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Dietary Monounsaturated Fatty Acids Promote Aortic Atherosclerosis in LDL Receptor–Null, Human ApoB100–Overexpressing Transgenic Mice

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Abstract—In mice with genetically engineered high levels of plasma low density lipoprotein (LDL), we tested the hypothesis that an increase in the dietary content of monounsaturated fatty acids but not of polyunsaturated fatty acids would promote atherosclerosis. The mouse model used was an LDL receptor–null, human apoB100–overexpressing strain. Six experimental groups of 19 to 38 mice of both sexes were established when the animals had reached 8 weeks of age. For the next 16 weeks, individual groups were fed either a commercial diet or prepared diets including fat as 10% of energy, with 5 different fatty acid enrichment patterns including the following: saturated (sat), *cis* and *trans* monounsaturated (mono), and n-3 and n-6 polyunsaturated (poly). Highly significant differences (ANOVA, $P < 0.0001$) in LDL cholesterol (in mg/dL) were found, with the rank order at 16 weeks being *trans* mono (mean, 1390) > sat (922) = *cis* mono (869) = n-6 poly (868) > n-3 poly (652) > commercial diet (526). Significant elevations in very low density lipoprotein cholesterol were also found in the *trans* and *cis* mono and sat groups, and triacylglycerol concentrations were also elevated in all groups. High density lipoprotein cholesterol concentrations were consistently low (20 to 50 mg/dL) in all groups. Highly significant differences (ANOVA, $P < 0.0001$) in atherosclerosis, quantified by measurement of aortic cholesteryl ester concentration (mg/g protein) among dietary fatty acid groups were found, with the order being *trans* mono (mean, 50.4) > sat (35.6) = *cis* mono (34.6) > n-6 poly (18.3) = n-3 poly (9.7) = commercial diet (7.8). Therefore, in this mouse model of hypercholesterolemia, dietary *cis* or *trans* monounsaturated fat did not protect against atherosclerosis development, whereas aortic atherosclerosis in either of the polyunsaturated fat groups was significantly less than in the saturated fat group. (*Arterioscler Thromb Vasc Biol.* 1998;18:1818-1827.)

Key Words: cholesterol ■ lipoprotein metabolism ■ polyunsaturated fat ■ saturated fat ■ *trans* fatty acids

The effects of dietary cholesterol and type of fat on coronary heart disease have been frequently studied. Generally, recommendations from organizations such as the American Heart Association have been to limit the amount of total and saturated fat in the diet, replace saturated with unsaturated fat, and limit dietary cholesterol. Data indicate that we have been better at increasing the amount of vegetable oil in our diet than in decreasing the percentage of energy intake as fat.¹ Decreased saturated fat and increased polyunsaturated fat intake may be a component of the overall 50% reduction in coronary heart disease rates that have occurred over the past 2 decades in the United States.² A significant body of evidence demonstrates that for linoleic acid, accumulation in adipose tissue³⁻⁵ and enrichment in plasma cholesteryl esters⁶⁻¹⁰ are both factors associated with decreased rates of premature complications of coronary heart disease. In addition, several studies have demonstrated that linoleic acid–rich diets directly protect against atherosclerosis development.^{11,12} In an attempt to make linoleic acid–en-

riched fats more suitable for a variety of cooking needs, hydrogenation has been used to increase the melting temperature and reduce the oxidizability of these preparations, in the process introducing higher amounts of *trans* fatty acids, a by-product of hydrogenation, into our diet. Data have been presented that *trans* fatty acids may detrimentally affect coronary heart disease risk,^{13,14} though again, establishing a firm basis for these risk factor associations with direct observations of effects on atherosclerosis remains to be achieved.¹⁵

Interestingly, in our society at the present time, monounsaturated fatty acids in the form of olive oil and canola oil instead of linoleic acid–rich corn and soybean oils, for example, are appearing more frequently as the cooking oils available for sale in grocery stores. This shift is apparently based, at least in part, on recommendations that monounsaturated fatty acids provide similar or improved risk factor relationships compared with those seen for polyunsaturated fatty acids,^{16,17} although protection against atherosclerosis

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development in studies of experimental coronary artery atherosclerosis, per se, has not been found.¹⁸ Diets enriched in n-3 fatty acids derived from fish oils have been found to be beneficial in limiting the development of experimental atherosclerosis,^{19,20} although effects on risk factors have led to some skepticism in favoring their use in protection against coronary heart disease.²¹

Simultaneous evaluation of the variety of different unsaturated dietary fats with direct comparisons to the effects of saturated fat on experimental atherosclerosis has not been attempted. With the development of genetically modified mouse models in recent years, it has become possible to use mice to evaluate some of the important factors affecting atherosclerosis development. One difficulty with the mouse as a model of human atherosclerosis is that the typical plasma lipoprotein spectrum in mice is quite different from that in humans, with a pattern of HDL as the predominant lipoprotein with low concentrations of LDL and VLDL, and drastic diets have been needed to induce arterial changes consistent with early atherosclerosis development.^{22,23}

The feasibility of using mice to evaluate dietary fatty acid effects on atherosclerosis was pioneered by Paigen and associates.²⁴ A mouse model (LDL receptor-null with human apoB100 overexpression) with LDL as the predominant lipoprotein has recently been developed by Hobbs and associates.²⁵ A mouse model of LDL-driven atherogenesis would appear to have advantages over other mouse models for evaluation of dietary fatty acid effects on atherosclerosis with extrapolation to humans, because at least a portion of the dietary effect in humans is on LDL particle composition. Therefore, we have used this mouse as a model to compare atherosclerosis outcomes in response to 5 different dietary fat types containing enrichments in saturated, *cis* and *trans* monounsaturated, and n-3 and n-6 polyunsaturated fatty acids, with all groups being compared with a standard commercial mouse diet (control) group. To the extent that dietary fatty acid-induced alterations in LDL cholesteryl ester composition participate in the overall atherogenicity of LDL, as has been hypothesized to be the case on the basis of outcomes in monkeys,^{12,18} a mouse model with LDL as the primary lipoprotein may permit the hypothesis to be tested.

