Effects of LDL enriched with different dietary fatty acids on cholesteryl ester accumulation and turnover in THP-1 macrophages

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Abstract **LDL enriched with either saturated, monounsaturated, n-6 polyunsaturated, or n-3 polyunsaturated fatty acids were used to study the effects of dietary fatty acids on macrophage cholesteryl ester (CE) accumulation, physical state, hydrolysis, and cholesterol efflux. Incubation of THP-1 macrophages with acetylated LDL (AcLDL) from each of the four diet groups resulted in both CE and triglyceride (TG) accumulation, in addition to alterations of cellular CE, TG, and phospholipid fatty acyl compositions reflective of the individual LDLs. Incubation with monounsaturated LDL resulted in significantly higher total and CE accumulation when compared with the other groups. After TG depletion, intracellular anisotropic lipid droplets were visible in all four groups, with 71% of the cells incubated with monounsaturated AcLDL containing anisotropic lipid droplets, compared with 30% of cells incubated with n-3 AcLDL. These physical state differences translated into higher rates of both CE hydrolysis and cholesterol efflux in the n-3 group. These data suggest that monounsaturated fatty acids may enhance atherosclerosis by increasing both cholesterol delivery to macrophage foam cells and the percentage of anisotropic lipid droplets, while n-3 PUFAs decrease atherosclerosis by creating more fluid cellular CE droplets that accelerate the rate of CE hydrolysis and the efflux of cholesterol from the cell.**—Lada, A. T., L. L. Rudel, and R. W. St. Clair. **Effects of LDL enriched with different dietary fatty acids on cholesteryl ester accumulation and turnover in THP-1 macrophages.** ***J. Lipid Res.*** **2003.** 44: **770–779.**

Supplementary key words triglyceride • physical state • fatty acyl composition

The association of a high-fat, high-cholesterol diet with atherosclerosis has made dietary modification an important method for reducing cardiovascular disease risk. Both epidemiological and dietary studies have shown the benefits of replacing saturated with unsaturated fats (1–7); however, there is debate over which type of fat, monounsaturated or polyunsaturated, should replace saturated fats in the diet. Support for the benefits of a monounsaturated fat diet is based primarily on epidemiological evidence from such studies as the Seven Countries study (1) and the possible positive effect a monounsaturated fat diet has on the plasma lipid profile (8–11). These studies demonstrated that replacing saturated fats with monounsaturated fats lowers LDL concentrations, whereas substituting polyunsaturated for saturated fats lowers both LDL and HDL concentrations.

The use of animal models has provided valuable insight into this debate through the generation of more definitive endpoints than would be possible in human studies. In studies with both African green monkeys and transgenic mice, despite the positive effects of monounsaturated fats on the plasma lipid profile, monounsaturated-fat fed animals developed similar levels of atherosclerosis as saturated fat-fed animals, while animals fed polyunsaturated fats developed less atherosclerosis (12, 13). These studies concluded that the monounsaturated-fat diet resulted in the production of a more atherogenic LDL particle, and that the atherogenicity of the LDL particle may be more important than absolute LDL and HDL concentrations (12, 13).

To better understand the mechanism underlying the differential effects of dietary fatty acids on atherosclerosis, we have examined the effects of fatty acid composition on macrophage cholesteryl ester (CE) metabolism. The hydrolysis of cellular CE and the subsequent efflux of cholesterol from the cell begin the process of reverse cholesterol transport, which can reduce macrophage CE concentrations and lead to the regression or remodeling of atherosclerotic lesions (14–18). The rate of hydrolysis of CE from cytoplasmic lipid droplets by neutral CE hydrolase depends on several factors, including the other lipid components of droplets, such as triglyceride (TG),

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Abbreviations: AcLDL, acetylated LDL; EC, esterified cholesterol; FC, free cholesterol; TG, triglyceride.

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and the degree of unsaturation of the acyl chains, both of which can affect the physical state of CE (19–22). CE can exist in several physical states, including crystalline, liquid-crystalline, and liquid, and can form two liquid-crystalline states, smectic and cholesteric (23–25). CE in a liquid (isotropic) state has been reported to be hydrolyzed faster than when in a liquid-crystalline (anisotropic) state, and the increased rate of hydrolysis can lead to an increase in the rate of cellular cholesterol efflux if an acceptor is present in the culture medium (19, 21, 22).

In the presence of high concentrations of cellular TGs, CEs become isotropic, and are hydrolyzed and effluxed more rapidly from cells than when very low levels of TG are present (21, 22). The fatty acyl composition of CEs also affects the physical state, with the more unsaturated the fatty acyl chain, the lower the transition temperature from an anisotropic to an isotropic physical state (19, 20, 22, 26–29). At body temperature (37°C), when CE fatty acids are mainly unsaturated, such as n-6 or n-3 polyunsaturated fatty acids, CE is in a liquid state, and when saturated or even monounsaturated fatty acids predominate, the physical state becomes liquid-crystalline (25, 29–32).

The effect of CE fatty acyl composition on cellular CE hydrolysis and cholesterol efflux, however, has proven difficult to demonstrate. In order to clearly demonstrate the effect of CE fatty acyl composition on CE physical state, hydrolysis, and cholesterol efflux in vitro, only minimal amounts of TG should be present. This will not only mimic the lipid composition of atherosclerotic lesions in vivo, which contain only 8–10% of total lipid as TG (33–38), but will minimize the effect of TG on CE physical state as well.

The present study was undertaken to determine the effects of different dietary fatty acids on macrophage CE accumulation, hydrolysis, physical state, and cholesterol efflux. For these studies, we utilized LDL isolated from the plasma of African green monkeys fed diets enriched with different fatty acids to produce macrophage foam cells with different fatty acyl compositions. The removal of cellular TGs prior to the measurement of CE hydrolysis and efflux (39) allowed for the study of the effect of fatty acyl composition alone without the confounding presence of cellular TG.

