Fatty acids differentially regulate hepatic cholesteryl ester formation and incorporation into lipoproteins in the liver of the mouse

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Abstract **These experiments tested the hypothesis that fatty acids (FAs) that drive cholesterol esterification also enhance sterol secretion and were undertaken using a mouse model where lipoprotein-cholesterol output by the liver could be assessed in vivo. The turnover of sterol in the animals was kept constant (~160 mg/d per kg) while the liver was enriched with the single FAs 8:0, 14:0, 18:1, or 18:2. Under these conditions, the steady-state concentration of cholesteryl ester in the liver varied 6-fold, from 1.2 to 7.9 mg/g, and the expansion of this pool was directly related to the specific FA enriching the liver (FA 18:1>18:2>8:0> 14:0). Secretion of lipoprotein-cholesterol varied 5-fold and was a linear function of the concentration of cholesteryl ester in the liver. These studies demonstrate that unsaturated FAs drive the esterification reaction and enhance lipoprotein cholesterol secretion by the liver under conditions where cholesterol balance across this organ is constant. Thus, individual FAs interact with cholesterol to profoundly regulate both the output and uptake of sterol by the liver, and these effects are articulated through the esterification reaction.**—Xie, C., L. A. Woollett, S. D. Turley, and J. M. Dietschy. **Fatty acids differentially regulate hepatic cholesteryl ester formation and incorporation into lipoproteins in the liver of the mouse.** ***J. Lipid Res.*** **2002.** 43: **1508–1519.**

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The major determinant of atherosclerosis in any population appears to be the steady-state concentration of total cholesterol (TC) in the plasma and, more particularly, the concentration of cholesterol carried in low density lipoprotein (LDL-C) and other apolipoprotein B (apoB)-containing lipoprotein fractions. The incidence of death due to coronary artery disease (CAD), for example, increases in a nearly linear relationship to the plasma TC between values of approximately 150 mg/dl and 400 mg/dl (1, 2). The relatively high concentration of circulating cholesterol and the high incidence of CAD seen in Western populations is related to the intake of both cholesterol and, particularly, triacylglycerol in these populations, although the manner in which these dietary constituents affect lipoprotein-cholesterol levels is still only partly understood.

The steady-state concentration of LDL-C is determined by the rate at which this lipoprotein fraction is formed within the plasma space, i.e., the LDL-C production rate, the level of LDL receptor (LDLR) activity in the liver, and the amount of apoE-containing lipoprotein that is competing with LDL for binding to the LDLR (3–8). In general, of these three parameters, the most powerful determinant of the plasma LDL-C concentration in both animals and humans is the LDL-C production rate term (6, 7, 9). The rate of LDL-C production, however, is not an independent variable since the magnitude of this rate constant is determined by both the rate of secretion of cholesterol in very low density lipoprotein (VLDL-C) and the level of hepatic LDLR activity. This is true because a portion of the metabolized remnants of VLDL contains apoE and is removed from the circulation by the LDLR before it can be converted to LDL (10). As a consequence of this complex relationship, it is nearly impossible in the intact animal or human to explore how intracellular events in the liver influence the rate of VLDL-C secretion

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Abbreviations: apoB, apolipoprotein B; apoE, apolipoprotein E; CAD, coronary artery disease; DPS, digitonin-precipitable sterols; ER, endoplasmic reticulum; FA, fatty acid; FPLC, fast protein liquid chromatography; GLC, gas liquid chromatography; LDL-C, cholesterol carried in low density lipoprotein; LDLR, LDL receptor; TC, plasma total cholesterol; SREBP, sterol regulatory element-binding protein; VLDL-C, cholesterol carried in very low density lipoprotein.

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by simply quantitating changes in the LDL-C production rate. Hence, investigators have been forced to use either isolated cells or the perfused liver preparation in order to examine the regulation of hepatic VLDL assembly and secretion (11, 12).

Nevertheless, previous studies have shown that both dietary cholesterol and triacylglycerol are important in the regulation of the plasma LDL-C concentration and, further, several lines of evidence suggest that the microsomal enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT) is key to this regulation. However, the manner in which this enzyme articulates the interaction between the dietary cholesterol and fatty acid (FA) reaching the liver, and the regulation of LDLR activity and VLDL-C secretion is poorly understood. Two isoforms of ACAT have recently been isolated (13–18). In the mouse, ACAT-1 functions primarily in the adrenal gland and macrophage while in both the monkey and mouse, ACAT-2 is found primarily in the liver and intestine (17, 19, 20). This enzyme does not appear to be transcriptionally regulated but, rather, it responds to the supply of the two substrates, unesterified cholesterol and FA, presumably in the vicinity of the endoplasmic reticulum (ER) (21–24).

Based upon these observations, it has been postulated that in the steady state ACAT distributes excess sterol entering the liver between a pool of unesterified cholesterol in the ER and a pool of cholesteryl ester (22). By suppressing the release of transcriptionally active sterol regulatory element-binding protein (SREBP) from the ER, this unesterified cholesterol suppresses hepatic LDLR activity (25–27). Increasing the flow of cholesterol into the hepatocyte progressively increases the size of these pools and proportionally reduces the activity of the LDLR. Thus, under conditions where the profile of FA in the liver is kept constant, hepatic LDLR activity varies inversely with the steady-state concentration of unesterified cholesterol in the ER and cholesteryl ester in the cell (28–30). This relationship is dramatically altered, however, when the liver is enriched with specific FAs that vary in their ability to be utilized by ACAT. Long chain-length saturated FAs, for example, suppress cholesteryl ester formation, force more unesterified cholesterol into the ER, and further suppress hepatic LDLR activity (7, 31). In contrast, long chain-length unsaturated FAs, which are the preferred substrate for ACAT, shift cholesterol from the unesterified to the esterified pool and restore LDLR activity (31). Thus, under conditions where the flow of sterol into the liver is kept constant, the content of specific FAs in the hepatocyte determines the distribution of intracellular cholesterol between the unesterified fraction in the ER and the cholesteryl ester pool. As a consequence, in this situation LDLR activity varies directly with the concentration of cholesteryl ester in the liver (3, 30–32).