Methods

Animals and Diets

The mice used in these studies were reared in our medical school animal facility. The original breeding pair was provided by Dr Helen Hobbs at the University of Texas Southwestern Medical Center in Dallas, and this mouse has been described in a separate publication.²⁵ In brief, this mouse strain is a hybrid cross between the LDL receptor $-/-$ mouse described by Ishibashi et al,²⁶ which itself is a hybrid of 129sv and C57BL/6 strains, and the human apoB100 transgenic mouse,²⁷ itself a hybrid of the SJL and C57BL/6B strains. Breeding demonstrated the LDL receptor knockout and apoB overexpression traits to be homozygous, and all animals maintained grossly elevated LDL levels that contained principally apoB100. When the animals reached 8 to 9 weeks of age, they were randomly assigned to 1 of the 6 dietary fat groups. Groups of between 20 and 38 animals containing at least 10 members of each sex were established and entered into the study as they became of age. A total of 167 animals were studied, including 93 males and 74 females. All animals were housed in our American Association for the Accreditation of Laboratory Animal Care-approved animal facility, and their care

was supervised by a veterinarian. All animal procedures were preapproved by the institutional animal care and use committee.

The diets used in the study were prepared on the basis of those fed previously to monkeys,^{28,29} and we added a group containing *trans* fatty acids in a fat blend provided to us through the generous offices of Dr Pete Huth of Kraft Foods and the Institute of Shortening and Edible Oils. Dietary ingredients are shown in Table 1. The diets contained 10% of energy as fat with the fatty acid compositions shown in Table 2. Cholesterol content in the prepared diets was consistently low at 0.005%. The cholesterol content of the commercial mouse food (Prolab 3000, PMI Feeds, Inc) used as a control was 0.06%, and the measured fat content of this food was 4.6% by weight.

At weeks 2, 8, and 16 of the experimental period, blood was taken from each mouse for measurement of lipids and lipoproteins. Ketamine HCl was administered (40 mg/kg) together with xylazine (8 mg/kg) to restrain the animals. Blood was taken via orbital bleeding and transferred to tubes containing 0.1% EDTA, 0.01% NaN₃, aprotinin (1 μ g/mL), and benzamide (1 mmol/L).³⁰ At the time of sacrifice, the animals were anesthetized with the ketamine (80 mg/kg) and xylazine (8 mg/kg) mixture, blood was drawn via heart puncture, and the mice were then euthanized in a CO₂ chamber. Plasma was promptly separated from cells by centrifugation at 5000g at 4°C.

Lipid, Lipoprotein, and Atherosclerosis Measurements

Lipoprotein separations were promptly begun on aliquots of fresh plasma isolated from each mouse. Whole plasma was injected onto a 30-cm Superose 6 chromatography column, which was subsequently run at 0.5 mL/min with 0.9% NaCl containing 0.05% EDTA, pH 7.4, and 0.05% NaN₃, as described.³¹ The average LDL particle size was measured from a standard curve constructed from known standards plotted against elution time.³¹ Fractions were collected and pooled according to the elution times for VLDL, LDL, and HDL, and aliquots of isolated lipoprotein fractions were used for enzymatic measurement of cholesterol³² and triacylglycerols.³³ Assays were monitored for accuracy and precision with simultaneous measurements in appropriate quality control serum samples.

In some cases for which purified lipoprotein fractions were needed for compositional analyses, plasma from mice in each diet group and of each sex was pooled and then ultracentrifuged for 24 hours at 50 000 rpm in a Ti 70.1 rotor (Beckman Instruments) at a density of 1.21 g/mL to isolate the combined lipoprotein fraction at the top of the tube. Fractions within a single lipoprotein class were then separated on agarose chromatography columns, and chemical compositions were determined for individual classes. For selected LDL samples, apolipoproteins were separated by polyacrylamide gel electrophoresis in the presence of SDS. In brief, aliquots of the lipoprotein sample containing 50 μ g of protein were lyophilized and resolubilized by boiling for 5 minutes in a 0.05 mol/L barbital buffer, pH 8.6, containing 2.5% SDS, 3% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. Samples containing 10 μ g of protein were then applied to a 4% to 30% polyacrylamide gel containing SDS, and electrophoresis was carried out as described previously.³⁴ The gels were then fixed and stained with Coomassie blue dye.

At the end of the study after the animals had been euthanized, the heart with attached aorta was removed from the body and placed in 10% neutral buffered formalin for subsequent processing. The end points of atherosclerosis quantified for each mouse in this study were aortic free cholesterol and cholesteryl ester concentrations, measured as mg/g protein. To obtain these end points, the tissue was placed on the platform of a dissecting microscope, and the adventitia was carefully and completely dissected and removed. The aortic intimal-medial preparation was then detached at the base of the heart and placed in a tube with 3 mL of chloroform-methanol, 2:1 vol/vol, containing 5 α -cholestane as an internal standard, and the lipids were extracted. The lipid extract was separated by filtration from the delipidated protein, and extracts were dried under N₂ at 60°C and then dissolved in hexane. Analysis of free and total cholesterol was carried out with 2 injections per sample on a DB 17 (0.53-mm ID \times 15 m \times 1 μ m) gas-liquid chromatography column (J&W Scientific) at 250°C and installed in a Hewlett-Packard 5890 gas chro-

TABLE 1. Experimental Diet Ingredients

| Ingredient | Diet Group | | | | |
|-------------------------------|------------|-----------------|----------|----------|-------------------|
| | Sat | <i>cis</i> Mono | n-6 Poly | n-3 Poly | <i>trans</i> Mono |
| (g/100 g Dry Weight) | | | | | |
| Diet fat | | | | | |
| Palm oil | 4 | 0 | 0 | 2 | 0 |
| Oleic acid-rich safflower oil | 0 | 4 | 0 | 0 | 0 |
| Safflower oil | 0 | 0 | 4 | 0 | 0 |
| Fish oil | 0 | 0 | 0 | 2 | 0 |
| <i>trans</i> Blend | 0 | 0 | 0 | 0 | 4 |
| Same for All Diets | | | | | |
| Alphacel | 7.4 | | | | |
| β -Sitosterol* | 0.0015 | | | | |
| Casein, USP | 8.0 | | | | |
| Cholesterol, crystalline | 0.0019 | | | | |
| Complete vitamin mixture† | 2.6 | | | | |
| Dextrin | 17.0 | | | | |
| Lactalbumin | 4.0 | | | | |
| Salt mix (Hegsted) | 5.0 | | | | |
| Sucrose | 17.0 | | | | |
| Wheat flour | 35.0 | | | | |

*Added to diets not containing safflower oil.

