-h incubation with MEM containing 0.3% fatty acid-free bovine serum albumin to equilibrate the radioactivity between the various cellular sterol pools.

Efflux of cellular FC

After washing the cell monolayers 3× with MEM containing HEPES (50 mm), FC efflux measurements were initiated by the application of 1.0 mL/well of the test medium, containing the acceptor diluted to the desired PL or protein concentration with MEM. The experiments were conducted in a 37°C incubator with an atmosphere of 95% air and 5% CO_2. The radioactivity in an aliquot of the medium was determined by liquid scintillation counting at specific time intervals to estimate the fraction of FC released into the medium; any cellular material was removed prior to counting by filtration through a 0.45-μm filter. Upon completion of the timecourse, all cell wells were washed with Dulbecco's phosphate-buffered salt solution (PBS) three times and the cellular lipids were extracted with isopropanol (27). From the extraction, the total amount of radioactive FC per well was determined by liquid scintillation counting. Unesterified (free) cholesterol (FC) mass was determined by gas–liquid chromatography analysis (28); cholesteryl methyl ether was utilized as an internal standard in this assay.

Bidirectional flux analysis

For bidirectional flux analysis, monkey HDL was labeled with [^{14}C]cholesterol by exchange of label from acid-washed Celite (29). Briefly, [^{14}C]FC was dried under nitrogen onto 20 mg of acid-washed Celite in a glass scintillation vial, HDL was added to this vial, and the mixture was gently rocked at 37°C overnight. After this incubation period, the HDL was filtered to remove the Celite and aliquots were taken for both radioactivity and FC mass determination. Mouse L-cell monolayers were grown to confluence in 24-well plates and then radiolabeled with [^{3}H]cholesterol as described above. Specific concentrations of the [^{14}C]HDL were incubated with the cells with 1 μg/mL of Sandoz compound 58035 present throughout the cell preparation and experiment to inhibit cholesterol esterification and to ensure that all of the radiolabel was present in the cells as FC. At each timepoint, an aliquot of the HDL-containing media was removed, filtered to remove any cellular material, and the [^{3}H]- and [^{14}C]cholesterol contents were determined by liquid scintillation counting; all radioactivity data were corrected for spillover of the two radiolabels between counting channels. The remaining media were aspirated and the monolayers were washed 3× with phosphate-buffered salt solution. The cellular lipids were extracted from the washed monolayers by incubation with isopropanol, and the [^{3}H]- and [^{14}C]cholesterol present in the monolayers was determined by liquid scintillation counting. Unesterified (free) cholesterol (FC) mass was determined by gas–liquid chromatography analysis as described above.

Data analysis

The fractional release of FC was determined experimentally and analyzed as described in detail for this system previously (6). The kinetic analysis was based on the assumption that the system is closed, and that all lipid therefore exists in either the cellular lipid pool or in the acceptor HDL pool. The equilibration of FC between these two pools was fitted to the single exponential equation $Y = Ae^{-Bt} + E$: Y represents the fraction of radiolabeled lipid remaining in the cells, t is the incubation time, A is a pre-exponential term that reflects the fraction of lipid that exists in the medium at equilibrium, B is a time constant representing the release of FC, and E is a constant that represents the fraction of labeled lipid that remains associated with the cells at equilibrium. These variables were derived by fitting the experimental data to the single exponential equation to give the best fit by nonlinear regression (Graph Pad Prism, Graph Pad Software Incorporated). The apparent rate constant for efflux (k_e), which is dependent on the acceptor particle concentration tested, was derived from these parameters. The apparent half time of efflux value in hours was then calculated as follows: $t_{1/2} = \ln 2/k_e$. The half time values were statistically compared by Student's *t*-test (Graph Pad Prism, Graph Pad Software Incorporated).

For bidirectional flux measurements, the fractional retention of [^{3}H]-labeled FC and the fractional uptake of [^{14}C]-labeled FC by the cells with respect to incubation time were fitted to a model for FC equilibration between two pools (27). This compartmental model assumes that the cell-associated and HDL-associated FC each forms a single kinetic pool, together making up a closed system. The rate constants for FC efflux and influx were calculated from fitting the timecourses (Graph Pad Prism, Graph Pad Software Incorporated) to exponential equations which are of the form $Y = Ae^{-Bt} + E$.

RESULTS

Efflux of cellular FC to HDL obtained after animals received a fatty acid-specific, low cholesterol diet

At the start of the study, the diets of African green monkeys were supplemented with one of four specific fats (fish oil, monounsaturated, polyunsaturated, or saturated) while a low level of cholesterol intake was maintained. Release of FC from radiolabeled mouse L-cell fibroblasts to 100 μg PL/ml HDL obtained from the monkeys at this stage was measured, and the calculated FC efflux over the course of a 6-h experiment is presented in **Fig. 1**. Similar results were obtained when rat Fu5AH hepatoma cells were utilized (data not shown). It is apparent that any modification of the HDL species by the fat-specific diets did not affect the ability of the lipoproteins to accept cellular FC as HDL from all diet groups resulted in efflux of approximately 25% of the cellular FC.