While these studies leave little doubt that the level of hepatic LDLR activity is determined by dietary cholesterol and FA acting in concert to regulate the concentration of unesterified cholesterol in the ER, there is less quantitative information on the consequence of altering the pool of cholesteryl ester on the rate of VLDL-C secretion and, ultimately, on the rate of LDL-C production. Thus, as the LDL-C production rate is the major determinant of the steady-state plasma LDL-C concentration, and as the VLDL-C secretion rate is an important determinant of the production of this atherogenic particle, these studies were undertaken to define the role of specific long chain-length FAs in regulating hepatic ACAT activity and the rate of cholesterol secretion in the VLDL particle. Specific experiments were designed *1*) to establish a mouse model where the VLDL-C secretion rate could be measured in vivo, *2*) to determine the dietary conditions under which net cholesterol balance across the liver was constant when specific FAs were fed, *3*) to quantitate the effects of each of these single FAs on steady-state hepatic cholesteryl ester levels and rates of sterol secretion in VLDL, and *4*) to determine the changes that take place in lipoprotein composition with the feeding of each of these FAs. These studies, therefore, provide the first detailed description of how each of these long chain-length fatty acids affect VLDL-C secretion, in vivo, and are complementary to data already published on how these same long chain-length FAs alter hepatic LDLR activity (30–32).

MATERIALS AND METHODS

Theoretical considerations

These studies were carried out in a mouse model where the steady-state concentration of cholesterol in plasma apoB-containing lipoproteins was taken as a direct measure of the rate of hepatic cholesterol secretion in VLDL particles. The theoretical basis for this model was as follows. Unesterified and esterified cholesterol, triacylglycerol, and apoB are assembled in the liver and secreted into blood as VLDL. The rate of total cholesterol secretion by this mechanism in the steady state is the VLDL-C production rate, J^{VLDLC}. After being metabolized in the periphery, a portion of the resulting VLDL remnants is cleared from the plasma by the LDLR while the remainder is converted to LDL-C at a velocity known as the LDL-C production rate (J^{LDLC}). Clearly, J^{LDLC} can be influenced by changes in either J^{VLDLC} or the level of LDLR activity. The LDL-C is also removed from the plasma by the LDLR located primarily in the liver (33–36) at a rate that is determined by the concentration of LDL-C in the plasma (C^{LDLC}), and by the maximal transport velocity (J^m) and apparent Michaelis constant (K_{m^*}) for the LDLR (4, 37). Because of competition from apoE-containing remnants, the K_{m^*} for LDL interacting with the LDLR is very high (~100 mg/dl) in all species so that this transport process is never saturated under physiological conditions (4, 5, 8). There is also a receptor-independent transport process that can remove LDL-C from the plasma at a rate designated P* (4, 33, 34). Thus, under normal circumstances, the steady-state concentration of LDL-C in the plasma is given by the following expression (37):

$$C^{LDLC} = \frac{J^{LDLC} - J^m - P^*K_{m^*} + \sqrt{(J^{LDLC} - J^m - P^*K_{m^*})^2 + 4P^*K_{m^*}J^{LDLC}}}{2P^*}$$

However, when LDLR activity is abrogated, the J^m and K_{m^*} terms equal zero and this expression simplifies so that C^{LDLC} becomes directly proportional to J^{LDLC}/P^*. Thus, as P* is not regu-

lable (33) in the absence of receptors, the concentration of cholesterol carried in the apoB-containing lipoproteins in the plasma is a direct, linear function of the rate of VLDL-C secretion from the liver in the steady state. This study, therefore, was largely carried out in mice lacking LDLR activity where the rate of cholesterol secretion from the liver could be judged directly from the concentration of sterol in the apoB-containing plasma lipoproteins.

Animals

The mice used in these studies were all males and were either control animals with normal LDLR activity ($LDLR^{+/+}$) or homozygous LDLR knockouts ($LDLR^{-/-}$) (36, 38). After weaning and genotyping, all experimental animals were fed ad libitum a cereal based, low cholesterol (0.02%, w/w), low fat (4.0%, w/w) rodent diet (No. 7001, Harlan Teklad, Madison, WI) until 3 months of age. The fatty acids in this diet have previously been described (39). At 3-months, the mice were placed on the specific experimental diets for 3 weeks, after which the various measurements were carried out during the fed state at the mid-dark phase of the light cycle as described (36).

Diets

The experimental diets were formulated using triacylglycerols containing a single FA that included trioctanoate (FA 8:0), trimyristate (FA 14:0), trioleate (FA 18:1), and trilinoleate (FA18:2) (Sigma Chemical Co., St. Louis, MO, and Nu-Chek-Prep, Inc., Elysian, MN). Most experiments were carried out using four different diets that were designated as the FA 8:0, FA 14:0, FA 18:1, and FA 18:2 diets. These were all prepared using a meal form of rodent diet (No. 8604, Harlan Teklad) to which was added 0.2% cholesterol (w/w) and 5% FA 8:0 triacylglycerol (w/w). The four different experimental diets were then formulated from this common mixture by adding an additional 15% of the FA 8:0, FA 14:0, FA 18:1, and FA 18:2 triacylglycerol (w/w). In one experiment, the total triacylglycerol level of these four diets was kept constant at 20%, but the added cholesterol concentration was varied in increments from 0% to 1.0% (w/w). In another experiment, the meal form of the rodent diet was mixed with either no cholesterol or with 3% cholesterol (w/w) alone.

Isolation and radiolabeling of LDL

Plasma was harvested from donor $LDLR^{-/-}$ mice maintained on the low cholesterol (0.02%, w/w) rodent diet (No. 7001, Harlan Teklad). The LDL fraction was isolated by preparative ultracentrifugation in the density range of 1.020–1.055 g/ml and then radiolabeled with either ^{125}I-cellobiose (TCB) or ^{131}I (35, 40, 41). These labeled LDL fractions were contaminated with small amounts of apoE-containing HDL, which were removed by passing the lipoprotein solution over a heparin sepharose CL-6B column (Pharmacia Biotech, Uppsala, Sweden) (42). After dialysis, these radiolabeled preparations were passed through a 0.45 μm Millex-HA filter (Millipore Products, Bedford, MA) immediately prior to injection into the recipient experimental animal. All fractions were used within 48 h of preparation.

Measurement of LDL clearance in vivo

Mice were lightly anesthetized with diethyl ether and then given xylazine-20 (Butler Company, Columbus, OH) subcutaneously, and a catheter was inserted into a jugular vein. After awakening, each animal was given a bolus of ^{125}I-TCB-labeled LDL followed by a continuous infusion of the same preparation at a rate calculated to maintain a constant specific activity in the plasma. Five minutes before the termination of the 4 h infusion period, a bolus of ^{131}I-LDL was administered to each of the animals. The animals were exsanguinated at 4 h and the liver was removed. The remaining carcass was cut into small pieces. Liver, carcass, and plasma samples were assayed for their content of ^{125}I and ^{131}I. These were used to calculate a rate of clearance of LDL by the liver and carcass. These clearance rates were expressed as the ml of plasma cleared of its LDL content per day per liver or carcass per kg body weight (ml/d per kg).