†Vitamin mix is complete and, in addition, includes 2 mg/100 g diet for each of α -tocopherol, γ -tocopherol, and tenox 20A as additional antioxidants.

matograph equipped with an HP 7673A automatic injector using on-column injection and a flame ionization detector. Cholesteryl ester (esterified cholesterol \times 1.67) was calculated as the difference between free and total cholesterol, as measured before and after saponification and reextraction of the nonsaponifiable sterol into hexane. The delipidated tissue protein was then digested and dissolved in 1N NaOH, and total protein was determined.³⁵

Statistical Evaluations

The data were first evaluated for main effects (sex and dietary fat) by 2-way ANOVA. For post hoc analyses to identify individual group differences (where they were found), 1-way ANOVA for individual diets was then used with post hoc analyses by Fisher's protected least significant difference test. Statistical significance was considered at $P \leq 0.05$. The outcomes for post hoc analyses are indicated in the tables.

Results

The body weights of the animals were measured when the animals were 8 to 9 weeks old, and at the time of sacrifice, 16

weeks into the experimental diet period, when all of the mice were 24 to 25 weeks of age. At the start of the study, the males weighed 28.2 ± 2.0 g (mean \pm SD) and the females, 23.2 ± 1.9 g. On average, the males weighed ≈ 5 g more than the females, and this was also true at the end of the study, when the overall average weight for males was 35.8 ± 2.6 g and for females, 30.3 ± 1.8 g. Thus, during the course of the study, both males and females gained ≈ 7 g, and this increase was true in each of the diet groups. Much of this weight gain appeared to be due to an increase in adipose tissue, because the carcasses contained significant amounts of fat at sacrifice. No apparent health problems were found in any of the diet groups.

Time-related total plasma cholesterol (TPC) responses to the different diets are shown in Figure 1. At 2 weeks into the study, TPC values already had begun to show diet-specific

TABLE 2. Dietary Fatty Acid Compositions

| Diet | Fatty Acids, % of Energy | | | | | |
|-------------------|---------------------------------|--|--|-------------------------------------|-------------------------------------|-------|
| | Saturated (14:0, 16:0, 18:0) | <i>cis</i> Monounsaturated (16:1, 18:1) | <i>trans</i> Monounsaturated (18:1t; 18:2t) | n-6 Polyunsaturated (18:2, 20:4) | n-3 Polyunsaturated (20:5, 22:6) | Other |
| Sat | 4.8 | 3.6 | ND | 1.4 | 0.03 | 0.2 |
| <i>cis</i> Mono | 1.2 | 6.8 | 0.02 | 1.8 | 0.06 | 0.2 |
| n-6 Poly | 1.3 | 1.2 | ND | 7.2 | 0.03 | 0.3 |
| n-3 Poly | 3.9 | 2.7 | 0.1 | 1.1 | 1.4 | 0.8 |
| <i>trans</i> Mono | 2.7 | 1.7 | 2.8 | 1.9 | 0.03 | 0.8 |
| Commercial | 1.6 | 2.2 | ND | 5.3 | ND | 0.9 |

ND indicates not detected. All values represent averages for duplicate determinations of at least 2 diet preparations. Fatty acid underlined is the major fatty acid within each class.

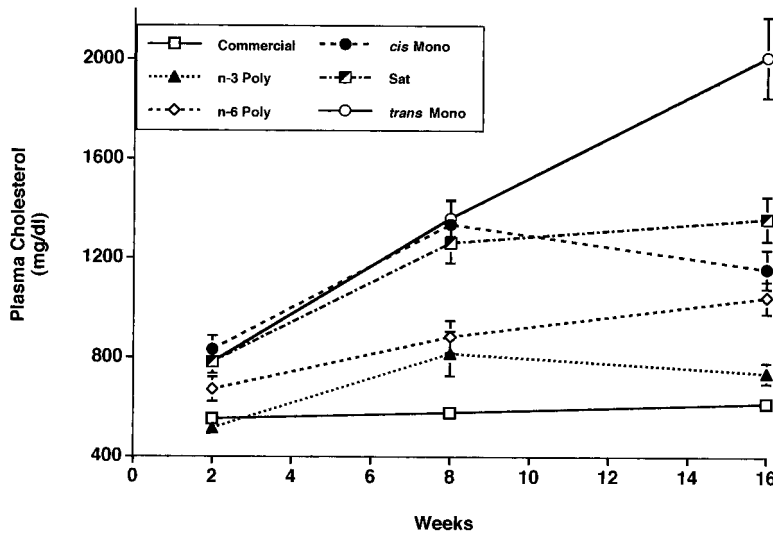


Figure 1. TPC responses to the diets at 2, 8, and 16 weeks of diet exposure. All animals were fed the diet starting at 8 to 10 weeks of age, with group sizes of 19 for the commercial diet, 27 for n-3 poly, 32 for n-6 poly, 25 for *cis* mono, 27 for sat, and 29 for *trans* mono.

differences, with the sat and *cis* and *trans* mono groups already being higher (near 800 mg/dL) than the n-6 poly (near 650 mg/dL) and n-3 poly and commercial diet groups (near 550 mg/dL). This trend continued and became even more marked at 8 weeks into the study, with TPC in the 2 poly groups being between 800 and 900 mg/dL, which was higher than the TPC of ≈600 in the commercial diet group; even higher TPC values near 1300 mg/dL were found in the *cis* and *trans* mono and sat groups. At 16 weeks, the TPC in the *trans* mono group was clearly highest, at ≈2000 mg/dL, with the average TPC in the sat and *cis* mono groups near 1300 mg/dL, and that in the n-6 poly group near 1100 mg/dL. The n-3 poly group remained lower, near 700 mg/dL, but the commercial diet group had not changed and remained near 600 mg/dL after 16 weeks of diet treatment. In all cases, the values for males and females were averaged together after no statistically significant differences between the sexes were found.

After 16 weeks of diet treatment, the distribution of cholesterol and triacylglycerol among lipoprotein fractions was determined (Table 3). Plasma cholesterol concentrations were high and dietary fat dependent, as discussed above. The concentration of cholesterol in VLDL was highest in the *trans* group, elevated in the sat and *cis* mono group, and lower in both poly groups and in the commercial diet group. LDL cholesterol was surprisingly dependent on the type of dietary fat fed. It was lowest in the commercial diet and n-3 poly groups; equivalently higher in the n-6 poly, *cis* mono, and sat groups; and highest in the *trans* mono group. The cholesterol concentrations in HDL were low in all groups, although they were somewhat higher in the n-6 poly group.