METHODS

Materials

Triacsin D was a generous gift from Dr. Satoshi Omura, The Kitasato Institute, Tokyo, Japan. CP113 was a gift from Pfizer, Inc., Groton, CT. RPMI 1640 cell culture medium, penicillin-streptomycin, L-glutamine, vitamins, and PBS were purchased from Cellgro by Mediatech, Herndon, VA. FBS was purchased from Atlanta Biologicals, Norcross, GA, and was heat inactivated at 56°C for 30 min before use. β-mercaptoethanol, Falcon culture dishes (35 mm), acetic anhydride, isopropanol, hexane, ether, iodine, glycerol, sodium azide, gluteraldehyde, microscope slides, 25 mm circular cover slips, and TLC plates were purchased from Fisher Scientific, Suwanee, GA. Glucose, phorbol 12-myristate 13-acetate (PMA), ethylenediamine-tetra acetic acid (EDTA), benzamidine, essentially fatty acid-free BSA, and 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical, Inc., St. Louis, MO. Aprotinin was purchased from Calbiochem Corporation, San Diego, CA. Triglycerides-GB enzymatic assay kit was purchased from Roche Diagnostics, Indianapolis, IN. [1,2,^{3}H(N)]cholesterol was purchased from NEN Life Science Products, Boston, MA. Cyto-Scint scintillation cocktail was purchased from ICN Biomedicals, Inc., Irvine, CA, and stigmasterol was purchased from Steraloids, Wilton, NH.

Cell culture

The human THP-1 monocyte-macrophage cell line was used for these studies. Cells were grown in the monocytic form in suspension at 37°C in 5% CO_2-95% air in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2-β-mercaptoethanol (50 μM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), glucose (1.5 mg/ml), and vitamins. This medium without FBS will be referred to as base medium. To convert THP-1 monocytes to the macrophage phenotype, 1×10^6 cells were plated into 35 mm culture dishes in base medium containing 10% FBS and 50 ng/ml PMA. For all experiments, PMA was added to the medium for the entire experiment to ensure that the cells remained fully differentiated into the macrophage phenotype. The cells were incubated with PMA for 72 h before use in experiments.

Lipoprotein isolation

LDL was isolated from the plasma of African green monkeys fed diets enriched in either saturated, monounsaturated, n-6 polyunsaturated, or n-3 polyunsaturated fats as previously described (40, 41). Briefly, the saturated fat diet contained 29% of the fatty acids as palmitic acid, the monounsaturated fat diet contained 58% of the fatty acids as oleic acid, the n-6 polyunsaturated fat diet contained 58% of the fatty acids as linoleic acid, and the n-3 polyunsaturated fat diet contained 9% and 7% of the fatty acids as eicosapentaenoic acid and docosahexaenoic acid, respectively. Each diet group contained 11–13 animals, four animals from the saturated, monounsaturated, and n-6 groups, and five animals from the n-3 group were used for these studies. Twenty to thirty milliliters of blood from each animal was drawn into tubes containing EDTA (1 mg/ml), and plasma was separated by centrifugation at 2,750 rpm for 30 min. Twenty microliters of a protease inhibitor cocktail consisting of 0.02 mg/ml aprotinin, 7.6 mg/ml benzamidine, and 1.25% sodium azide was added per ml of plasma. The plasma from the animals in each group was pooled, and LDL was isolated from pooled plasma by sequential ultracentrifugation. The plasma was overlayered with saline-EDTA at 1.006 g/l and centrifuged for 20 h in a Beckman L8-55R ultracentrifuge at 36,000 rpm in a SW-40 rotor to remove VLDL. The bottom layer was adjusted to 1.08 g/l with solid KBr and overlayered with saline-EDTA that had been adjusted to 1.063 g/l with solid KBr and centrifuged for 24 h at 36,000 rpm in a SW-40 rotor to float the LDL. The LDL was isolated by tube slicing and dialyzed extensively against a solution containing 0.9% NaCl and 0.01% EDTA. The isolated lipoproteins were sterilized by filtration through a Millipore filter (0.45 μm) and stored at 4°C under N_2. LDL cholesterol content was determined by the method of Allain et al. (42), and LDL-TG content was determined by the method of Fossati and Prencipe (43). LDL molecular weight was determined through the chromatographic separation of lipoproteins using a standard curve made from known standards (44). LDL from each group was acetylated by the acetic anhydride method of Basu et al. (45), and the percent acetylation was assessed by the 2,4,6-trinitrobenzene sulfonic acid assay (46).

Modulation of cellular lipid composition

THP-1 macrophages were maintained in culture in the presence of PMA for 3 days before they were loaded with lipid by incubation with acetylated LDL (AcLDL). Cells were incubated with 100 μg AcLDL protein per ml in base medium containing 1% FBS for 3 days as indicated in the figure legends.

Incubation with triacsin D and albumin was used to modulate the cellular TG concentration as described previously (39). Briefly, THP-1 macrophages were loaded with lipids through incubation with AcLDL as indicated above. The AcLDL-containing medium was removed, and the cells were washed with base medium and then incubated with medium consisting of 12.5 μM triacsin D in base medium containing 400 μM BSA. This will be referred to as "triglyceride removal medium."

Measurement of cellular lipid and protein content

After removing the culture medium, the cells were washed three times with cold PBS, allowed to dry, and the lipid extracted overnight with 2 ml isopropanol containing 20 μg stigmasterol as an internal standard. The isopropanol extract was analyzed for free cholesterol (FC) and total cholesterol (TC) content by gas-liquid chromatography by the procedure of Ishikawa et al. (47) as modified by Klansek et al. (48). Esterified cholesterol (EC) mass was calculated as the difference between TC and FC. Cellular TG concentrations were determined by enzymatic assay on a portion of the isopropanol extract using a kit from Roche Diagnostics (Triglycerides-GB, Cat. No. 450032). Protein was measured in cells and lipoproteins by the method of Lowry et al. (49). Cell viability was determined by a colorimetric assay, which measures the reduction of MTT to form a blue formazan product (50), as modified by Denizot and Lang (51).

LDL and cellular lipid fatty acid analysis

Total LDL fatty acid composition was determined using 0.2 mg of LDL protein from each group. Lipids were first extracted by the method of Bligh and Dyer (52), and then the fatty acids were saponified and methylated for analysis by gas-liquid chromatography (53). For analysis of cellular lipids, a portion of the isopropanol extract was subjected to TLC, and the phospholipid (PL), TG, and CE bands were recovered. The fatty acids from each band were saponified and methylated for analysis by gas-liquid chromatography (53).