Measurement of cholesterol synthesis rates in vivo

Each animal was injected ip with approximately 20 mCi of [^{3}H]water and, after 1 h, was anesthetized and exsanguinated. Aliquots of plasma were taken for measurement of the specific activity of the plasma water. The liver and remaining carcass were saponified and digitonin-precipitable sterols (DPS) were isolated as described (35, 43). The rates of sterol synthesis in each of these tissues were then calculated as the nmol of [^{3}H]water incorporated into the DPS per hour per liver or carcass. These rates were then converted to an equivalent mg quantity of cholesterol assuming that 0.69 ^{3}H atoms were incorporated into the sterol molecule per carbon atom entering the biosynthetic pathway as acetyl CoA (41, 44, 45). These rates were expressed as the mg of cholesterol synthesized per day per kg body weight (mg/d per kg).

Measurement of intestinal cholesterol absorption, fecal bile acid excretion, and cholesterol 7α-hydroxylase activity

Cholesterol absorption was measured by a modified fecal dual-isotope ratio method using [4-^{14}C]cholesterol (NEN Life Science Products, Boston, MA) and [5,6-^{3}H]sitostanol (stigmastanol) (American Radiolabeled Chemicals, Inc., St. Louis, MO) as described with the stools being collected from each animal over a 3-day period immediately following dosing with the labeled sterols (46, 47). In the experiments involving fecal bile acid excretion measurements, stools were collected during the last 3 days of the 3-week feeding period, and their content of bile acid was determined enzymatically using [carboxyl-^{14}C]cholic acid (NEN Life Science Products) as an internal standard (41, 48). These data were expressed as the equivalent mg of cholesterol excreted as bile acid each day per kg body weight (mg/d per kg). The activity of cholesterol 7α-hydroxylase was measured in hepatic microsomal preparations by a high performance liquid chromatography method as described (49).

Tissue fatty acid abundance

Total hepatic lipids were extracted with chloroform-methanol (2:1, v/v) (50). Hepatic cholesteryl ester and triacylglycerol were isolated after passing a portion of the extract through a Bond Elut LRC column (10 cc/500 mg) (Varian, Harbor City, CA) (51). The total lipid extract and the cholesteryl ester and triacylglycerol fractions were subjected to methyl esterification, and the relative abundance of the individual fatty acids in each fraction was determined by gas liquid chromatography (GLC) (52).

Cholesterol, cholesteryl ester, and triacylglycerol concentrations in plasma and tissues

The total cholesterol concentration in plasma was measured enzymatically (Kit No. 1127771, Boehringer Mannheim, Indianapolis, IN). Plasma lipoproteins were isolated using two different procedures. First, lipoproteins were separated by simultaneously centrifuging plasma at the densities of 1.020 and 1.063 g/ml. The cholesterol content in the top and bottom half of each tube was quantitated by GLC using stigmastanol (Sigma Chemical Co.) as an internal standard. Second, plasma lipoproteins were separated by fast protein liquid chromatography (FPLC) using a Superose 6 column (53). Two milliliter fractions were collected and their cholesterol content was determined en-

zymatically. The livers and remaining carcasses were saponified, and their total cholesterol content was determined by GLC. Unesterified and esterified cholesterol in the liver and various lipoprotein fractions were separated on Sep-Pak Vac RD (500 mg) columns (Waters, Milford, MA) and the concentration of cholesterol in each fraction was quantitated by GLC. Triacylglycerol concentrations in liver were measured using a modified method as described (54). Liver total lipid was extracted with chloroform-methanol (2.1, v/v). Aliquots of these extracts and a triolein standard were dried under air and then dissolved in *tert*-butanol. A solution of Triton X-114-methanol (1:1, v/v) was added and the triacylglycerol concentration was measured enzymatically. Lipoprotein protein was measured using BSA as the standard (55).

Calculations

Cholesterol turnover was calculated as the sum of the amounts of cholesterol that were absorbed and synthesized each day per kg body weight (mg/d per kg). All data are presented as mean values ± 1 SEM. The two tailed, unpaired Student's *t*-test was used to determine whether the differences between mean values were statistically significant ($P < 0.05$). The relationships between hepatic cholesteryl ester and apoB-containing lipoprotein cholesterol levels were expressed as linear regression curves. The differences in the values for the slope and intercept of these regression curves for each of the four experimental diets were analyzed by an F test ($P < 0.05$).

RESULTS

Plasma and tissue cholesterol balance in the LDLR$^{+/+}$ and LDLR$^{-/-}$ mice

Several preliminary studies were necessary to establish the appropriate dietary conditions to be used in all subsequent experiments and to confirm that deletion of LDLR activity altered plasma, but not tissue, cholesterol balance as previously reported (36). Furthermore, it was also essential to determine the level of cholesterol feeding required to suppress sterol synthesis in the liver. In the live animal, the processes of hepatic cholesterol synthesis, cholesteryl ester formation, and LDLR activity are not coordinately regulated (56). Rather, an increase in net cholesterol inflow to the liver from the intestine is compensated for solely by a commensurate suppression of hepatic sterol synthesis. Only when hepatic synthesis is suppressed nearly to zero is there expansion of the cholesteryl ester pool and down-regulation of LDLR activity (41, 57). In preliminary experiments, mice were fed increasing amounts of dietary cholesterol to steady state at 3 weeks, and these three parameters were then evaluated. These experiments revealed that a dietary cholesterol concentration of 0.2% (w/w) in this strain of mice was sufficient to suppress hepatic cholesterol synthesis to essentially zero and to minimally elevate the level of hepatic ester in the steady state. Thus, all subsequent experiments were carried out in 3-month-old male mice fed a diet containing 0.2% cholesterol, 5.0% FA 8:0 triacylglycerol, and an additional 15% of either the FA 8:0, 14:0, 18:1, or 18:2 triacylglycerol.