Plasma triacylglycerol concentrations were also very high and diet dependent (Table 3). The highest value was in the *trans* mono group, and the lowest value was in the commercial diet group. The other groups were intermediate and not

TABLE 3. Dietary Fatty Acid Effects on Plasma and Lipoprotein Lipid Concentrations

| Diet Group | n | Total Cholesterol, mg/dL | | | |
|------------------------|----|--------------------------|----------|-----------|--------|
| | | WP | VLDL | LDL | HDL |
| Commercial | 19 | 616±31* | 63±9* | 526±32* | 27±5*† |
| n-3 Poly | 27 | 736±44* | 65±13* | 652±36* | 20±2* |
| n-6 Poly | 32 | 1074±65‡ | 152±19‡ | 868±55‡ | 54±9‡ |
| <i>cis</i> Mono | 25 | 1252±70‡† | 342±31† | 869±45‡ | 42±4‡† |
| Sat | 38 | 1360±56† | 408±28† | 922±29‡ | 32±3*† |
| <i>trans</i> Mono | 29 | 2005±167§ | 585±50§ | 1390±124† | 30±2*† |
| Triacylglycerol, mg/dL | | | | | |
| Commercial | 19 | 592±44* | 118±17* | 468±32 | 5±1 |
| n-3 Poly | 27 | 717±99*‡ | 186±59* | 525±56 | 7±1 |
| n-6 Poly | 32 | 782±75*‡ | 235±33*‡ | 549±49 | 7±1 |
| <i>cis</i> Mono | 25 | 901±86‡ | 418±42† | 477±32 | 6±1 |
| Sat | 38 | 916±86‡ | 340±47†† | 568±47 | 7±2 |
| <i>trans</i> Mono | 29 | 1180±83† | 623±55§ | 546±40 | 10±1 |

WP indicates whole plasma. All values are mean±SEM. Values in a column under cholesterol or triacylglycerol with different symbols are significantly different at P<0.05.

TABLE 4. Dietary Fatty Acid Effects on Mouse LDL Particle Composition and Size

| Diet Group | Percentage, wt/wt | | | | | LDL MW, g/ μ mol |
|-------------------|-------------------|------------------|------------------|------------------|------------------|-------------------------|
| | FC | CE | TG | PL | Pro | |
| Commercial | 8.50 \pm 0.05 | 27.39 \pm 2.33 | 20.03 \pm 2.13 | 21.33 \pm 0.25 | 22.76 \pm 0.28 | 2.43* \pm 0.09 |
| n-3 Poly | 9.08 \pm 0.49 | 29.05 \pm 2.31 | 21.34 \pm 3.54 | 18.93 \pm 0.38 | 21.61 \pm 0.76 | 2.34* \pm 0.05 |
| n-6 Poly | 9.11 \pm 0.29 | 33.47 \pm 1.96 | 15.30 \pm 1.55 | 21.70 \pm 0.57 | 21.42 \pm 0.53 | 2.73† \pm 0.05 |
| <i>cis</i> Mono | 10.14 \pm 0.17 | 35.29 \pm 2.23 | 13.77 \pm 2.11 | 21.58 \pm 0.15 | 19.21 \pm 0.45 | 3.00‡ \pm 0.04 |
| Sat | 9.84 \pm 0.20 | 34.74 \pm 2.48 | 13.51 \pm 1.77 | 21.52 \pm 0.57 | 20.39 \pm 0.48 | 2.71† \pm 0.04 |
| <i>trans</i> Mono | 10.02 \pm 0.21 | 34.25 \pm 2.05 | 14.07 \pm 1.71 | 22.44 \pm 0.37 | 19.23 \pm 0.81 | 2.47* \pm 0.08 |

FC indicates free cholesterol; CE, cholesteryl esters; TG, triacylglycerols; PL, phospholipids; and Pro, protein. All values are mean \pm SEM. There is a statistically significant difference if the symbol is different. Percentage compositions were determined on 2 LDL pools for males and 2 for females of each diet group. The average LDL particle size was measured for each mouse of the study during chromatographic separation, and group sizes are as in Table 3.

different from each other. The triacylglycerol values in VLDL were highest in the *trans* and *cis* mono groups. The sat group also had an elevated VLDL triacylglycerol concentration, whereas the VLDL triacylglycerol in the poly groups remained lower and similar to the commercial diet value. Triacylglycerol concentrations in LDL were high but not diet dependent. Triacylglycerol concentrations in HDL were low and not diet dependent.

Plasma LDL particle size and composition have been shown to be diet dependent in other species, and these characteristics were compared in these mice. LDL particle size was estimated as molecular weight during gel filtration chromatography³¹ of individual animal samples in each group, and the results are shown in Table 4. In general, the LDL particles were small, with the average in the commercial diet group being 2.43 g/ μ mol (or 2.43 \times 10⁶ Da). In most of the groups fed the prepared diets, except for the n-3 poly group, the particles were somewhat larger, with the average being between 2.5 and 2.7 \times 10⁶ Da. In the *cis* mono group, the LDL particles were the largest and averaged 3.0 \times 10⁶ Da. The percentage compositions are also shown in Table 4. LDLs used for this evaluation represent the material from the LDL peaks isolated from the Superose column, which were pooled from 3 to 4 animals into a total of 4 pools (2 for males and 2 for females) for each diet group.

The LDL particles of each pool were then floated at a density of 1.063 g/mL in the ultracentrifuge to remove extraneous proteins. The compositions of LDL from each diet group were similar, and there were not major differences among LDLs from the different diet groups. The protein, phospholipid, and free cholesterol percentages were quite similar in LDLs from each of the diet groups and together made up \approx 50% of the mass of the particle. The core lipid was somewhat different in composition, with the triacylglycerol percentage being higher in the commercial diet and n-3 poly groups than in that of other groups, wherein the cholesteryl ester made up close to 35% of the mass.