Cholesterol efflux

AcLDL was labeled with [^{3}H]FC (10 μCi/mg AcLDL protein) as described previously (54). THP-1 cells were lipid loaded through an incubation with 100 μg/ ml [^{3}H]FC-labeled AcLDL for 3 days in base medium containing 1% FBS. Cells were washed with base medium and equilibrated in medium containing 1% BSA for 24 h. To compare cholesterol efflux in cells with different TG concentrations, 2 ml of efflux medium consisting of 10% FBS and 1.25 μg/ml of the ACAT inhibitor, CP113, were added to one set of dishes following the equilibration period. To a second set of dishes, TG removal medium was added for 24 h, after which efflux medium was added for 24 h. During the 24 h efflux period, at time points of 4, 8, 12, and 24 h, 200 μl aliquots of the efflux medium were taken. The aliquots were centrifuged to pellet any cell debris, and a 100 μl aliquot of the supernatant fluid was taken for scintillation counting. In addition, 50 μl of the supernatant fluid was used to determine the percent FC in the efflux medium after extraction of lipids by the method of Bligh and Dyer (52) and separation by TLC. Cellular isopropanol lipid extracts were analyzed for cholesterol and TG mass, and for FC and CE [^{3}H]DPM after separation by TLC. Percent cholesterol efflux was calculated by dividing total [^{3}H]FC in the efflux medium by the sum of [^{3}H]FC and EC present in the cells at zero time. Percent CE hydrolysis after 24 h was calculated by dividing the increase of [^{3}H]FC in both the cell and media by the [^{3}H]CE present in cells at 0 time.

Polarizing light microscopy

THP-1 cells were plated on 25 mm round glass cover slips in 35 mm culture dishes. For microscopy, cells were first fixed in 2% gluteraldehyde in PBS for 10 min at room temperature. The cover slip was then placed inverted onto a drop of glycerol on a glass slide. Cells were examined at room temperature using a neofluor (N.A. 1.3) oil immersion 100× objective with both phase contrast and polarizing optics on a Zeiss Axioplan microscope. Polarizing filters were crossed at 180°C, and cells were examined for the appearance of a formée cross, which is a positive sign of birefringence and is consistent with lipid droplets being in the liquid-crystalline or anisotropic physical state (24, 25, 27, 29, 33, 35). To evaluate the percentage of cells that contained formée crosses, three slides from each diet group were prepared as described above. An observer was blinded to the experimental groups then randomly selected 10 fields from each slide and first counted the number of cells in the field using a 100× objective. The two polarizing filters were then crossed, and the cells in the same field that contained formée crosses were counted. For each diet group, the percent of cells containing formée crosses was calculated by dividing the number of cells containing formée crosses by the total number of cells present. In addition, within an individual cell the percentage of lipid droplets that displayed formée crosses was determined. Using two slides from each diet group, 10 cells from each slide were randomly selected. Cellular lipid droplets within a single cell were first counted using a 100× objective, and then formée crosses visible with polarizing microscopy in the same cell were counted. With these numbers, the percentage of lipid droplets within single cells containing formée crosses was determined.

Statistical analysis

All data are presented as the mean of triplicate samples ± SD. ANOVA was used to determine if a difference was present among groups, and the Student-Newman-Keuls test was used to determine differences between groups. Differences less than $P = 0.05$ were considered to be significant.

RESULTS

In order to study the effects of dietary fatty acids on macrophages in vitro, we utilized LDL isolated from the plasma of African green monkeys fed diets enriched in either saturated, monounsaturated, n-6 polyunsaturated, or n-3 polyunsaturated fatty acids. The plasma from the animals in each diet group was pooled, the LDL was isolated, and the fatty acid composition of a sample from each group of LDL was determined (**Table 1**). LDL from the saturated group (Sat) had a slightly higher percentage of 14:0 and 18:0 than the other groups, and a higher percentage of 16:0 than the monounsaturated (Mono) and n-6 polyunsaturated (n-6) groups. 18:1 Fatty acids accounted for the highest percentage of fatty acids in the Mono group, more than double the percentage of 18:1 found in the other diet groups. Similarly, 18:2 fatty acids accounted for the highest percentage of fatty acids in the n-6 group, and this percentage of 18:2 was the highest among the four groups. The n-3 polyunsaturated group (n-3) con-

TABLE 1. Percent total fatty acyl composition of LDL isolated from African green monkeys consuming different dietary fats

Diet Group	Fatty Acids [a]							
	14:0	16:0	18:0	18:1	18:2	20:4	20:5	22:6
	% of total fatty acids							
Sat	1.5	20.4	10.0	20.6	37.7	3.3	–	1.2
Mono	0.3	14.7	8.1	49.2	22.2	2.6	–	0.9
n-6	0.5	13.9	7.7	20.8	53.3	1.4	–	0.4
n-3	0.6	25.0	8.7	21.2	9.9	4.3	16.7	6.3

LDL was isolated from the pooled plasma from four or five animals in each diet group as described in Methods. Lipids were extracted and the fatty acids in the lipid extract were then saponified, methylated, and separated by gas-liquid chromatography. The values for each fatty acid are a percentage of total fatty acids present. The percentages shown account for 93–98% of the total fatty acids identified, and identified fatty acids not included each accounted for 0.1–4% of total fatty acids. The fatty acid composition of the LDL from the four diet groups was measured on three separate LDL preparations with similar results.

[a] Fatty acids are designated by chain length: number of double bonds.

tained 16.7% and 6.3% of the n-3 polyunsaturated fatty acids 20:5 and 22:6, respectively. While n-3 fatty acids were not the primary fatty acids in the n-3 group, there was greater enrichment in these fatty acids compared with the other diet groups, which only contained marginal amounts.