Having established these dietary conditions, the next experiment was undertaken to be certain that deletion of LDLR activity dissociated regulation of net cholesterol balance across the plasma space from that across the tissues and, further, to demonstrate that this dissociation was present when all four FA diets were fed. As illustrated in **Fig. 1A**, total receptor dependent and independent LDL clearance in the four groups of LDLR$^{+/+}$ animals was very high and averaged 431 to 468 ml/d per kg body weight in animals receiving the FA 8:0, 18:1, and 18:2 diets and was 374 ml/d per kg in the mice fed the FA 14:0 ration. As expected, the great majority of this clearance took place in the liver (35, 36, 58). In contrast, clearance rates in the LDLR$^{-/-}$ mice were only about 40 ml/d per kg in all diet

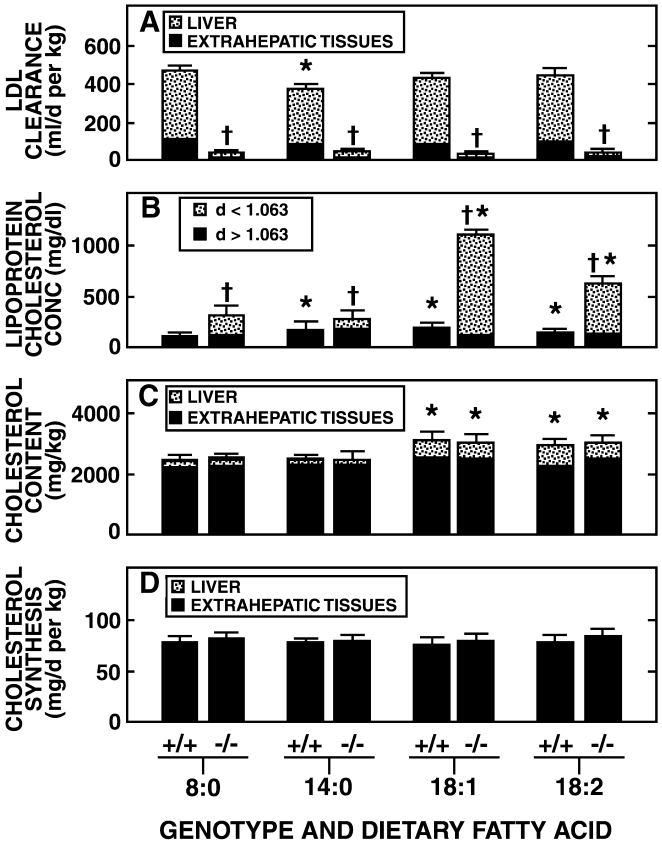

Fig. 1. LDL clearance rates, plasma lipoprotein cholesterol concentrations, tissue cholesterol contents, and sterol synthesis rates in LDL receptor (LDLR)$^{+/+}$ and LDLR$^{-/-}$ mice fed diets containing single fatty acids. Three-month-old, male LDLR$^{+/+}$ and LDLR$^{-/-}$ animals were fed diets containing 0.2% cholesterol, 5% FA 8:0 triacylglycerol, and 15% of either the FA 8:0, 14:0, 18:1, or 18:2 triacylglycerol for 3 weeks. A: Rate of LDL clearance in the liver and extrahepatic tissues of these animals. These rates are expressed as the ml of plasma cleared entirely of its LDL content per day (d) per kg body weight. B: Concentration of cholesterol in lipoproteins with densities that are either less than or greater than 1.063 g/ml. C: Total cholesterol content of the liver and extrahepatic tissues in these animals expressed as mg of sterol per kg body weight. The rates of cholesterol synthesis measured in vivo in these animals are shown in D, and these are expressed as the milligram of cholesterol synthesized per day per kg body weight. All values represent means ± 1 SEM for 8 to 10 mice in each group. * Value was significantly different ($P < 0.05$) from that seen in the same genotype animal fed the control FA 8:0 diet. † Data point in the LDLR$^{-/-}$ animals was significantly different ($P < 0.05$) from that in the LDLR$^{+/+}$ mice in the same diet group.

groups and only about half of this receptor independent uptake occurred in the liver. The plasma total cholesterol concentrations were all relatively low in the animals with normal LDLR activity (Fig. 1B) and varied from 105 to 151 mg/dl. However, in the LDLR$^{-/-}$ mice these values were much higher and were influenced dramatically by the type of fatty acid in the diet. These differences were reflected almost entirely in the concentration of cholesterol in the apoB-containing lipoprotein fraction ($d < 1.063$ g/ml) while the HDL-C concentrations ($d > 1.063$ g/ml) were similar in all groups. In contrast to these findings in the plasma compartment, tissue cholesterol content (Fig. 1C) and rates of whole animal cholesterol synthesis (Fig. 1D) were virtually identical in the LDLR$^{+/+}$ and LDLR$^{-/-}$ animals fed any of the four experimental diets. As is apparent however, feeding the FA 18:1 and 18:2 diets did expand the cholesterol pool in the livers of both genotypes, and this expansion was due entirely to higher steady-state levels of cholesteryl esters (Fig. 1C). Thus, taken together, these preliminary studies established that *1*) hepatic sterol synthesis was suppressed essentially to zero by the amount of cholesterol present in these diets (Fig. 1D); *2*) abrogation of LDLR activity did not alter either the content of cholesterol in the liver and extrahepatic compartment (Fig. 1C) or the rate of sterol synthesis in the extrahepatic tissues (Fig. 1D); and *3*) this abrogation did, however, markedly alter the concentration of cholesterol carried in the lower density lipoproteins (Fig. 1B) as would be predicted when plasma cholesterol levels became a dependent variable of VLDL-C secretion.

Cholesterol balance across the liver and extrahepatic tissues of the LDLR$^{-/-}$ mice

If this model was to be used to explore the role of specific fatty acids in determining the ACAT-driven equilibrium between unesterified and esterified cholesterol in the liver and the rate of VLDL-C secretion, it was essential to also establish that these various diets enriched the liver with specific fatty acids but did not alter net cholesterol balance across this organ. The second set of studies, therefore, was undertaken using only 3-month-old male LDLR$^{-/-}$ mice that were placed on these same defined diets. As illustrated in **Fig. 2A**, in animals receiving the control FA 8:0 diet, the most abundant FAs in the hepatic total lipid fraction were the FA 16:0 (21%), 18:2 (18%), 18:1 (16%), and 18:0 (10%). FA 18:1 was the most abundant acid in the triacylglycerol (31%) (Fig. 2B) and cholesteryl ester (40%) (Fig. 2C) fractions. In the other diet groups, all three hepatic lipid fractions became significantly enriched with the specific fatty acid present in the respective diets. For example, while FA 14:0 could barely be detected in the liver of animals fed the control diet, this compound accounted for 10% to 24% of the lipids after feeding the FA 14:0 diet. This enrichment was much more dramatic, however, after feeding the FA 18:1 and 18:2 diets. For example, the FA 18:1 (67%) and 18:2 (62%), respectively, became the predominant long chain-length lipid in the cholesteryl ester fraction (Fig. 2C) in the mice fed the FA 18:1 and 18:2 diets.