The apolipoprotein composition of the LDL particles was also examined, and a gel showing some of the data is shown in Figure 2. The LDL peak was separated into large, medium, and small fractions by pooling the material in the front, middle, and back of the LDL peak from the column. The major apolipoprotein in all fractions was apoB100, and lesser

amounts of apoB48 were apparent. Small amounts of apoE were visible in the fraction containing larger LDL particles, and in 1 preparation, a small amount of apoA-I was also seen in the fraction containing the smaller LDL particles; for the most part, however, apoB100 was the principal apolipoprotein on the LDL particles. The small amounts of apoE and apoA-I could represent the presence of particles distinct from the apoB-containing LDL particle.

The cholesteryl ester composition of LDL was also examined, and the data are shown in Table 5. The pattern was characteristic of rodent species and different from that typical of primates. The commercial diet group had 35% as cholesteryl linoleate, 15% as cholesteryl palmitoleate and oleate, and 10% as cholesteryl palmitate and stearate. Approximately 25% was cholesteryl arachidonate. A similar distribution was seen in the n-6 poly group, although the percentage of cholesteryl linoleate was \approx 10% lower and the percentage of

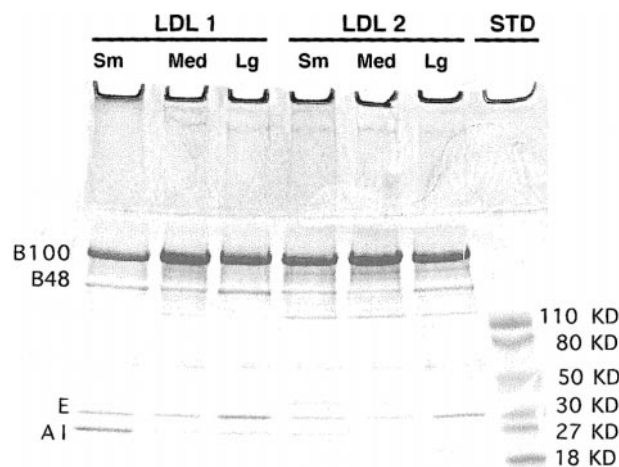


Figure 2. SDS-polyacrylamide gel electrophoresis of the apolipoproteins isolated from LDL size fractions. LDLs isolated from the Superose column were pooled for 3 to 4 animals in the n-6 poly and sat groups, and ultracentrifugation at $d=1.063$ g/mL was performed. The LDL that floated to the top of the ultracentrifuge tube was then reappplied to the Superose 6 column, and material that eluted in the front, middle, and back of the LDL peak was combined separately and extracted. The protein components were solubilized in SDS-containing buffer and run on a 4% to 20% polyacrylamide gradient gel. The migration positions of apoB100, apoB48, apoE, and apoA-I are marked, as are the positions for the molecular weight standards.

TABLE 5. Dietary Fatty Acid Effects on Mouse LDL Cholesteryl Ester Composition

| Diet Group | Cholesteryl Ester Fatty Acid, wt % | | | | | | | | |
|-------------------|------------------------------------|-------------|------------|-------------|--------------|-------------|---------------|------------|------------|
| | Sat | | | Mono | | n-6 Poly | | n-3 Poly | |
| | 16:0 | 18:0 | 22:0 | 16:1 | 18:1 | 18:2 | 20:4 | 20:5 | 22:6 |
| Commercial | 7.96*±0.23 | 2.60*†±0.44 | ... | 2.82*±0.18 | 12.04*±1.66 | 35.34*±1.51 | 26.52*±0.50 | ... | 3.10*±0.16 |
| n-3 Poly | 12.92†±1.15 | 3.39†±0.76 | ... | 7.07†§±0.47 | 12.09*±2.10 | 11.63†±0.90 | 9.05†±0.68 | 27.89±1.68 | 7.58†±0.34 |
| n-6 Poly | 8.33*±0.51 | 1.71*±0.24 | ... | 5.06‡±0.27 | 16.28*†±2.43 | 23.81‡±1.37 | 36.23‡±0.88 | ... | 1.19‡±0.08 |
| <i>cis</i> Mono | 11.40†±1.05 | 2.42*†±0.31 | 2.33*±0.18 | 6.48†±0.19 | 31.79†§±5.05 | 12.13†±0.91 | 25.00* ±2.91 | ... | 1.09‡±0.11 |
| Sat | 12.16†±0.35 | 2.48*†±0.17 | 3.66†±0.39 | 7.94§±0.36 | 33.60†§±2.41 | 12.78†±1.11 | 19.94§±0.99 | ... | 1.35‡±0.06 |
| <i>trans</i> Mono | 16.74‡±1.40 | 4.32‡±0.43 | 2.26*±0.14 | 7.03†§±0.37 | 24.73‡§±3.82 | 13.71†±1.58 | 21.56§ ±1.22 | ... | 0.47§±0.28 |

All values are mean±SEM. There is a statistically significant difference if the symbol is different. Cholesteryl ester fatty acid compositions were measured on the LDL pools described in Table 4.

cholesteryl arachidonate ≈10% higher. The n-3 poly group was distinctive, in that >40% of the cholesteryl esters contained long-chain, highly polyunsaturated fatty acids, the majority being n-3 polyunsaturated fatty acids. The remaining groups had only ≈12% to 13% cholesteryl linoleate, between 20% and 25% cholesteryl arachidonate, and as the major cholesteryl ester, cholesteryl oleate, with a significant amount of cholesteryl palmitoleate as well. This pattern of monounsaturated fatty acid enrichment of cholesteryl esters is typical of the pattern in primates fed saturated and monounsaturated fat, although the actual percent is lower in mice due to the higher percentage of cholesteryl arachidonate. The *trans* fatty acids appeared to interfere to some extent with the accumulation of *cis* monounsaturated fatty acids in cholesteryl esters, although this outcome was not due to accumulation of *trans* fatty acids in cholesteryl esters, since <1% of the LDL cholesteryl esters in the mice of this group were identified as containing a *trans* fatty acid.