In addition to the fatty acid composition, the LDL was also characterized for its lipid content. **Table 2** displays the TC, FC, EC, and TG composition of the LDL from each diet group, in addition to the average LDL molecular weight. Since the TC, FC, EC, and TG analysis was done on a pooled sample of LDL from each diet group, statistical comparisons were not possible. However, the trends were consistent with the molecular weight values, which were determined separately for each animal in the diet groups. LDL from the Mono group had the highest TC, FC, and EC content. Surprisingly, the Sat group contained almost twice as much TG compared with the Mono and n-6 groups, which were equivalent. The n-3 group had the lowest TG content, consistent with the known ability of n-3 fatty acids to lower plasma TG (55–59). Mono LDL also had the highest molecular weight, although only reaching significance compared with the Sat group, but these results agree with past reports that feeding a monounsaturated fat diet results in the production of a more atherogenic LDL particle based on its increased molecular weight and increased cholesterol oleate content (12, 13).

The pooled LDLs from each diet group were acetylated, labeled with [^{3}H]FC, and incubated for 3 days with THP-1 macrophages. The fatty acid compositions of cellular lipids are shown in **Table 3**. Cellular CEs in the Mono group were enriched in 18:1 fatty acids to a similar percent as the Mono LDL. Cellular CEs from the n-6 group had 20.6% of fatty acids as 18:2, which was lower than the 53.3% found in the n-6 LDL, but this percentage was higher than the level of 18:2 fatty acids found in cells in the other diet groups. Similarly, for the n-3 group enrichment of CEs with 20:5 and 22:6 fatty acids was lower than in the LDL

TABLE 2. Lipid composition and molecular weight of LDL isolated from African green monkeys consuming different dietary fats

Diet Group	TC	FC	EC	TG	LDL Mw
	mg/mg				*g/μmol*
Sat	2.15	0.45	1.70	0.122	2.79 ± 0.47[b]
Mono	2.54	0.63	1.91	0.065	4.13 ± 0.39[a]
n-6	2.23	0.51	1.72	0.065	3.53 ± 0.70[a,b]
n-3	2.05	0.44	1.61	0.056	3.33 ± 0.36[a,b]

EC, esterified cholesterol; FC, free cholesterol; TC, total cholesterol; TG, triglyceride. LDL was isolated as described in Table 1. Total lipids were extracted, and TC, FC, and EC mass were measured as described in Methods. The values shown are expressed as mass of the indicated lipid per mg of LDL protein. LDL molecular weight (LDLMw) was determined for each animal individually, and the value shown is the mean and standard deviation of the animals in each group. There were four animals in the Sat, Mono, and n-6 groups, and five in the n-3 group. The grand group means for LDL molecular weight for all animals within each diet group after 49 months on diet were: Sat: 3.55 ± 0.14; n = 12, Mono: 4.20 ± 0.18, n = 11; n-6: 4.04 ± 0.10, n = 13; n-3: 2.98 ± 0.15, n = 12. The lower molecular weight for the Sat group shown in the table combined with the elevated TG suggests that these animals may not have been fully fasted. The lipid composition of three separate preparations of LDL was measured, and values did not differ from those shown above by more than 3%.

[a,b] Significant differences ($P < 0.05$) are represented by different letters.

TABLE 3. Fatty acyl composition of lipids from THP-1 cells incubated with AcLDL isolated from African green monkeys consuming different dietary fats

Lipid Diet Group	Fatty Acids[a]							
	14:0	16:0	18:0	18:1	18:2	20:4	20:5	22:6
	% of total fatty acids							
CE								
Sat	4.8	36.6	10.2	25.2	13.1	1.2	–	2.1
Mono	4.2	29.0	10.4	42.5	6.8	0.9	–	1.6
n-6	5.6	37.9	9.5	20.0	20.6	0.8	–	1.5
n-3	5.8	39.9	6.9	24.8	4.1	1.5	5.5	4.5
TG								
Sat	3.4	43.6	11.0	17.9	15.7	0.6	–	0.5
Mono	3.0	38.8	10.0	29.7	10.9	0.5	–	0.4
n-6	3.0	44.2	11.5	16.6	18.6	0.4	–	0.4
n-3	3.9	56.2	9.9	16.6	3.7	0.6	–	0.9
PL								
Sat	1.8	31.3	15.9	13.8	25.9	–	–	4.5
Mono	1.5	21.9	12.0	21.5	19.2	7.3	1.8	4.1
n-6	1.3	25.4	13.9	11.7	28.8	5.7	1.5	3.0
n-3	1.7	30.3	11.6	14.0	6.8	6.9	10.5	5.8

AcLDL, acetylated LDL; CE, cholesteryl ester; PL, phospholipid. THP-1 cells were incubated with 100 μg/ml AcLDL from each of the four diet groups for 3 days in medium containing 1% FBS. Cells were then harvested, and cellular lipids were extracted by overnight incubation with isopropanol. Extracted lipids were separated by TLC, and the CE, TG, and PL bands were isolated. The fatty acids in each band were then saponified, methylated, and separated by gas-liquid chromatography. The values for each fatty acid are a percentage of total fatty acids present. The percentages shown account for 88–96% of the total fatty acids identified, and identified fatty acids not included each accounted for 0.1–4% of total fatty acids. The data shown are from a single analysis of one experiment; however, the percent composition of CE, TG, and PL from two other experiments done in an identical fashion using separate isolates of LDL, did not differ by more than 5%.

[a] Fatty acids are designated by chain length: number of double bonds.

used, but the enrichment with these fatty acids was greater than in the other groups. For all four groups, 16:0 represented the main fatty acid found in TGs. In the Mono group, there was a higher percentage of 18:1 fatty acids in TGs than in the other groups, and to a lesser extent, 18:2 fatty acids were the highest in the n-6 group. In contrast, the enrichment of n-3 LDL with 20:5 and 22:6 fatty acids was not reflected in cellular TGs. However, cellular PLs were enriched with a higher percentage of these n-3 fatty acids than was seen in CEs. There was also enrichment in PLs in 18:2 fatty acids for the n-6 group and 18:1 fatty acids for the Mono group.