Characterization of HDL after animals received the fat-specific, cholesterol-enriched diet

During the next stage of the study, monkeys were fed diets enriched in cholesterol with one of the four specific dietary fats (**Table 1A**). Sizing the HDL from each diet group by gel filtration chromatography (**Fig. 2**) indicated that the average sizes of the HDL particles were comparable. The mean hydrodynamic diameter of the HDL particles was 10.5 ± 0.3 nm and Student's *t*-test indicated that there were no significant differences between diet groups. The HDL subspecies size distribution was determined by proton NMR and five distinct HDL subfractions were evident (**Table 2**). The average HDL diameter was found to be similar among the fat-specific diet groups with a slightly increased diameter in the polyunsaturated and fish oil groups; these results are consistent with the gel filtration chromatography determinations (Fig. 2) showing the same average size for the HDL from the four diet groups because the small variations detected by NMR are within the resolution of the column. Further characterization of the HDL particles revealed that the fat-specific diets administered did not dramatically alter the distribution of HDL PL classes in the African green monkey; the phosphatidylcholine contents of HDL were 82, 85, 79, and 77% for the saturated, monounsaturated, polyunsaturated, and fish oil groups, respectively. This is consistent with previous findings that fat-specific diets do not affect the HDL PL head group distribution in cynomolgus monkeys (30). The ratio of FC/PL in the HDL also was not influenced by diet; no significant differences were detected between fat-specific diet groups (Table 2).

Treatment with the fatty acid-specific diets led to dramatic changes in the overall distribution of fatty acids within the PL acyl chains (Table 1B), as determined by GLC. The monounsaturated diet resulted in significantly more 18:1 acyl chains (24% of the total chains as compared to 16% for saturated and 8% for either fish oil or polyunsaturated diets) while the fish oil diet resulted in 20:5n–3 (21%), 22:5n–3 (4%), and 22:6n–3 (14%) acyl

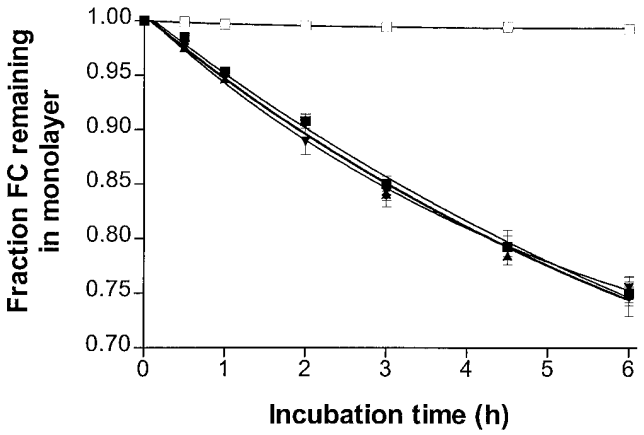

Fig. 1. FC efflux from mouse L-cell fibroblasts to African green monkey HDL following consumption of a fat-specific, low cholesterol diet. Mouse L-cell fibroblasts were grown to confluence in 22-mm tissue culture wells. Two days prior to the experiment, the cells were labeled with 2 μCi [^{3}H]cholesterol/ml minimal essential media supplemented with 1% fetal bovine serum. Twelve h prior to initiation of the experiment, the cells were incubated with 0.3% bovine serum albumin to equilibrate the label between sterol pools. The experiment involved the application of 1 mL of African green monkey HDL at 100 μg PL/mL MEM to the radiolabeled cell monolayers. The plates were incubated in a 5% CO_2 incubator at 37°C, and at specific timepoints from 0 to 6 h, an aliquot of the media was removed, filtered, and analyzed for [^{3}H]cholesterol content. The symbols in the above graph indicates the dietary fat enrichment of the animal from which the HDL were obtained: fish oil (■), monounsaturated (▲), polyunsaturated (▼), and saturated (◆), and represent the mean fraction of FC remaining in the monolayers ±1 SD (n = 6 measured in triplicate for each diet group). Flux to control media was measured in triplicate and is indicated by the open squares.

TABLE 1. Composition of the fatty acid-specific diets and resulting HDL

Diet	16:0	18:0	18:1	18:2	20:5	22:6	Other
	A: Dietary fatty acids[a]						
	% (w/w)						
Fish oil	33.5 (0.6)	3.8 (0.2)	26.8 (0.7)	7.5 (1.1)	7.8 (1.3)	5.7 (1.0)	14.9
Mono.	6.4 (0.2)	2.2 (0.1)	75.0 (0.4)	15.7 (0.4)	0.0 (0.0)	0.0	0.7 (0.0)
Poly.	7.7 (0.2)	2.4 (0.1)	12.9 (0.3)	75.7 (0.5)	0.0 (0.0)	0.0	1.3 (0.0)
Sat.	43.2 (0.3)	4.1 (0.1)	37.9 (0.3)	11.8 (0.2)	0.0 (0.0)	0.0 (0.0)	3.0
	B: HDL PL fatty acids[b]						
Fish oil	22.3	14.7	7.5	5.9	21.2	13.5	14.8
Mono.	18.0	13.4	24.3	22.0	0.1	0.5	21.6
Poly.	16.9	16.3	7.6	44.5	0.0	0.3	14.2
Sat.	23.1	13.4	16.0	30.2	0.13	0.6	16.4

[a]Shown is the percentage of each fatty acid present in the fat-specific diets. Values are the mean of six batches of diet with the exception of the saturated diet group which is the mean of seven batches; numbers in parentheses represent 1 SE.

[b]Shown is the percentage of each fatty acid present in the PL of HDL from monkeys fed the fat-specific diets. Values are from a single analysis of pooled diet-specific HDL samples; SE from this analysis typically represents approximately 2% of the values (14).