The variety of plasma lipoprotein responses to the different dietary fatty acid challenges was somewhat unexpected, given the low energy percent of fat in the diet. Nevertheless, the atherogenic response in the aorta was considered the primary indicator of beneficial or detrimental effects of any particular fatty acid on atherosclerosis, and the lipoprotein responses suggested that differences would be present, so atherosclerosis quantification in each of the animals in the study was performed. Whereas most studies in mice have used a tissue histology-based system for atherosclerosis evaluation, the method is labor intensive, the tissue available for evaluation is small, and the accuracy of this type of evaluation for determining the overall extent of aortic atherosclerosis is unknown. Therefore, we measured the accumulation of free cholesterol and cholesteryl ester in the whole aorta as a chemical end point that quantitatively monitors the extent of atherosclerosis. With a 2-way ANOVA, we found no differences between males and females; thus, the following analyses were done after combining the data from all of the animals in the study. The group averages for aortic free cholesterol (mg/g protein) are shown in Figure 3A. Aortic free cholesterol concentration was significantly different among diet groups ($P<0.0001$). It was the same, at about 20 mg/g, in the commercial diet and the 2 poly groups, but this value was significantly ($P<0.008$) elevated in the *cis* mono

(29.8 mg/g) and sat (30.5 mg/g) groups and was even higher in the *trans* mono group (41.9 mg/g) ($P<0.0001$ compared with all other groups).

The atherogenic response as measured by aortic cholesteryl ester concentration showed even more exaggerated differences among diet groups (Figure 3B). The lowest values were in the commercial diet and n-3 poly groups, 7.8 and 9.7 mg/g, respectively. The cholesteryl ester concentration in the n-6 poly group was significantly higher ($P<0.05$) than that in the

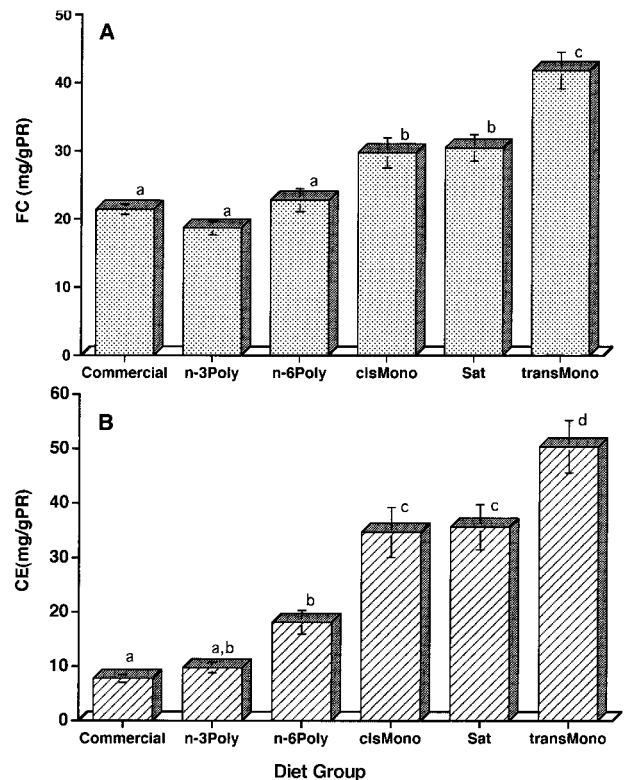


Figure 3. Aortic concentrations of free cholesterol (FC) (A) and cholesteryl ester (CE) (B) of mice fed different dietary fats for 16 weeks. Dietary fat designations are as defined in Methods. The aortas from individual mice of each group were cleaned, extracted, and quantified for FC and CE concentrations, and the mean±SEM values for each diet group were plotted. Statistically significant differences were determined by ANOVA with post hoc analysis and are indicated by the letter over the bar; bars with different letters are significantly different from each other.

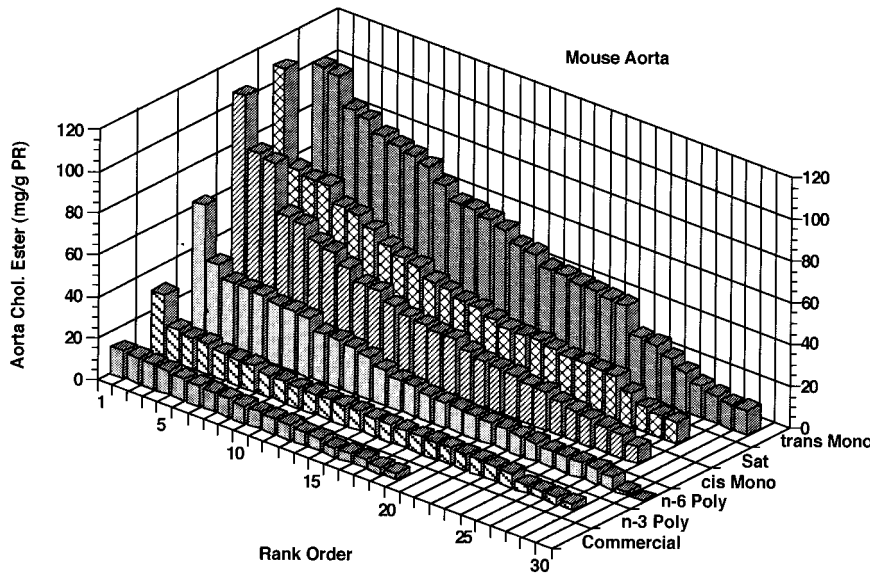


Figure 4. Rank order of aortic cholesteryl ester concentration in each diet group as a comparison of the atherosclerosis extent across the range of variability within each diet group. PR indicates protein.

commercial diet group, at 18.2 mg/g, and was barely higher ($P=0.07$) than the value in the n-3 poly group, but the averages in the *cis* mono group, at 34.6 mg/g, and sat group, at 35.6 mg/g, were each higher ($P<0.001$) than either of the poly groups. The highest value was found in the *trans* mono group (50.4 mg/g), and this value was significantly higher than either the sat or *cis* mono group ($P<0.002$), as well as higher than that of any of the other groups. Thus, the outcome found for both free and esterified cholesterol concentrations, as indicators of atherosclerosis extent, was similar. The greatest accumulation of both forms of cholesterol occurred in the *trans* mono group, the next highest occurred in the sat and *cis* mono groups, and less accumulation occurred in the poly groups, which were not much different from the group fed the commercial diet. A very similar outcome was obtained when the analyses were done on cholesterol and cholesteryl ester as measured in mg/g of wet tissue (data not shown). The data expressed as mg/g protein are thought to be more accurate, owing to the small amounts of tissue (≈ 2 mg per aorta) and the potential weighing errors of wet tissue that could occur.