One way in which the different LDLs could affect the progression of atherosclerosis is by inducing a greater accumulation of cholesterol within macrophages. To test this, THP-1 cells were incubated with AcLDL from the four diet groups under identical conditions for 3 days, and cellular cholesterol and TG mass were measured (**Table 4**). In all four groups, the EC concentrations were markedly increased over that of the control cells, thus resembling macrophage foam cells of atherosclerotic lesions. Mono LDL caused significantly higher TC and EC accumulation, and n-3 LDL caused significantly lower EC accumulation compared with the three other groups. There were no significant differences among the Sat, n-6, and n-3 groups in TC accumulation, and no differences among any of the groups in FC accumulation. Despite a wide range in TG content of the LDLs, TG accumulation among the Sat, Mono, and n-6 groups was not significantly different. TG accumulation in the n-3 group was less than in the others, reaching statistical significance with the Sat and Mono groups. This reflected the reduced TG content of the n-3 LDL; however, the increased TG content of the Sat LDL did not cause elevated TG accumulation.

Since the extent of acetylation can influence binding to the scavenger receptor and cellular uptake, the percent acetylation of the four LDLs used in each experiment was measured. They ranged from 73–76% acetylated, as determined by the TNBS assay (data not shown). Thus, differences in acetylation cannot explain the differences in EC accumulation.

TABLE 4. Lipid composition of THP-1 cells incubated with AcLDL isolated from African green monkeys consuming different dietary fats

Group	TC	FC	EC	TG
		μg/mg		
Control	9.8 ± 0.4^a	7.9 ± 0.9^a	1.9 ± 1.1^a	30.5 ± 2.2^a
Sat	95.3 ± 4.5^b	34.5 ± 0.9^b	60.9 ± 3.6^b	83.3 ± 4.1^b
Mono	106.9 ± 3.7^c	37.1 ± 1.5^b	69.8 ± 2.3^c	84.7 ± 8.2^b
n-6	95.4 ± 1.4^b	36.1 ± 1.8^b	59.2 ± 3.0^b	77.8 ± 3.3^{b,c}
n-3	90.7 ± 2.2^b	37.3 ± 3.2^b	53.5 ± 1.1^d	69.1 ± 6.3^c

THP-1 cells were incubated with 100 μg/ml AcLDL from each of the four groups for 3 days in medium containing 1% FBS. Cells were harvested and analyzed for TC, FC, EC, and TG mass as described in Methods. "Control" represents cells harvested before the AcLDL incubation. Values shown are expressed as μg lipid per mg cell protein. The data are expressed as mean ± SD, n = 3. Significant differences ($P < 0.05$) versus control, Sat, Mono, n-6, and n-3 groups are represented by different letters. These results are representative of a total of three experiments done in an identical fashion using two different LDL preparations from the same animals.

Another way in which dietary fatty acids could affect macrophage foam cell development is by influencing the efflux of cholesterol from the cell. To examine efflux, cells were incubated for 3 days with [^{3}H]FC-labeled AcLDL from each of the four diet groups, and then incubated for 24 h with medium containing 1% BSA in order to allow time for cholesterol-specific activity to equilibrate between the FC and CE pools. This was followed by incubation for 24 h in medium containing 12.5 μM triacsin D and 400 μM BSA to deplete the cells of TG (39). Cholesterol efflux was compared among the four diet groups by measuring the appearance of [^{3}H]FC in the medium both immediately following the equilibration period and after TG removal. The lipid composition of the cells after AcLDL incubation is given in Table 4, and the 1% BSA equilibration and TG removal periods had little effect on cholesterol mass (data not shown). It should be noted that although ACAT is inhibited during the triacsin D incubation, FC did not accumulate in the cells due to the relatively slow rate of hydrolysis of CEs compared with TGs (39). The TG concentrations during the course of the experiment are shown in **Fig. 1**. Except for the Sat group, there was a slight, but nonsignificant reduction in

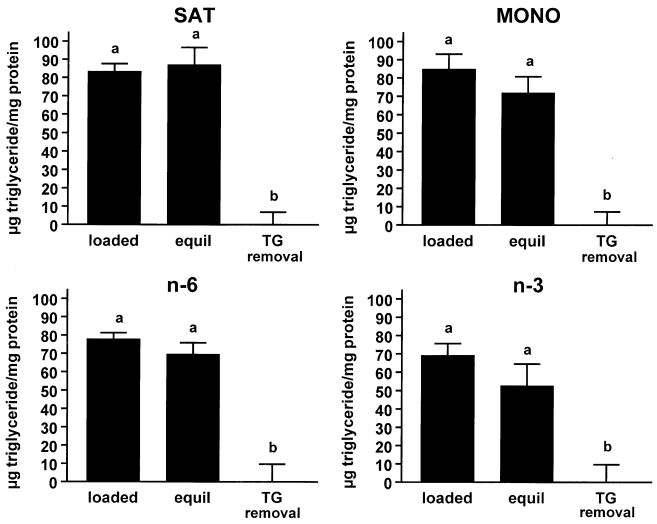

Fig. 1. Triglyceride (TG) concentration of THP-1 cells after acetylated LDL (AcLDL) incubation, equilibration, and TG removal. THP-1 cells were incubated with 100 μg/ml [^{3}H]free cholesterol (FC)-AcLDL from each of the four diet groups for 3 days in medium containing 1% FBS. Cells were then allowed to equilibrate in medium containing 1% BSA for 24 h. Following equilibration, cholesterol efflux was examined in one set of dishes. To a second set of dishes, TG removal medium containing 12.5 μM triacsin D and 400 μM BSA was added for 24 h, after which cholesterol efflux was examined. After each stage, cells were harvested and analyzed for TG mass as described in Methods. "Loaded" represents cells harvested after the initial 3 day AcLDL incubation. The data for TG shown in the "loaded" bars are the same as is shown in Table 4, and are included here for comparison purposes. "Equil" represents cells harvested after 24 h incubation in 1% BSA, and "TG removal" represents cells harvested after 24 h incubation with triacsin D and BSA. The data are expressed as mean ± SD, n = 3. Significant differences ($P < 0.05$) are represented by different letters.

TG following the equilibration period, whereas incubation with triacsin D and BSA resulted in the removal of virtually all of the cellular TG.