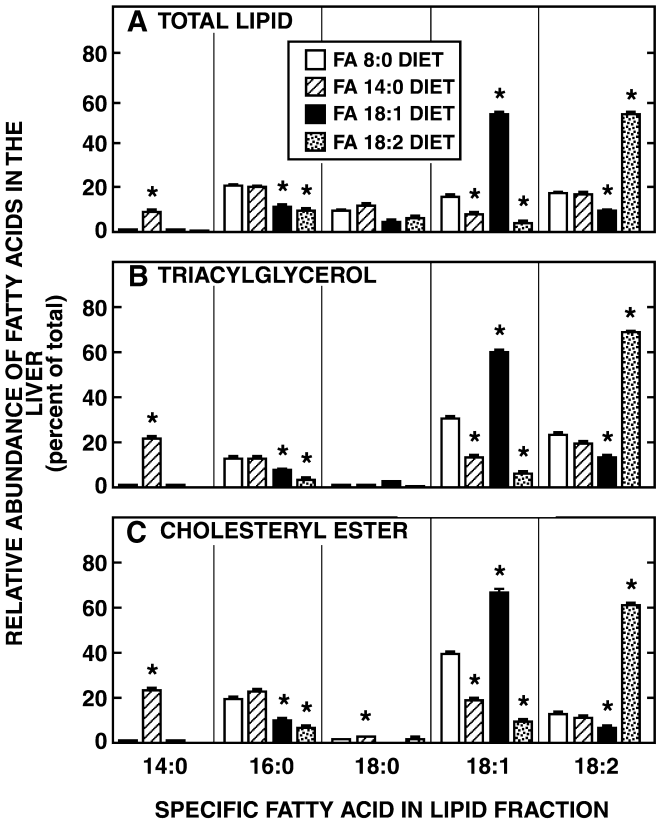

Fig. 2. Relative abundance of specific fatty acids in the total lipid, triacylglycerol, and cholesteryl ester fractions of the livers of LDLR$^{-/-}$ mice that were fed four different triacylglycerol diets for 3 weeks. The diets utilized in these studies were the same as those described in Fig. 1. At the end of this feeding period the lipids in the livers of these animals were extracted and separated into a total lipid fraction (A), a triacylglycerol fraction (B), and a cholesteryl ester fraction (C). Subsequently, the relative abundance of each fatty acid in these three fractions was quantified. All values are means ± 1 SEM for 10 to 12 mice in each diet group. * Fatty acids with relative abundance in the animals fed the FA 14:0, 18:1, and 18:2 diets significantly different ($P < 0.05$) from that in the mice fed the control FA 8:0 diet.

That these changes, however, did not alter net cholesterol balance across the liver or whole animal is illustrated by the data shown in **Figs. 3** and **4**. Using LDLR$^{-/-}$ animals fed these same four experimental diets, the absolute rates of dietary cholesterol intake and the percentage of this dietary sterol load that was absorbed was quantified. As is apparent in Fig. 3A, there was no significant difference in the rate of cholesterol absorption in the four experimental groups, and these rates all averaged 74 to 84 mg/d per kg body weight. The absolute rates of cholesterol synthesis were also measured in parallel groups of mice. Hepatic synthesis was suppressed in all groups, but the extrahepatic tissues synthesized cholesterol at rates of 77 to 82 mg/d per kg (Fig. 3B). Again, there were no significant differences among the four dietary groups so that net cholesterol turnover in these whole animals (Fig. 3C) was the same regardless of which dietary fatty acid was fed.

There was also no difference in bile acid synthesis in

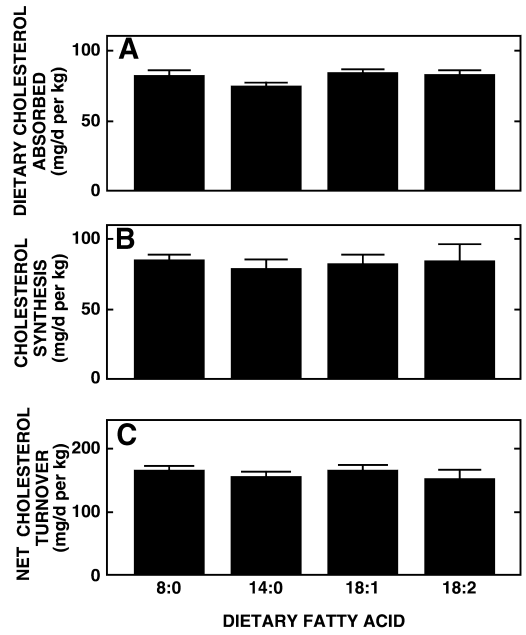

Fig. 3. Dietary cholesterol absorption, cholesterol synthesis, and net cholesterol turnover in LDLR$^{-/-}$ mice fed the four different FA diets. The diets were the same as those described in Fig. 1 and were fed for 3 weeks. At the end of this time, different groups of animals were utilized for measuring the rates of dietary cholesterol intake and absorption (A), whole animal cholesterol synthesis (B), and net cholesterol turnover (C). In all three cases the data are expressed as milligram of cholesterol absorbed, synthesized, or turned over each day per kg body weight. All values represent means ± 1 SEM for 12 to 14 mice in each diet group. There were no significant ($P > 0.05$) differences between the mice fed the FA 14:0, 18:1, or 18:2 diet and the control animals fed the FA 8:0 diet.

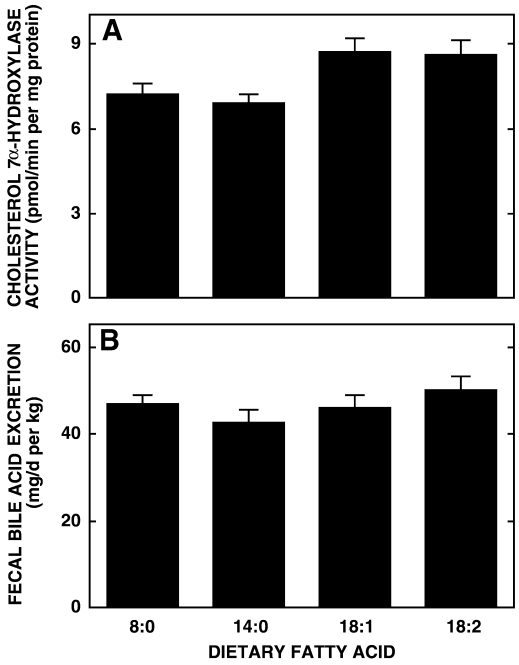

Fig. 4. Hepatic cholesterol 7α-hydroxylase activity and fecal bile acid excretion in LDLR$^{-/-}$ mice fed the four different FA diets. The diets were the same as described in Fig. 1 and were fed for 3 weeks. At the end of this time, hepatic microsomal cholesterol 7α-hydroxylase activity (A) and the rate of fecal bile acid excretion (B) were measured. The data represent means ± 1 SEM for measurements carried out in 10 to 12 mice in each diet group. There were no significant differences ($P > 0.05$) in the values found in the groups fed the FA 14:0, 18:1, or 18:2 diet and the control animals fed the FA 8:0 diet.