The individual animal data for cholesteryl ester concentration are plotted in rank order from least involved to most involved in Figure 4 for each of the diet groups to illustrate the degree of difference among the groups. These data indicate that the diet group differences identified by the statistical analyses are present across the entire spectrum of response, from low to high responders, and are not confined to a particular segment of the response spectrum.

Finally, the data shown in Figure 5 indicate that the data from 5 of the groups all described a similar log-linear relationship, with a correlation coefficient of $r=0.7$ between TPC and aortic cholesteryl ester concentration. The data for individual groups are also indicated and were shown to essentially fit the line in Figure 4 when each data set was plotted separately. The data comparing plasma LDL and VLDL cholesterol concentrations were also plotted (not shown), and similar relationships were seen, although the correlation coefficients were not as high as for TPC. We

interpret this finding to mean that both lipoprotein fractions are contributing to the development of atherosclerosis, although LDL appears to be the more important, presumably because it is present in much higher concentrations. Interestingly, the data for the *trans* mono group did not fit the relationship shown in Figure 5 (data not shown). The scatter in the data from this group, if plotted as in Figure 4, was large ($r=0.24$), and the regression line for the data of this group was displaced, such that at any aortic cholesteryl ester concentration, the TPC concentration was apparently higher. This result is probably related to the distinct pattern of increase in plasma cholesterol between 8 and 16 weeks for this particular diet, as shown in Figure 1 for the *trans* mono group. The pattern suggests that the extended increase induced by *trans* monounsaturated fat in plasma cholesterol during the 8- to 16-week interval may not have been associated with an immediate and proportional effect on atherosclerosis, although the study needs to be carried out

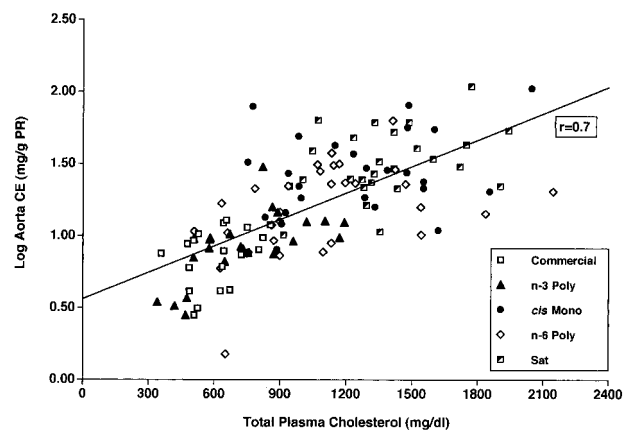


Figure 5. Relationship between TPC and the logarithm of aortic cholesteryl ester (CE) concentration (mg/g protein [PR]) across 5 diet groups. The best-fit regression line for all of the data is plotted, and the correlation coefficient for these data is shown. The correlation coefficients for the individual diet groups were as follows: commercial diet, 0.40; n-3 poly, 0.65; *cis* mono, 0.11; n-6 poly, 0.60; and sat, 0.52.

for a longer time to determine the real consequences for atherosclerosis.

Discussion

The data of this study illustrate the strongly positive effects of polyunsaturated fat compared with saturated fat to reduce the development of atherosclerosis in a mouse model that has LDL as the predominant lipoprotein in the circulation. This observation has also been made in several studies of coronary artery atherosclerosis in primates^{11,12,18,19} and is reproduced in the aortas of the mice of this study. The n-3 polyunsaturated fat appeared to be more effective than the n-6 polyunsaturated fat, although only 1 level of either polyunsaturated fat was studied here (7.2 energy% of n-6 versus 1.4 energy% of n-3 fatty acids), and therefore, the dose response is not known. Nevertheless, because almost 5 times more n-6 than n-3 polyunsaturated fatty acids were fed in the diet and because the n-3 polyunsaturated fatty acids kept aortic cholesteryl ester concentrations lower (Figures 3 and 4), the data suggest that n-3 fatty acids are more potent in reducing atherosclerosis.

The reduction in aortic atherosclerosis was not found when either *cis* or *trans* monounsaturated fatty acids were fed. Rather, just as much atherosclerosis was seen when *cis* monounsaturated fat diets were fed (6.8 energy%) as when saturated fat was fed, and significantly more atherosclerosis was seen when the *trans* monounsaturated fatty acids were fed as 2.8 energy% (Figures 3 and 4). The average plasma LDL cholesterol concentration was very similar in the mice fed n-6 polyunsaturated fat, saturated fat, and *cis* monounsaturated fat, yet the amount of atherosclerosis was greater in the *cis* monounsaturated and saturated fat groups. The HDL cholesterol concentrations were slightly higher in the group fed n-6 polyunsaturated fat, but the LDL to HDL cholesterol ratios were very high in all groups (in the range of 15 to 30), and it is not clear that HDL at these ratios can offer much protection in the face of such high plasma LDL concentrations. The VLDL cholesterol levels were lower in the n-6 polyunsaturated fat group, and even though these lipoproteins were present in lower concentrations than LDL, this could account for some of the difference in atherosclerosis.

The observation in these LDL receptor-null, human apoB-overexpressing mice that *cis* monounsaturated fatty acids in the diet promote as much atherosclerosis as saturated fatty acids and more atherosclerosis than polyunsaturated fatty acids is similar to an earlier observation on coronary artery atherosclerosis in primates.¹⁸ This is an important outcome when one considers that monounsaturated fats, often in the form of olive oil, are widely promoted as being healthful and effective for protection against heart disease.^{16,17} Although effects to modify lipoprotein risk factor profiles to be apparently less atherogenic were seen in response to dietary monounsaturated fat in monkeys and humans,^{17,18} effects to reduce atherosclerosis did not follow. In the present study in mice, the difference between the n-6 polyunsaturated fat group and the *cis* monounsaturated fat group in TPC and LDL cholesterol concentrations was not statistically significant, yet the former dietary fat still protected against atherosclerosis while the latter did not. Clearly, some other factor in response

to *cis* monounsaturated fatty acids but not in response to polyunsaturated fatty acids appears to promote atherosclerosis, and as a result, the amount of atherosclerosis in animals fed monounsaturated fat was comparable to that when saturated fat was fed.