Lipid droplet physical state was examined both before and after TG removal using polarizing light microscopy. As expected, before TG removal, lipid droplets from all diet groups were entirely in an isotropic physical state, as indicated by the absence of formée crosses under polarizing microscopy. After TG removal, formée crosses were visible in all four groups, indicating a change in physical state, but the relative number of cells that displayed formée crosses was different for each diet group (**Fig. 2**). The Mono group had the highest percentage of cells that displayed formée crosses (71%), significantly higher than the n-6 and n-3 groups. The n-3 group displayed the lowest percentage (30%), significantly lower than the other three groups. The n-6 group was intermediate, significantly higher than the n-3 group, but significantly lower than the Mono group, with a nonsignificant decrease compared with the Sat group. Our previous studies indicated that within one individual cell, lipid droplet physical state was uniform (39); thus, in the data in Fig. 2, the cells that were counted as containing formée crosses had virtually all their lipid droplets in an anisotropic state.

Cholesterol efflux was compared in the presence of an ACAT inhibitor to prevent reesterification of hydrolyzed CEs. Using parallel sets of dishes, efflux medium containing 10% FBS as the cholesterol acceptor was added either following the equilibration period or after the TG removal period. In addition, control cells incubated with labeled AcLDL from the Sat group were treated in a similar fashion, and 1% BSA was used as an acceptor during the efflux periods. For all groups, 95% or greater of the [^{3}H]cholesterol in the media during efflux was FC (data not shown). **Figure 3A** shows the percent cholesterol efflux over a 24 h time course for cells that did not undergo TG removal. Among the four diet groups, there were no differences in the percent efflux at any of the time points. When cholesterol efflux was compared after cellular TG had been depleted, there was significantly greater efflux in the n-3 group over the three other groups at 8 h, 12 h, and 24 h (Fig. 3B). Comparison of Figs. 3A and B reveals that for the Sat, Mono, and n-6 groups, the percent efflux was identical with or without cellular TG, and in the n-3 group, the percent efflux was the same through the first 12 h, with a significant increase at 24 h in cells that contained no TG. Thus, except for the n-3 group, cellular TG had no effect on cholesterol efflux. This experiment was repeated, and similar results were found, with TG not affecting cholesterol efflux and with a slight, but significant, increase in efflux in the n-3 group over the three others after TG removal (data not shown). The FC- and CE-specific activities for the four groups at the end of the equilibration and TG removal periods are shown in Fig. 3C. The AcLDLs only contained ^{3}H-labeled FC, and the nearly equivalent specific activities of the FC and CE fractions demonstrate that LDL-derived cholesterol had been processed through the lysosomes and esterified by ACAT, forming an intracellular pool of CE. There was little change in FC- and EC-specific activity from the equilibration to the TG removal period, due primarily to the presence of triacsin D in the TG removal medium, which inhibited cholesterol reesterification (39).

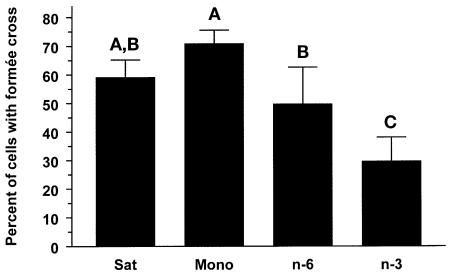

Fig. 2. Differences in cellular lipid droplet physical state among the four diet groups. THP-1 cells were plated in 35 mm dishes containing a 25 mm glass cover slip and incubated with 100 μg/ml AcLDL in medium containing 1% FBS for 3 days followed by incubation in medium containing 12.5 μM triacsin D and 400 μM BSA for 24 h. Cells were then rinsed three times with balanced salt solution and fixed in 2% gluteraldehyde solution in PBS for 10 min at room temperature. The cover slip was then removed from the dish and placed inverted onto a drop of glycerol on a glass slide. For each diet group, three such slides were prepared from three separate dishes. Cells were viewed with phase contrast and polarizing light microscopy using a 100× objective. For each slide, 10 random fields were selected, and, using phase contrast, the number of cells present within each field was counted. Switching to polarizing optics, the number of cells in the same field containing formée crosses was then counted. These numbers were used to determine the percent of cells in each group that contained formée crosses. The individual counting the cells was blinded to which group a particular slide belonged. The data are expressed as the mean ± SD from the results from three slides in which 10 fields were counted. Significant differences ($P < 0.05$) are represented by different letters.

The percent CE hydrolysis before and after TG removal for each diet group is shown in **Table 5**. Prior to TG removal, CE was hydrolyzed at a similar rate for all diet groups (17.2–20.5% hydrolyzed/24 h). After TG removal, the rate of CE hydrolysis was significantly increased in all diet groups (21.4–31.7% hydrolyzed/24 h). The greatest change after TG removal was an increase in CE hydrolysis in the n-3 group from 17.4% to 31.7%. This was significantly higher than in the other groups, consistent with the significantly greater efflux of [^{3}H]FC from the n-3 group. In all cases, cholesterol efflux measured in the same cells was greater or equal to the amount of CE hydrolyzed. This can be explained by the efflux of preexisting [^{3}H]FC as well as the efflux of [^{3}H]FC derived from hydrolysis of [^{3}H]CE and by the presence of FC on the FBS used in the efflux medium providing a source of FC for exchange with [^{3}H]FC.

DISCUSSION

In order to study the effect of dietary fatty acids on macrophage CE accumulation, physical state, hydrolysis, and cholesterol efflux, we utilized LDL from African green monkeys fed diets enriched with saturated, monounsatu-