any of these groups. The levels of hepatic cholesterol 7α-hydroxylase in the animals fed the FA 8:0, 14:0, 18:1, and 18:2 diets were not significantly different (Fig. 4A), nor were the absolute rates of bile acid synthesis and excretion (Fig. 4B). Thus, in these LDLR$^{-/-}$ mice, net cholesterol turnover averaged about 160 mg/d per kg (Fig. 3C) while approximately 45 mg/d per kg of this turnover occurred through bile acid formation (Fig. 4B). Although not quantified in these studies, the remaining sterol presumably was excreted as neutral fecal sterols, skin sterols, and steroid hormones (58). Thus, under conditions where the fatty acid composition of the lipid pools in the liver varied markedly (Fig. 2), there were no differences in the rates of net sterol turnover in the experimental animals fed the four different fatty acid diets. Differences in hepatic VLDL-C secretion, therefore, presumably were associated with changes in the steady-state concentration of hepatic cholesteryl ester and not with a change in net cholesterol flux across the liver.

Effect of specific fatty acids on hepatic VLDL secretion

Having established that net cholesterol balance in the LDLR$^{-/-}$ mice was unaltered by the different diets, it was next possible to explore how enrichment of the liver with these specific fatty acids shifted the steady-state distribution of hepatic cholesterol into the ester pool and drove VLDL secretion. As shown in **Fig. 5A**, in LDLR$^{-/-}$ mice fed to steady state, the concentration of cholesteryl ester in the liver varied markedly in the different diet groups, even though hepatic cholesterol balance in these animals was not different. These variations, therefore, reflected differences in the ability of the specific fatty acids to drive the equilibrium of the ACAT reaction in the direction of esterification. The mice that were enriched with the FA 14:0 had hepatic cholesteryl ester concentrations (1.2 ± 0.1 mg/g) that were marginally lower than those fed the control diet (1.4 ± 0.1 mg/g). In contrast, enriching the liver with either the FA 18:1 or 18:2 increased hepatic cholesteryl ester 6-fold, to 7.9 ± 0.7 mg/g and 7.0 ± 0.6 mg/g, respectively. There were also differences in the triacylglycerol concentration in these livers (Fig. 5B). All three long chain fatty acids increased the hepatic triacylglycerol level, compared with the control group, but the degree of elevation varied significantly among the three.

Most importantly, the concentration of cholesterol in apoB-containing plasma lipoproteins was marginally suppressed in the animals fed FA 14:0 but was markedly elevated in the FA 18:1 and 18:2 groups (Fig. 5C). The distribution of cholesterol in the plasma of these groups is shown in more detail in **Fig. 6**. The highest levels of cholesterol were seen in the animals fed the FA 18:1 diet

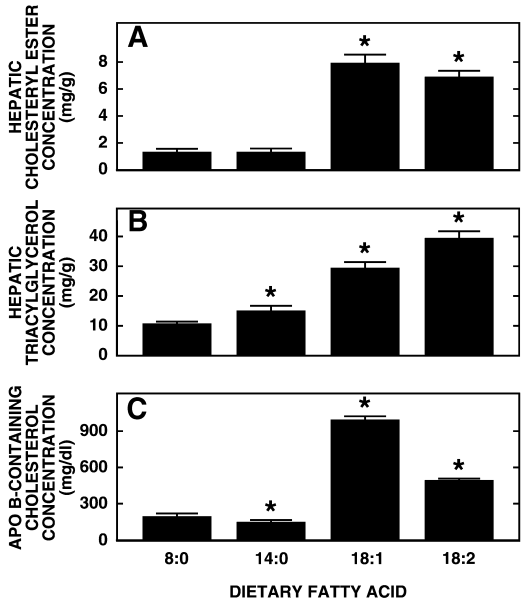

Fig. 5. Concentration of cholesteryl ester and triacylglycerol in the liver and apoB-containing lipoprotein cholesterol in the plasma of LDLR$^{-/-}$ mice fed the four different FA diets. The diets were the same as described in Fig. 1 and were fed for 3 weeks. At the end of this time the steady-state concentrations of cholesteryl ester (A) and triacylglycerol (B) were measured in the livers. Plasma was also harvested from the animals and the apoB-containing lipoproteins (d < 1.063 g/ml) were isolated (C). The data represent means ± 1 SEM for measurements carried out in 10 mice in each group. * Values that were significantly different ($P < 0.05$) from the corresponding value in the control animals fed the FA 8:0 diet.

where all of the apoB-containing fractions were markedly elevated above control values. A similar profile was seen in the mice fed the FA 18:2 diet, although these values were less elevated than those seen with the FA 18:1. In contrast to these alterations in the VLDL-C, IDL-C and LDL-C fractions, the concentration of cholesterol in HDL was relatively unaffected by enrichment of the liver with these three different long-chain fatty acids. The concentration of total cholesterol in the apoB-containing lipoprotein fractions (d < 1.063) is summarized in Fig. 5C, and these data demonstrate that relative to the control mice (190 ± 12 mg/dl), enriching the liver with the FA 14:0 significantly reduced the secretion of lipoprotein cholesterol (144 ± 15 mg/dl) while both the FA 18:1 (979 ± 15 mg/dl) and 18:2 (435 ± 10 mg/dl) markedly increased this outflow from the liver. It is notable, however, that while the levels of cholesteryl ester and triacylglycerol were very high in the livers enriched with the FA 18:2 (Fig. 5A, B), the secretion of cholesterol into the plasma was only half as great as seen after enrichment of the liver with the FA 18:1 (Figs. 5C, 6).