On the basis of data in monkeys, we speculated that another factor promoting atherogenesis could have been the remarkable enrichment of the lipoprotein particles with cholesteryl oleate.^{12,18} We examined the livers of monkeys in isolated liver perfusion studies and found a high correlation between cholesteryl ester accumulation in apoB-containing particles secreted during perfusion and the extent of coronary artery atherosclerosis in the liver donor animals, suggesting that the accumulation of cholesteryl esters in LDL and the associated increase in atherosclerosis might be in response to stimulated hepatic secretion.³⁶ The stimulation of hepatic cholesteryl ester secretion was apparently due to a stimulation of hepatic acyl coenzyme A:cholesterol acyltransferase by oleic acid.^{37,38}

In the mice of these studies, enrichment of LDL with cholesteryl oleate was most pronounced in the *cis* mono and sat groups, although the lipid milieu in the core of the LDL particles of these mice was quite different from that found in monkeys and humans. A high percentage of triacylglycerol was found in the mouse LDL (Table 4), and significant quantities of cholesteryl arachidonate (and n-3 fatty acid cholesteryl esters in the n-3 poly group) were also found (Table 5). These lipids should modify the proatherogenic effect of the cholesteryl oleate enrichment, if effects on the physical state of the lipoprotein core are important in atherosclerosis, as has been proposed.³⁹ Triacylglycerols and polyunsaturated cholesteryl esters both have the effect of reducing the transition temperature of the neutral lipids in the core of the LDL particle.⁴⁰ Perhaps this factor contributes to the apparently modest rate of atherosclerosis development in our mouse model, in which plasma cholesterol concentrations were >500 mg/dL in the commercial diet-fed animals, for example, whereas the degree of cholesteryl ester accumulation in the aorta at 24 weeks of age was measurable but not remarkable (Figures 3 and 4). For example, in the African green monkey coronary arteries of our earlier publication,¹⁸ average cholesteryl ester concentrations in mg/g protein were 57, 179, and 137 in the polyunsaturated, monounsaturated, and saturated fat groups, respectively, values higher than those found in the mice of the present study (Figure 3). On the other hand, if cholesteryl oleate enrichment of LDL were to promote cholesteryl ester accumulation in the artery wall by forming a higher-melting-point lipid "phase" during lysosomal processing of lipoprotein particles, for example, then enrichment of LDL with cholesteryl oleate might still be part of the effect to increase atherosclerosis through delayed clearance of arterial cholesteryl esters.⁴¹

Clearly, more work is needed on the mechanism(s) by which an atherosclerosis-promoting effect of dietary monounsaturated fat would occur. What is clear when comparing the present studies in mice with the earlier studies in monkeys¹⁸ is that a lack of protection against atherosclerosis development by monounsaturated fat occurred in 2 experimental animal models with quite different lipoprotein profiles

and lipoprotein lipid compositions. It seems important to discern whether a similar outcome is present in humans before more recommendations are made to consume more fat enriched with monounsaturated fatty acids.

Animals fed polyunsaturated fat have LDL particles that are greatly enriched in the polyunsaturated fatty acid content in their cholesteryl esters⁴² and phospholipids,⁴³ and this result also appears to be true in the present study in mice (Table 5). Numerous studies, including our own,⁴⁴ have demonstrated that LDL particles enriched in polyunsaturated fatty acids are more easily oxidized in vitro. To the extent that LDL oxidation promotes atherosclerosis, data on dietary polyunsaturated fat protection against atherosclerosis present a paradox. It appears that other factors, possibly including LDL cholesterol concentration and/or particle composition, are more important in promoting atherosclerosis development in this circumstance. In the present studies in mice, in which the degree of atherosclerosis was less in the n-6 poly group (Figures 3 and 4) even though LDL cholesterol concentrations were not different among the n-6 poly, *cis* mono, and sat groups (Table 3), it remains a distinct possibility that LDL particle composition is an important factor in determining the extent of atherogenesis. Perhaps the LDLs from mice fed either *cis* mono or sat diets interact with the matrix within the artery more effectively than do LDLs from the polyunsaturated fat groups, as has been demonstrated in monkeys.⁴⁵ Alterations in the metabolism of cholesteryl esters and other lipids within the artery wall in response to dietary fatty acid composition, such as an effect of monounsaturated fatty acids to promote acyl coenzyme A:cholesterol acyltransferase activity, might also be involved. Clearly, more data are needed before we will fully understand the relative importance of the various effects of individual dietary fatty acids on atherosclerosis.

The effect of *trans* fatty acids to promote even more atherosclerosis than do saturated fatty acids was not anticipated on the basis on the literature.^{46–49} In the current study, *trans* fatty acids accounted for only 2.8% of energy, yet they dramatically promoted plasma cholesterol concentration increases more than any other type of fat (Figure 1 and Table 3). The mechanism by which plasma cholesterol was increased is unknown, but it seems likely that this increase was at least in part responsible for the worse atherosclerosis (Figures 3 and 4). The fact that the proportionality between TPC and aortic atherosclerosis was not high among animals in the *trans* mono group may be due to the fact that plasma cholesterol concentrations rose to such high levels during the last 8 weeks of the study that the impact of this increase was not fully registered in the arterial response measured at 16 weeks. Perhaps if the diet had been continued for a longer time, the extent of atherosclerosis would have been more highly correlated with the plasma cholesterol concentration. Alternatively, it is possible that *trans* fatty acids have a unique effect in promoting hypercholesterolemia but that the proportionality of atherosclerotic lesion development to plasma cholesterol concentration is different, due to an effect on the lipoprotein profile or on the artery wall, which was not delineated by the analyses performed in the present study.

Finally, these studies can be compared with those of Sanan et al,²⁵ wherein mice similar to those used in this study were studied after 26 weeks on a commercial diet. The range of cholesteryl ester concentrations observed for the commercial diet group was between 2 and 20 mg/g protein (Figure 4), whereas the range of values for the proportion of aortic surface area involved with lesion in the comparable group of mice of the Sanan study was between 2% and 50%. This result indicates that significant lesion development occurred during the 6-month study, even in mice fed the commercial diet, and that this animal model is indeed an excellent one for studying the factors affecting atherosclerosis in the mouse. We have shown the effects on atherosclerosis of individual dietary fatty acids in this study in mice that parallel those of our studies of coronary artery atherosclerosis in monkeys.¹⁸ This fact suggests that identification of factors important in the development of atherosclerosis in this LDL receptor–null, human apoB100–overexpressing mouse model could be appropriate for understanding the relevant factors in this disease process in humans.

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