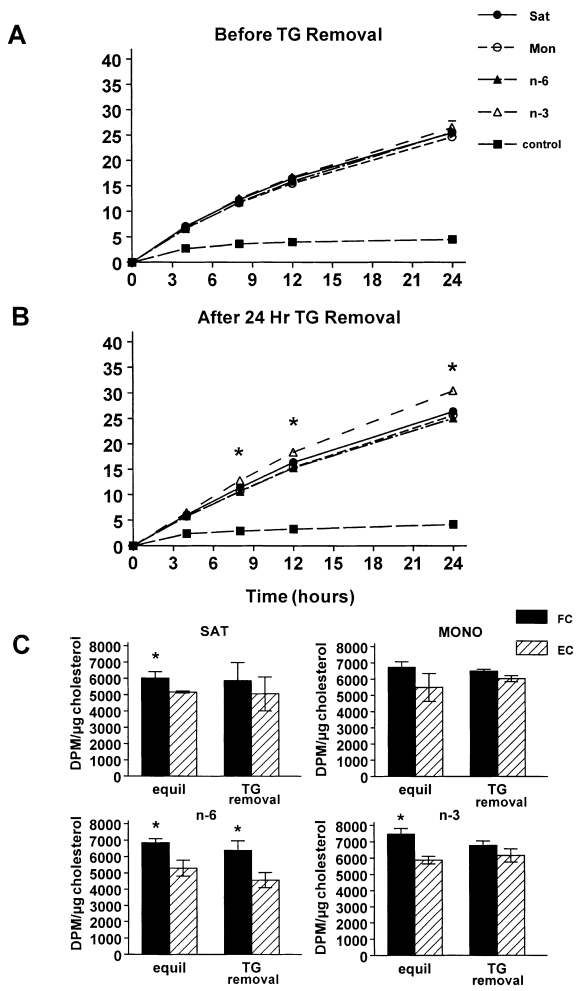

Fig. 3. Comparison of cholesterol efflux among the four diet groups both before and after TG removal. THP-1 cells were incubated with 100 μg/ml [^{3}H]FC-AcLDL from each of the four groups for 3 days in medium containing 1% FBS, and then equilibrated in medium containing 1% BSA for 24 h. A: The percent efflux from cells, which did not undergo TG removal, to medium containing 10% FBS and 1.25 μg/ml CP113 for up to 24 h. B: The percent efflux for cells that, following equilibration, were first incubated with 12.5 μM triacsin D and 400 μM BSA for 24 h to deplete them of TGs, followed by efflux to medium containing 10% FBS and 1.25 μg/ml CP113. Control cells were incubated with labeled AcLDL from the Sat group, treated as described above, and during efflux, incubated for up to 24 h in medium containing 1% BSA and 1.25 μg/ml CP113. Percent efflux was calculated by dividing the amount of [^{3}H]FC DPM appearing in the medium at each time point by the total [^{3}H]DPM present in the cells at zero time. A: The mean total DPM/mg cell protein present at 0 time for each group was: Sat: 520,264; Mono: 658,850; n-6: 542,535; n-3: 579,111. B: The values at 0 time were: Sat: 485,941; Mono: 600,282; n-6: 498,328; n-3: 499,805. C: The FC- and cholesteryl ester (CE)-specific activities after the 1% BSA equilibration and TG removal periods. The data are expressed as mean ± SD, n = 3. Significant differences ($P < 0.05$) in A and B among the four diet groups are represented by an asterisk. Where error bars are not evident, they are contained within the symbol. Significant differences ($P < 0.05$) in C between FC- and CE-specific activity after each stage are represented by an asterisk.

TABLE 5. Percent hydrolysis of CE and efflux of FC after 24 h

Diet Group	Percent EC Hydrolysis After 24 h	Percent Cholesterol Efflux After 24 h
Before TG removal		
Sat	20.5 ± 0.72^d	25.5 ± 0.4
Mono	17.2 ± 1.51^d	24.7 ± 0.8
n-6	18.4 ± 0.82^d	25.6 ± 0.5
n-3	17.4 ± 4.73^d	26.5 ± 1.3
After TG removal		
Sat	22.7 ± 0.88^{a,b}	26.4 ± 0.4^b
Mono	23.9 ± 0.88^a	25.7 ± 0.5^b
n-6	21.4 ± 0.65^b	25.1 ± 0.9^b
n-3	31.7 ± 1.12^c	30.5 ± 0.8^a

P-1 cells were treated during the efflux phase of the experiment as described in the legend to Fig. 3. The percent CE hydrolysis after the 24 h efflux period was calculated by dividing the FC produced through CE hydrolysis by the EC present at 0 time. The percent CE hydrolysis and FC efflux data are calculated from the 24 h time point from the experiment shown in Fig. 3. The mean DPM/mg cell protein of EC present at 0 time for determinations done with no TG removal for each group are: Sat: 347,737; Mono: 438,586; n-6: 384,600; n-3: 404,300. Values for determinations done following TG removal are: Sat: 328,569; Mono: 426,024; n-6: 361,682; n-3: 347,704. The data are expressed as mean ± SD, n = 3.

^{a,b,c} Significant differences ($P < 0.05$) among groups are represented by different letters.

^{d} A significant difference ($P < 0.05$) between CE hydrolysis before and after TG removal within each group.

rated, n-6 polyunsaturated, or n-3 polyunsaturated fatty acids. Previous studies have shown that monkeys consuming these diets have different rates of progression of atherosclerosis, trends thought to be influenced by the properties of the LDL itself (12, 60). Using LDL isolated from each of these four groups of animals, we were able to produce macrophage foam cells enriched with the different classes of fatty acids.

THP-1 macrophages incubated with n-3 AcLDL accumulated less CE than with the other groups of LDL, consistent with the lower CE content of the n-3 LDL. Incubation of THP-1 macrophages with Mono AcLDL resulted in greater TC and CE accumulation than with the other groups of LDL, corresponding to the increased LDL TC and CE content per particle and the greater molecular weight of the Mono LDL. Previous studies have demonstrated that larger LDL particles are more effective at promoting CE accumulation within cells in culture (61–63). This ability to deliver more cholesterol per particle to macrophages could enhance the development of atherosclerosis by inducing greater macrophage CE accumulation in the intima of arteries.

After TG depletion, cellular anisotropic lipid droplets were visible in all four groups, with the n-6 and n-3 groups having fewer cells with anisotropic droplets than the Sat and Mono groups. With the removal of virtually all cellular TG, the resulting differences in the physical state of lipid droplets must be due solely to CE fatty acyl composition. These data agree with the results of others, which have demonstrated that CEs containing polyunsaturated fatty acids are isotropic, while those containing saturated or monounsaturated fatty acids are anisotropic at 37° (19–22, 26–29).