In a parallel study the apoB-containing lipoprotein fraction (d < 1.063 g/ml) from each experimental group was further separated into fractions of d < 1.020 g/ml and d = 1.020–1.063 g/ml, and these were then subjected to an additional wash. As shown in **Fig. 7A**, the ratio of cholesteryl ester to protein in the VLDL and IDL fractions was marginally reduced in the animals fed the FA 14:0 diet, but was markedly elevated in those mice fed the FA 18:2 and, especially, FA 18:1 diets. These differences were also seen in the LDL fractions (Fig. 7B), although the eleva-

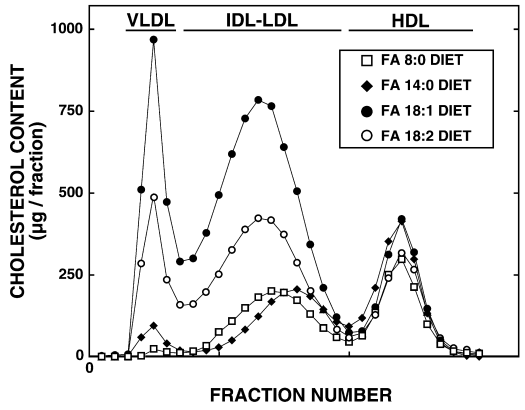

Fig. 6. Cholesterol content in the major lipoprotein fractions of LDLR$^{-/-}$ mice fed the four different FA diets for 3 weeks. The diets utilized in this study were the same as described in Fig. 1. At the end of this feeding period, plasma was pooled from five animals in each group and the lipoproteins were separated by fast protein liquid chromatography.

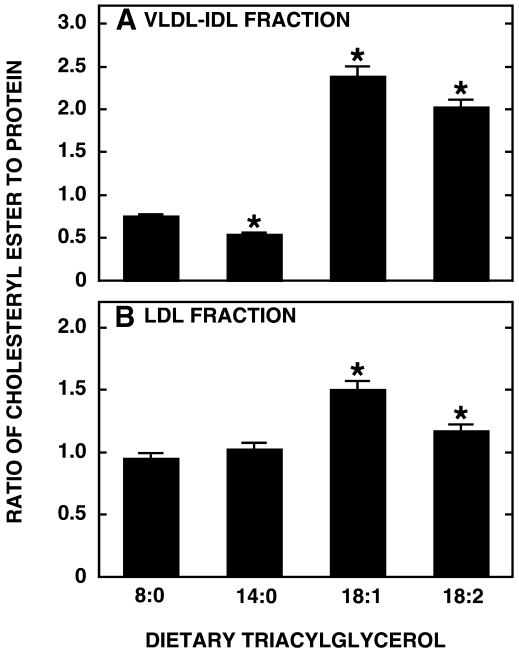

Fig. 7. Ratio of cholesteryl ester to protein in lipoproteins of two different density classes in LDLR$^{-/-}$ mice fed the four FA diets. The diets were the same as those described in Fig. 1 and were fed for 3 weeks. At the end of this feeding period, plasma was harvested and lipoproteins were separated into VLDL-IDL (d < 1.020 g/ml) and LDL (d = 1.020–1.063 g/ml) fractions. After washing, the concentration of cholesteryl ester and total protein in each of these fractions was quantified, and the ratio of these two values is shown in A and B. These values represent means ± 1 SEM for determinations in 10 mice from each diet group. * Indicates that the value was different ($P < 0.05$) from that found in the control animals fed the FA 8:0 diet.

tions were less striking. Given that the clearance rates (Fig. 1A) and lipoprotein protein concentrations in the plasma were similar in all four dietary groups, it was concluded from this experiment that the major effect of these specific fatty acids was articulated through changes in the concentration of cholesteryl ester in each lipoprotein particle. Thus, relative to the control group, FA 14:0 marginally suppressed this ratio while the FA 18:1 and 18:2 increased the amount of cholesteryl ester in the secreted particles by 3- to 4-fold (Fig. 7).

Relationship of hepatic cholesteryl ester to VLDL-C secretion

Even though FA 18:1 and 18:2 raised hepatic cholesteryl ester levels to nearly equal values when fed with 0.2% cholesterol (Fig. 5A), the rate of cholesterol secretion appeared to be only half as great when the liver was enriched with FA 18:2 as with 18:1 (Fig. 5C). A more detailed analysis of this observation, and of the relationship between these two parameters, was carried out by feeding large groups of LDLR$^{-/-}$ mice the same experimental diets except that the concentration of added cholesterol fed to each of the 76 to 99 animals in each group was systematically varied in small increments from 0% to 1.0%. In this manner the liver of each animal was enriched with a constant amount of FA 14:0, 18:1, or 18:2, but in this experiment the mass of cholesterol flowing into the liver varied in each of the animals.

As shown in **Fig. 8**, there were striking differences in the relationship between apoB-containing lipoprotein cholesterol secretion and the steady-state hepatic cholesteryl ester concentration when the liver was enriched with the different long-chain fatty acids. Three major conclusions were derived from these experiments. First, in the animals fed the FA 8:0 and 14:0 diets, titration of the individual animals with 0% to 1.0% dietary cholesterol maximally raised the steady-state cholesteryl ester concentrations to only about 4 mg/g (Fig. 8A, B). However, when the liver was enriched with either the FA 18:1 or 18:2, these concentrations reached values that were 6-fold higher (Fig. 8C, D). Second, while the intercepts of the linear regression curves fitted to these data were not different for the FA 8:0 (135 mg/dl) and 14:0 (126 mg/dl), these values were significantly higher ($P > 0.05$) for the FA 18:1 and 18:2. Furthermore, the intercept for the FA 18:1 (478 mg/dl) was twice as high as that for the FA 18:2 (212 mg/dl). Third, the slope of the regression curve fitted to the data from the animals fed the FA 18:1 diet (49 mg/dl per mg/g of cholesteryl ester) was significantly greater ($P < 0.05$) than that found after feeding the FA 18:2 diet (36 mg/dl per mg/g of cholesteryl ester). Thus, this experiment demonstrated that fatty acid enrichment of the hepatocyte could dictate the ACAT-driven equilibrium between free and esterified cholesterol over a very large range of cholesterol inflows into the liver.

A final experiment explored this relationship when the cholesteryl ester pool of the liver was expanded with dietary cholesterol alone when no dietary triacylglycerol was added to the diet. As shown in **Table 1**, feeding 3% cholesterol alone raised the cholesteryl ester concentration in the liver about 7-fold and this was associated with a 7-fold increase in apoB-containing lipoprotein cholesterol secretion by the liver. This increase occurred under circumstances where the hepatic triacylglycerol concentration was not significantly ($P > 0.05$) changed. Thus, cholesterol secretion in lipoproteins could be markedly driven by expansion of the cholesteryl ester pool either by increased net delivery of sterol to the liver or by redistributing a constant amount of cholesterol into the ester pool by enriching the liver with the appropriate fatty acid substrate for the ACAT reaction.