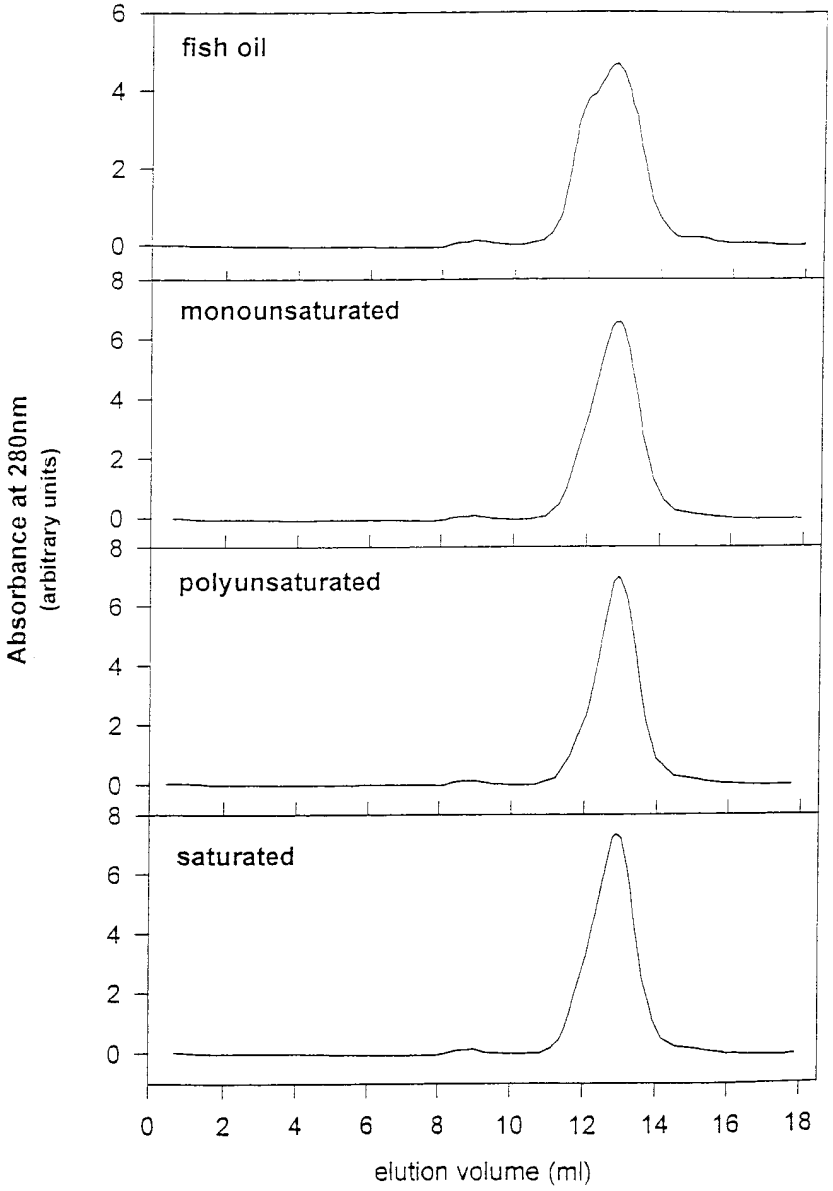

Fig. 2. Elution profiles of African green monkey HDL subjected to gel filtration chromatography. A 1 × 30-cm Superdex HR 200 (Pharmacia Biotech Inc.) gel filtration column was used to analyze HDL isolated from monkey sera after extended consumption of fatty acid-specific, cholesterol-enriched diets. Fractions of 0.5 ml were collected and the protein was detected by absorbance at 280 nm. The void volume of the column was 8.1 ml and the total volume was 20.1 ml; the column was calibrated with standard proteins and the hydrodynamic diameters of the particles were calculated from the HDL elution volumes.

chains, which were generally not present as components of the HDL PL from the other diet groups (<0.5% of the total chains present in the monounsaturated, polyunsaturated, or saturated diet groups). In addition, 18:2n–6 acyl chains were more enriched in the PL with the polyunsaturated-specific diet (45%) as compared to the fish oil (6%), monounsaturated (22%), and saturated diet groups (30%). The overall effect of the fatty acid-specific, cholesterol-enriched diet on the distribution of saturated and unsaturated acyl chains is summarized in **Table 3**. The four diets did not lead to differences in the proportion of saturated acyl chains present in the PL. However, there was a strong influence of the dietary fat supplementation on the occurrence of unsaturated bonds in the HDL PL acyl chains. The fish oil diet resulted in a much greater unsaturated/saturated carbon–carbon bond ratio with respect to the other diet groups and both the polyunsaturated and fish oil diets resulted in a greater fraction of polyunsaturated bonds as compared to the monounsaturated and saturated diet groups.

The effect of the fatty acid modifications on the fluidity of the various HDL particles was determined by measuring the polarization of the fluorescence from TMA-DPH located in the surface of the particles. The results, listed in Table 2, indicate a statistically significant effect of the introduction of polyunsaturated chains on the fluidity of the particles. However, the variation in fluorescence polarization observed with respect to diet group is minimal, ranging from 0.41 to 0.43; for comparison, complete melting of the PL acyl chains in discoidal HDL particles from the gel phase to the liquid crystal phase causes an increase in polarization value for this probe from 0.26 to 0.45 (24). Therefore, the variation between diet groups with respect to the fluidity of the HDL particle surface is minimal; in all cases the PL are in the liquid–crystalline state.