In the presence of a cholesterol acceptor, the rate-limit-

ing step in the efflux of stored CE from cells is thought to be their hydrolysis to FC (64, 65). Due to the effect of TG on CE physical state, and since TG makes up only a small fraction of lipid in the atherosclerotic artery (33–38), it was important to study the effect of CE fatty acyl composition where TG concentration was low. By including an ACAT inhibitor to prevent the reesterification of FC formed from the hydrolysis of CE, it was possible to measure the rate of CE hydrolysis and cholesterol efflux in the same cells simultaneously. The less ordered physical state of the CEs in the n-3 group (Fig. 2) correlated with an increased rate of CE hydrolysis (Table 5) and cholesterol efflux (Fig. 3), providing one possible mechanism for the protective effect of a fish oil diet on macrophage foam cell formation and atherosclerosis.

A variety of studies have addressed the issue of dietary fatty acids and cellular cholesterol efflux. Gillotte et al. (40) used HDL isolated from the plasma of African green monkeys fed diets enriched in saturated, monounsaturated, n-6 polyunsaturated, or n-3 polyunsaturated fats to study the effect of HDL PL modification on cholesterol efflux. No effect was found, indicating that dietary fats do not influence cholesterol metabolism by affecting HDL's ability to promote cholesterol efflux. The study of the effect of cellular CE fatty acyl composition on cholesterol efflux, however, has proven difficult. Adelman et al. (22) demonstrated that CE physical state can affect the rate of cholesterol efflux, and that both the presence of cellular TGs and CE fatty acyl composition can affect physical state. However, they were unable to show directly that cellular CE fatty acyl composition affected cholesterol efflux. In their studies, cells were enriched with free fatty acids in order to alter the cellular fatty acyl composition, which resulted in increased cellular TG synthesis and accumulation that make it difficult to interpret the cholesterol efflux results. In our present studies, we were able to remove virtually all cellular TG, resulting in a human macrophage cell line loaded with CEs enriched in different fatty acids. This allowed for the study of CE physical state within cells and cellular CE hydrolysis and efflux without the confounding effects of TG.

Our studies indicate that n-3 fatty acids increase macrophage cholesterol efflux by creating CE droplets with an altered physical state, which have a faster rate of hydrolysis. The extent to which hydrolysis of CEs is mediated by the physical state of the CE droplets within cells versus preferential hydrolysis of CEs containing n-3 fatty acids is unclear. The effects of n-3 fatty acids on cholesterol efflux are also not limited to the effects on CE physical state. Others have suggested that n-3 fatty acids can enhance cholesterol efflux by modifying the plasma membrane (66, 67). As shown in Table 3, a higher percentage of both 20:5 and 22:6 was found in cellular PL than in CE and TG in the n-3 group. The presence of these fatty acids in the membrane may be contributing to the enhancement of cholesterol efflux, making it possible that n-3 fatty acids influence cholesterol efflux by several mechanisms.

A recent study by Wang and Oram (68) demonstrated that unsaturated fatty acids increased ABCA1 turnover and reduced ABCA1-dependent efflux compared with saturated fatty acids. Although ABCA1-mediated PL and cholesterol efflux is essential for the formation of mature HDL particles that can then promote cholesterol efflux by other mechanisms, the extent of total cellular cholesterol efflux that occurs by this mechanism is unknown. In our studies, by using serum as the cholesterol acceptor, cholesterol efflux from a variety of mechanisms was measured, not just ABCA1. This may explain the differences in the results of Wang and Oram (68) compared with the current studies.

By examining cholesterol efflux before and after TG removal, we were also able to study the effects of TG on CE hydrolysis and cholesterol efflux. Except for the n-3 group, the removal of virtually all cellular TG did not appear to affect cholesterol efflux, and surprisingly led to higher rates of CE hydrolysis in all groups despite a physical state change from isotropic to anisotropic. This result differs from the results of Glick et al. (21) and Adelman et al. (22), who have shown enhanced CE hydrolysis and cholesterol efflux when TGs are present. The same group, however, using a mouse macrophage cell line reported that CE hydrolysis was faster in cells containing anisotropic droplets than in those containing isotropic droplets (69). This is in agreement with our results in macrophages, but in contrast to results using Fu5AH hepatoma cells (21, 22). It is possible that the different cell types could be responsible for this discrepancy.

In our original studies in which the method for TG depletion was developed, cholesterol efflux was compared in cells containing either 69% or 39% of neutral lipid as TG, and a higher rate of CE hydrolysis and cholesterol efflux was found in the cells containing the higher concentration of TG (39). Human LDL was used that contained more TG than the monkey LDL used in the present studies and, as a result, cellular TG concentrations were much higher, even after TG removal, than in the efflux study shown in Fig. 3. Thus, the current studies differ from our previous work because virtually all cellular TG had been eliminated after incubation with triacsin D and BSA, and this difference may explain the effects of TG on cholesterol efflux described in these studies and those shown previously (39). The cells used in the current studies, however, are more reflective of macrophage foam cells of the atherosclerotic artery, which contain little TG.

In summary, at least two mechanisms appear to play a role in the differential ability of LDL from Mono and n-3 diets to promote cholesterol accumulation in THP-1 macrophages. Mono LDL stimulated greater accumulation of CE, probably due to a greater number of CE molecules per particle and the enrichment of a greater percentage of cells with anisotropic CE lipid droplets. On the other hand, n-3 LDL stimulated less CE accumulation, had fewer cells with anisotropic lipid droplets, and exhibited a more rapid rate of CE hydrolysis and cholesterol efflux. The differences in cholesterol accumulation and the rates of CE hydrolysis and cholesterol efflux between the Mono and n-3 groups, although statistically significant, were relatively small. Nevertheless, these results are potentially im-

portant physiologically since these differences were found after only a 3 day incubation with AcLDL. When one considers that atherosclerosis is a disease that develops over a lifetime, the relatively small differences in the balance of CE accumulation by macrophages in the arterial wall of individuals consuming different fatty acids could have a major effect on development of atherosclerosis. The current findings provide a potential mechanism for the accelerated atherosclerosis in animals consuming monounsaturated fats versus diets enriched in polyunsaturated fatty acids (12, 13).

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