TABLE 2. Characteristics of HDL from African green monkey on fat-specific diets

	Fish Oil	Mono.	Poly.	Sat.	*P* value
			mg chol/dl		
HDL-Chol	23.3 ± 2.6[b,c]	38.3 ± 9.1[a,c]	19.2 ± 1.8[b]	37.7 ± 6.1[a,c]	0.03
HDL 1	11.4 ± 0.6	11.8 ± 1.8	11.3 ± 0.9	12.6 ± 1.5	NS
HDL 2	0.4 ± 0.3[b]	3.9 ± 1.4[a]	0.0 ± 0.9[b]	3.9 ± 1.5[a]	0.008
HDL 3	0.4 ± 0.2[b]	14.0 ± 3.4[a]	0.7 ± 0.2[b]	12.7 ± 1.9[a]	0.001
HDL 4	3.8 ± 1.3	2.2 ± 1.6	0.2 ± 0.1	2.9 ± 1.8	NS
HDL 5	7.3 ± 1.3	6.4 ± 3.7	7.0 ± 1.1	5.5 ± 3.0	NS
Average HDL diameter (nm)	8.6 ± 0.1[a]	8.1 ± 0.1[b]	8.4 ± 0.1[a,c]	8.1 ± 0.1[b,c]	0.03
HDL FC/PL Ratio (w/w)	0.115 ± 0.009	0.096 ± 0.004	0.107 ± 0.005	0.105 ± 0.003	NS
TMA-DPH polarization, 37°C	0.408 ± 0.001[b]	0.433 ± 0.004[a]	0.407 ± 0.002[b]	0.431 ± 0.003[a]	0.001

Values are mean ± SEM. Data were obtained from animals during the cholesterol-enriched (0.6 mg chol/kcal) dietary phase. HDL1-HDL5 represent subfraction cholesterol concentration (mg/dL) determined by proton NMR (36–38); HDL5 is the largest and HDL1, the smallest subfraction. Numbers of individual animals per diet group were: saturated, 13; monounsaturated, 12; polyunsaturated, 14; and fish oil, 12. The average HDL size was calculated as the weighted average for the five subfractions. *P* value was determined by analysis of variance with subsequent post-hoc analysis using Fisher's protected least significant difference test. Unlike letters indicate statistically significant difference at $P < 0.05$; NS, not statistically different at $P = 0.05$. HDL FC/PL ratio was determined for animals in each diet group at a later time during the cholesterol-enriched dietary phase, when dietary cholesterol had been reduced to 0.4 mg chol/kcal to reduce total plasma cholesterol of the animals. TMA-DPH fluorescence depolarization was determined on HDL isolated from a subset of animals (n = 4 per diet group) during the cholesterol-enriched dietary phase (0.4 mg chol/kcal).

Efflux of cellular FC to HDL obtained after animals received a fat-specific, cholesterol-enriched diet

FC efflux from mouse L-cell fibroblasts was determined for individual monkey samples (n = 24; 6 per diet group) or samples pooled together within the four diet groups. Although there was a general trend observed that the HDL of the monounsaturated and saturated diet group removed 1–5% more FC during a 6-h incubation than the other HDL, no consistent statistically significant differences in the ability of the HDL from each diet group to remove cellular FC were detected (**Fig. 3**). The bidirectional flux of FC between the [^{3}H]cholesterol-labeled monolayers and either a high concentration (100 μg PL/mL) (**Fig. 4**) or low concentration (50 μg protein/mL) of [^{14}C]cholesterol-labeled HDL was also determined. Again, despite HDL PL acyl chain variation, no differences were detected in either [^{3}H]cholesterol efflux or [^{14}C]cholesterol influx; this constancy was observed at both the high and low HDL concentrations utilized. Therefore, modification of HDL PL acyl chain composition did not alter the ability of the particle to participate in FC efflux and influx. The timecourses were fitted to a single exponential rate equation to obtain $t_{1/2}$ values of efflux and influx for each particle (**Table 4**). Comparison of the $t_{1/2}$ values by Student's *t*-test confirms the result indicated by Fig. 4: there are no significant differences among diet groups with respect to HDL-mediated FC efflux or influx.

In addition to providing halftimes of FC flux, the computer fitting of the data also generated equilibrium values of the fraction of total FC remaining in the cells; these values were 0.29 ± 0.1 and 0.28 ± 0.04 for the efflux and influx timecourses, respectively. These equilibrium values determined from the fitting are also comparable to the estimated equilibrium value of 25 ± 1% of the total FC associated with the monolayers as derived from the mass of FC in the monolayer and media (1.7 ± 0.1 μg and 4.9 ± 0.2 μg, respectively). No significant differences were observed among diet groups with respect to any of the equilibrium values computed. These manipulations demon-

TABLE 3. Characterization of the African green monkey HDL-PL acyl chain composition after consumption of a fat-specific, cholesterol-enriched diet

	PL acyl chain characteristics		
Fatty Acid-Specific Diet Group	Unsaturated/Saturated Bond Ratio[a]	Saturated Chains/Total Acyl Chains[b]	Polyunsaturated Chains/Total Acyl Chains[c]
Fish oil	0.172	0.376	0.535
Monounsaturated	0.089	0.328	0.409
Polyunsaturated	0.094	0.337	0.574
Saturated	0.089	0.369	0.458

[a] Determined by calculating the total number of unsaturated and saturated C–C bonds in the HDL-PL acyl chains as determined from the peak area of each acyl chain subspecies in GLC analysis.

[b] The ratio of saturated acyl chains with respect to the total acyl chains present was determined by GLC analysis of the acyl chain composition.

[c] The ratio of polyunsaturated chains (≥2 unsaturated bonds) with respect to the total acyl chains present was determined by GLC analysis of the acyl chain composition.

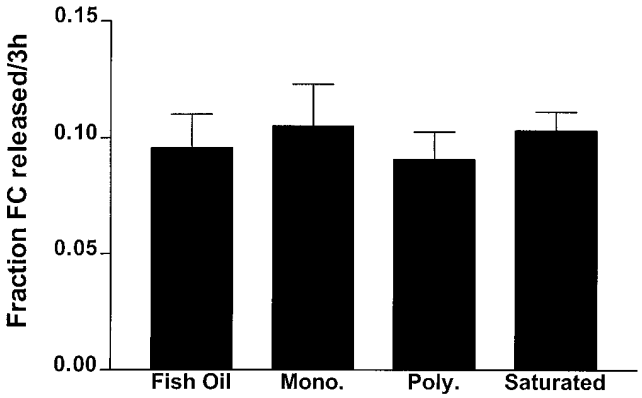

Fig. 3. FC efflux from mouse L-cell fibroblasts to African green monkey HDL after consumption of a fat-specific, cholesterol-enriched diet. Mouse L-cell fibroblasts were grown to confluence and radiolabeled as described in the legend to Fig. 1. The experiment involved the application of 1 mL of African green monkey HDL at 100 μg PL/mL MEM to the radiolabeled cell monolayers. The plates were incubated in a 5% CO_2 incubator at 37°C, and at specific timepoints from 0 to 6 h, an aliquot of the media was removed, filtered, and analyzed for [^{3}H]cholesterol content. The bars in the above graph represent the mean fraction of FC released from the monolayers after 3 h of incubation ±1 SD (n = 6 measured in triplicate for each diet group).

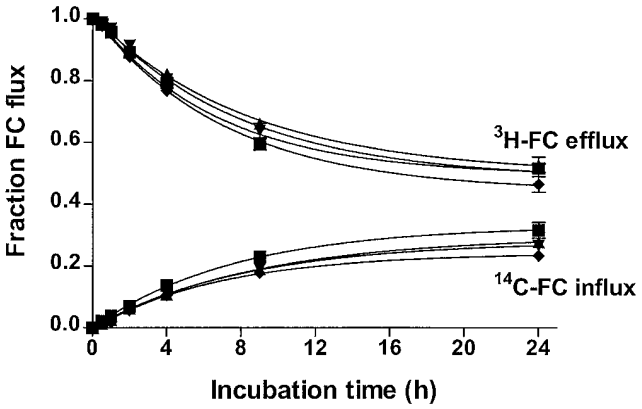

Fig. 4. Bidirectional FC efflux between mouse L-cell fibroblasts and African green monkey HDL. Mouse L-cell fibroblasts were grown to confluence in 24-well plates and radiolabeled as described in the legend to Fig. 1. The experiment involved the application of 0.5 ml of [^{14}C]FC-labeled African green monkey HDL at 100 μg PL/mL MEM to the radiolabeled cell monolayers and all media was supplemented with 2 μg/mL of the ACAT inhibitor 58035 to prevent the esterification of cellular FC. The plates were incubated in a 5% CO_2 incubator at 37°C, and at specific timepoints from 0 to 24 h, an aliquot of the media was removed, filtered, and analyzed for [^{3}H]cholesterol and [^{14}C]cholesterol content. The symbols in the above graph represent the mean fraction of FC efflux from or influx to the monolayers during the timecourse ±1 SD, and also demonstrate the enrichment of the diet prior to obtaining the monkey HDL (fish oil (■), monounsaturated (▲), polyunsaturated (▼), and saturated (◆); n = 3 for each diet group).

strate that this system measuring dual-labeled FC flux accurately traces the FC exchange between the cell monolayers and HDL.

FC efflux to rHDL composed of monkey HDL PL and human apoA-I

To directly investigate the effect of the diet-induced PL acyl chain modification, HDL samples were pooled within their four diet group subdivisions and delipidated. The isolated PL was combined with human apoA-I at a starting mol ratio of 72:1 (PL:protein) to form rHDL particles of similar size. To ensure homogeneous preparations of similar size, the rHDL prepared from the PL of each diet group was further isolated from unreacted protein or phospholipid by gel filtration chromatography, and circular dichroism measurements showed the apoA-I had a similar α-helix content in all four rHDL preparations (% α-helix = 75 ± 1). The rHDL, with an average size of 8.3 ± 0.2 nm, were incubated with radiolabeled L-cell monolayers at concentrations of 25, 50, or 100 μg PL/mL for measurement of a 6-h FC efflux timecourse. Comparable to the results of FC efflux to intact monkey HDL, the timecourses of FC flux to the prepared rHDL present at 100 μg/mL were all similar (**Fig. 5**); the same phenomenon was observed with the lower concentrations (data not shown). In Fig. 5, at 6 h of incubation, there is some suggestion that the rHDL containing the fish oil diet group PL may be less efficient than the others, however this indication is not observed in a comparison of the half-times of efflux (**Table 5**). Taken together, these results demonstrate that dietary modification of African green monkey HDL PL does not influence the ability of the HDL particle to remove cellular FC.

Investigation of the influence of diet on FC efflux to monkey sera

Having found no effect of HDL-PL acyl chain modification on the ability of the particles to accept cellular FC, we questioned whether sera isolated during the high fat, cholesterol-enriched diet period would display variances among fat-specific diet groups (n = 49; 12 per diet group with 13 in the polyunsaturated group). The sera were diluted to 5% and incubated with mouse L-cell fibroblasts for periods up to 6 h (**Fig. 6**). During this time frame, there is very little contribution to cholesterol movement by influx of FC from the sera, thus the radiolabeled FC movement reflects the FC efflux from the monolayers to the sera. Figure 6 demonstrates that while there is variability among individual sera samples, the differences were not defined by the diet group of the monkey. In contrast, the wide range of efflux values within groups is more closely correlated with the range of HDL cholesterol or phospholipid values in the sera samples. A significant positive correlation with cholesterol levels was determined for the monounsaturated ($r^2 = 0.63$, $P < 0.05$) and saturated groups ($r^2 = 0.71$, $P < 0.05$). The fish oil and polyunsaturated groups may have similar effects; however, the cholesterol levels within these groups did not vary markedly. Similarly, efflux was correlated with HDL-PL in the monounsaturated dietary group ($r^2 = 0.90$, $P < 0.05$). These results taken together with the isolated HDL experiments suggest that efflux to sera is more directly a function of the HDL-particle levels and that di-

TABLE 4. Halftimes of bidirectional FC flux between mouse L-cell fibroblast monolayers and African green monkey HDL

FC Movement[a]	Halftimes ($t_{1/2}$)[b]			
	Fish Oil	Monounsaturated	Polyunsaturated	Saturated
Efflux to HDL	10.6 ± 3.3	8.7 ± 2.1	9.3 ± 2.7	10.9 ± 1.7
Influx from HDL	17.4 ± 3.1	21.6 ± 6.0	21.8 ± 3.4	22.3 ± 2.3

Values represent the mean halftime of 6 timecourses ±1 SD. Timecourses are not statistically different among diet groups as determined by Student's *t*-test.

[a] Determined by [^{3}H]FC movement from the monolayers to HDL (100 μg PL/mL) and [^{14}C]FC movement from the HDL to the monolayers.

[b] Efflux and influx halftimes are derived by fitting the experimental timecourse of 24 h to a single exponential equation.

etary modification of the HDL acyl chains does not influence the FC efflux process.

DISCUSSION

The objective of the current study was to investigate the role of dietary fatty acids in offering protection from the onset of atherosclerosis. Experiments were specifically designed to determine whether a fat-specific diet might have an effect on HDL-mediated cellular FC efflux in the first step of reverse cholesterol transport. As discussed below, the results demonstrate that the diet-induced HDL fatty acid modifications apparently do not influence the ability of the lipoprotein to accept cellular FC.

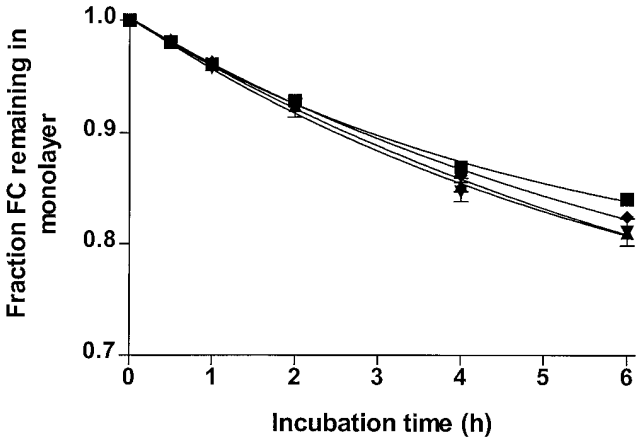

Fig. 5. FC efflux from mouse L-cell fibroblasts to rHDL consisting of human apoA-I and monkey HDL phospholipid. HDL were delipidated to isolate the PL components which were combined with human apoA-I to form rHDL by the cholate/Biobead method. Mouse L-cell fibroblasts were grown to confluence in 22-mm tissue culture wells and radiolabeled as described in the legend to Fig. 1. The experiment involved the application of 1 mL of rHDL at 100 μg PL/mL MEM to the radiolabeled cell monolayers. The plates were incubated in a 5% CO_2 incubator at 37°C, and at specific timepoints from 0 to 6 h, an aliquot of the media was removed, filtered, and analyzed for [^{3}H]cholesterol content. The symbols in the above graph represent the mean fraction of FC efflux from or influx to the monolayers during the timecourse ±1 SD, and also demonstrate the enrichment of the diet prior to obtaining the monkey HDL (fish oil (■), monounsaturated (▲), polyunsaturated (▼), and saturated (◆); n = 3 for each diet group).

Characterization of isolated monkey HDL

The acyl chain modifications induced by the fat-specific diets are consistent with those observed previously in cynomolgus monkeys under similar dietary conditions (30). Very distinct effects of diet are observed upon examination of the lipid acyl chain classes present in the isolated HDL particles. The diet enriched in monounsaturated fatty acids resulted in an HDL PL enrichment of 18:1 acyl chains as compared to the other diet groups, and the fish oil diet led to the noteworthy introduction of n–3 polyunsaturated fatty acids. The percent of saturated acyl chains present in the HDL PL was not affected by diet (Table 3). However, the diets rich in polyunsaturated fatty acids due to either safflower (n–6) or fish oil (n–3) did result in an increased percentage of polyunsaturated chains as compared to the other diet groups and a dramatic result of diet was observed for the fish oil diet group when the unsaturated/saturated bond ratio was calculated. The increase in unsaturated bonds observed as a result of this diet leads to a bond ratio of 0.17 as compared to values of approximately 0.09 for the HDL PL of the other diet groups. Overall these results indicate that the fat-specific diets did lead to distinct HDL PL modifications. The average HDL size was the same, as demonstrated by the elution profiles in Fig. 2 and the proton NMR measurements listed in Table 2. Constancy with respect to HDL size as well as the PL subclass distribution and FC/PL ratios eliminates these potentially complicating factors in the FC efflux measurements.

TABLE 5. Halftimes of efflux from L-cell fibroblast monolayers to discoidal rHDL

Diet Group of Recombinant HDL PL[a]	Halftimes of Efflux[b]
	h
Fish oil	15.2 ± 0.8
Monounsaturated	15.4 ± 2.2
Polyunsaturated	14.2 ± 2.1
Saturated	15.9 ± 0.8

[a] Recombinant HDL were prepared with PL isolated from African green monkey HDL and human apoA-I. The discoidal particles were used in the FC efflux experiment at a concentration of 100 μg PL/mL.

[b] Efflux halftimes were derived by fitting the experimental timecourse of 6 h to a single exponential rate equation. Values are the average of triplicate measurements ±1 SD. Timecourses are not significantly different as determined by comparing halftimes by Student's *t*-test.

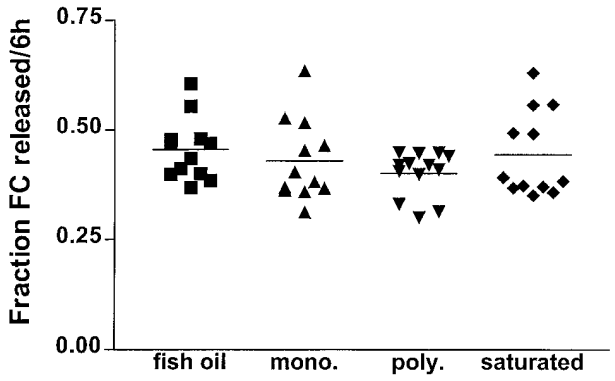

Fig. 6. Efflux of cholesterol from L-cell fibroblasts to 5% monkey sera. Mouse L-cell fibroblasts were grown to confluence in 24-well tissue culture plates in preparation for the measurement of cholesterol efflux. Two days prior to the experiment, the cells were labeled with 1 μCi [^{3}H]cholesterol/mL minimal essential media supplemented with 1% fetal bovine serum. Twelve h prior to initiation of the experiment, the cells were incubated with 1% bovine serum albumin to equilibrate the cholesterol pools. African green monkey sera, diluted to 5% with MEM, was incubated at 0.5 mL/well with the prepared monolayers, and at specific timepoints ranging from 0 to 6 h, an aliquot was removed from the media for liquid scintillation counting analysis. The symbols represent the mean FC efflux to each monkey sample, determined in triplicate at 6 h; horizontal bars demonstrate the mean FC efflux value for each diet group. Student's *t*-test indicated no significant differences among the diet groups.

Cholesterol efflux to diet-modified HDL

As shown in Figs. 3 and 4, there was no significant difference in the ability of the HDL particles from different diet groups to accept cellular FC. This suggests that diet-induced acyl chain modification of HDL did not influence the function of the particles in cellular FC efflux. As bidirectional flux of FC between HDL and cell monolayers has been described (27), we also examined whether influx of FC from the HDL may be influenced by the dietary fat modifications. Again, no detectable differences in HDL functionality were observed. These results, also supported by the computer-generated half-times of efflux and influx (Table 4), demonstrate that overall FC exchange was not influenced by modification of the HDL PL.

Sola et al. (11) have reported that human HDL_3 isolated after individuals received a monounsaturated fatty acid-enriched diet induces greater FC efflux from fibroblasts than lipoproteins isolated after individuals received saturated-, polyunsaturated-, or fish oil-specific diets. The reason for the discrepancy from our results is not entirely clear. The conflicting results do not appear to be a result of variability in the effect of diet on HDL PL acyl chain modification in humans versus non-human primates. The African green monkey HDL were dramatically influenced by the fat-specific diet as compared to the human study; for instance, the level of polyunsaturated fatty acyl chains present in the monkey HDL ranged for the various diet groups from 41 to 57% (Table 3), whereas the human HDL varied only from 46 to 49% (11). Examination of the specific acyl chains present in the HDL lends further support to the dietary effects being greater in the experimental model of the current work. It may be significant that the prior work, suggesting a relationship between the fluidity of HDL and the ability of the particle to incorporate cellular FC (11), utilized a probe that monitors the microenvironment within the core of the HDL particle whereas the TMA-DPH probe utilized in the current work measures the fluidity of the PL layer at the particle surface (24). However, the polarization values reported by Sola and colleagues (11, 31) with human HDL did not display any greater variability among diet-specific groups than that measured in the current study with monkey HDL. As far as the measurement of cellular cholesterol efflux is concerned, Sola et al. (11) incubated isolated HDL_3 with human fibroblasts at 50 μg protein/ml and analyzed FC efflux at a single 24-h timepoint; [^{3}H]cholesterol efflux values ranged from 35% (monounsaturated diet group) to 12% (fish oil group). In the current study, detailed kinetics (0–24 h) were measured for the bidirectional FC exchange between mouse fibroblasts and HDL utilized at several concentrations, including that used in the human HDL application. No disparities were detected in the ability of the HDL to remove FC, regardless of diet group, at any of the concentrations or timepoints. Differences between the two studies are more likely due to aspects of the human HDL particles other than the acyl chain content. Variables such as the heterogeneous particle size and HDL_3 cholesteryl ester content appear to have contributed to the monounsaturated fat induction of efflux observed with the human HDL, rather than the acyl chain modifications. Furthermore, the human HDL samples had variable PL/protein ratios, which suggests that the species were not incubated with the monolayers at a constant particle number, an important determinant of efficient efflux (7).

Construction of rHDL and measurement of FC efflux

The effect of HDL PL modification on cellular FC efflux was directly examined through constructing rHDL comprised of isolated monkey HDL PL and human apoA-I. PL was isolated from HDL from each diet group and successfully combined with apoA-I to form rHDL discoidal particles of identical size. The apoA-I associated with the PL in a similar manner to form the complexes, despite variations in the PL acyl chain composition, as circular dichroism measurements indicated the same α-helix content for each rHDL species. Therefore, this experiment eliminated variables that could affect HDL-mediated efflux, and any differences observed in rHDL-mediated efflux would evidently be a direct result of variations in acyl chain composition. Upon incubation of these well-characterized complexes at both low and high concentrations with mouse L-cell fibroblast monolayers, no differences were observed between the abilities of the acceptor particles to remove cellular FC. To our knowledge this approach has not been carried out previously and clearly demonstrates that the diet-induced HDL PL acyl chain modifications are not sufficient to affect the FC efflux from L-cell monolayers to HDL particles.

Previously we have combined synthetic PL of varying

acyl chain content with apoA-I to form well-defined rHDL particles (8). These complexes demonstrated an inverse relationship between the degree of acyl chain saturation or length with efficient FC efflux, presumably due to changes in the physical state of the lipid from a quasi-crystalline packing to a liquid–crystalline packing (8). The acyl chain modifications due to diet as presented in the current study are subtle as compared to those introduced through the use of various synthetic PL and thus did not result in the same influence of acyl chain content on the ability of the particles to incorporate cellular FC. For instance, the halftime of efflux decreased from 33 h to 11 h when a double bond was introduced into half of the acyl chains through the use of rHDL constructed with 1-palmitoyl 2-stearoyl phosphatidylcholine (16:0, 18:0) or 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) (16:0, 18:1), respectively. The diet-induced modifications of the current investigation did not alter the percentage of saturated chains present (Table 3) and no change in $t_{1/2}$ for efflux was measured (Table 5). However, when the unsaturated/saturated bond ratio was modified by constructing rHDL of POPC or 1,2-dioleoyl phosphatidylcholine (18:1, 18:1) (ratio of 0.03 and 0.06, respectively), no changes in FC efflux rates were observed (8). This 2-fold alteration in bond ratio is consistent with that introduced as a result of the fish oil diet as compared to the other diet groups (Table 3), demonstrating consistent results between the two studies. Therefore, although dietary fatty acids were significantly incorporated into the HDL PL, the diet-induced modifications did not alter the liquid–crystalline state of the phospholipid, and consequently did not affect the ability of the particles to remove cellular FC.

Cholesterol efflux to diet-modified sera

As no variability in HDL-mediated FC efflux was detected, sera were incubated with the cell monolayers to determine whether diet influences other aspects of the first step of reverse cholesterol transport. To simplify the system, we focused on [^{3}H]cholesterol movement during the first 6 h of sera exposure, when FC movement should predominantly be a result of FC efflux. Again, no differences among diet groups were detected, reinforcing the conclusion that dietary modification of lipoproteins does not effect the initial FC efflux. In the monounsaturated and saturated diet groups, which exhibit a distinct spread of HDL cholesterol levels in the sera samples, a significant correlation was observed between HDL cholesterol concentration and cellular FC efflux. Similarly, the monounsaturated diet group has a wide range of HDL PL levels among samples which is positively correlated with initiation of FC efflux. These results suggest that the quantity of HDL particles present is a greater determinant of efficient FC efflux than the PL-acyl chain composition of these species.

Role of diet-induced acyl chain modification in atherosclerosis

The results from this study indicate that a cholesterol-enriched, fat-specific diet does not affect HDL PL in a manner that enhances or inhibits the ability of the lipoprotein to accept cellular FC. Therefore, the protection offered against atherosclerosis by particular fatty acids must influence another aspect of cholesterol metabolism.

With respect to the role of HDL in reverse cholesterol transport, acyl chain modification may have a greater influence on the reactivity of the lipoprotein with lecithin:cholesterol acyltransferase (LCAT) or the exchange and transfer of cholesteryl ester mediated by cholesteryl ester transfer protein (CETP). In fact, n–3 fatty acids have been shown to decrease the reactivity of HDL PL with LCAT in similar monkey models (14, 30). The sera samples of the current study displayed some variations in LCAT reactivity with respect to diet group (data not shown); further experiments need to be carried out to confirm the trends observed. Data from fish oil-fed animals also suggest decreased recognition of n–3 cholesteryl esters by CETP (14). Therefore, steps in RCT subsequent to the initial efflux of cellular FC may be more affected by the fat-specific diets. In vivo, however, the fat-specific diets will also result in modification of the plasma membrane PL (32). Cellular changes of this nature have been suggested to play a role in determining the efficiency of FC efflux (33, 34), thus the in vivo combination of cellular acyl chain modification as well as HDL PL alterations may lead to a greater dietary influence on cellular FC efflux.

The protective or damaging effects of specific dietary fats may also affect other aspects of the progression of atherosclerosis. Despite the negative effects of fish oil-enriched diets on reverse cholesterol transport as described above, polyunsaturated fat protects African green monkeys from coronary artery atherosclerosis (13, 35). This anti-atherogenic effect has been explained by dietary-induced decreases in LDL concentration, size, and cholesteryl ester content. In this manner, fatty acids may play a central role in protection against coronary heart disease.

Overall, this study clearly demonstrates that dietary alteration of HDL PL acyl chains does not influence the ability of the lipoprotein to accept cellular FC. Acyl chain modification of HDL and other lipoproteins appears to have a greater impact on the functionality of these species at other steps in cholesterol metabolism. Further in vivo studies will clarify the advantages and disadvantages of specific fatty acid-enriched diets as related to the onset of atherosclerosis.

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