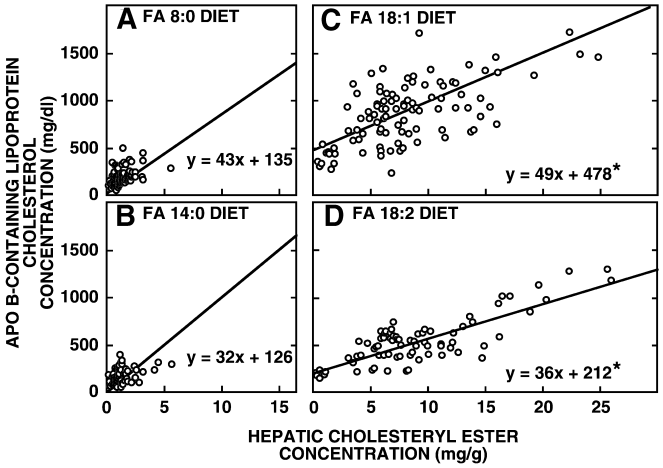

Fig. 8. The concentration of cholesterol in apoB-containing lipoproteins as a function of the concentration of hepatic cholesteryl ester in LDLR$^{-/-}$ mice fed a constant amount of the four different triacylglycerols and a variable amount of cholesterol. Each experimental group consisted of 76 to 99 LDLR$^{-/-}$ mice that were individually fed diets containing 5% FA 8:0 triacylglycerol and an additional 15% of either the FA 8:0 (A), FA 14:0 (B), FA 18:1 (C), or FA 18:2 (D) triacylglycerol. In addition, the cholesterol concentration in these individual diets was systematically varied in small increments from 0% to 1% (w/w). In this manner, each animal received a constant amount of one of the four triacylglycerols and cholesterol throughout the 3 week feeding period, but the concentration of cholesterol in the diet varied in each of the animals. At the end of this time, the concentration of cholesterol in the plasma apoB-containing lipoproteins (d < 1.063 g/ml) and in hepatic cholesteryl ester was determined in each mouse. Linear regression equations were calculated and are shown for each of the experimental groups. Data from individual animals are plotted. * Intercepts in the animals fed the FA 18:1 and 18:2 diets that were significantly different ($P < 0.05$) for that in the animals fed the 8:0 diet. The slope in the animals fed the FA 18:2 diet (D) was also significantly different ($P < 0.05$) when compared with the slope found in the animals fed the FA 18:1 (C).

DISCUSSION

These studies provide direct evidence that dietary FAs differentially regulate the steady-state level of cholesteryl ester in the liver, and these levels, in turn, dictate the rate of sterol incorporation into VLDL particles and secretion into the plasma. Importantly, these measurements were

TABLE 1. Concentration of hepatic cholesteryl ester and triacylglycerol and of cholesterol carried in plasma apoB-containing lipoproteins in mice fed additional dietary cholesterol alone

Added Dietary Cholesterol	Hepatic Cholesteryl Ester Concentration	Hepatic Triacylglycerol Concentration	Plasma ApoB-Cholesterol Concentration
%	*mg/g*	*mg/g*	*mg/dl*
0	0.52 ± 0.06	8.90 ± 0.79	94 ± 14
3	3.80 ± 0.97*	10.61 ± 1.89	664 ± 85*

LDL receptor$^{-/-}$ mice were fed diets containing either 0% or 3% added cholesterol but no added triacylglycerol. After 3 weeks the concentration of hepatic cholesteryl ester and triacylglycerol and of cholesterol carried in plasma apoB-containing lipoproteins ($d < 1.063$ g/ml) was measured. These data represent means ± 1 SEM for 12 mice in each diet group.

*Data for the animals fed 3% added cholesterol that were significantly different ($P < 0.05$) from those receiving no added cholesterol.

carried out in a mouse model where the rates of cholesterol absorption and synthesis were shown to be constant and unaffected by the different FAs fed in the experimental diets (Figs. 1 and 3). The effect of the different lipids on cholesteryl ester levels in the liver, therefore, reflected the ability of these different FAs to drive the esterification reaction and was not due to a change in net sterol balance across the liver. Apparently, under steady-state conditions, an equilibrium is established between the concentration of unesterified cholesterol in the membranes of the endoplasmic reticulum and the size of the adjacent pool of cholesteryl ester. This equilibrium is dictated, in part, by the specific FAs that are available in the cytosol to support the ACAT reaction.

Thus, under these circumstances, feeding the experimental diets containing single FAs resulted in enrichment of the hepatic lipid pools with either the FA 14:0, 18:1, or 18:2 (Fig. 2A). With this enrichment, there was a marked increase in the incorporation of each of these respective FAs into both the triacylglycerol (Fig. 2B) and cholesteryl ester (Fig. 2C) fractions. However, the absolute concentration of cholesteryl ester achieved in the liver at steady state varied markedly and presumably reflected the rate at which each of these acids could be utilized by ACAT. Thus, the level of cholesteryl ester found after feeding these diets followed the pattern FA 18:1>18:2>8:0>14:0 (Fig. 5A). In parallel with these findings, the rate of secretion of cholesterol carried in apoB-containing lipoproteins manifested this same pattern.

While these investigations were deliberately carried out under conditions where cholesterol balance across the hepatocyte was kept constant, it was clear in other studies that the FA profile in the liver also had a continuous effect on the size of the ester pool even when net cholesterol balance across the liver was varied. For example, in the animals fed a constant amount of FA 8:0 or 14:0, increasing the dietary sterol intake 50-fold from approximately 28 to 1,400 mg/d per kg increased the ester pool to about 4 mg/g and had a modest effect on the output of sterol in the apoB-containing lipoproteins (Fig. 8A, B). However, when the liver was enriched with either FA 18:1 or 18:2, the concentration of cholesteryl ester at steady state was increased several fold, as was the amount of sterol secreted into the plasma (Fig. 8C, D).

These experiments illustrate how powerful the interaction is between dietary FA and cholesterol in regulating VLDL-C secretion. In the mice fed only 0.2% cholesterol along with the FA 18:1, the level of cholesteryl ester in the liver and the rate of sterol secretion in the apoB-containing lipoproteins was elevated about 6-fold (Fig. 5A, C). In contrast, in those animals fed 15 times more dietary cholesterol, but no FA 18:1, the level of cholesteryl ester in the liver and the rate of VLDL-C secretion into the plasma was not increased to the same extent (Table 1). Thus, the combination of small amounts of cholesterol and large amounts of triacylglycerol had a far greater effect on cholesterol balance within the liver than did the administration of much larger amounts of cholesterol alone.

These findings confirm earlier observations where VLDL-C secretion was assessed in vitro using isolated hepatocytes or the perfused liver. Increasing the level of cholesteryl ester in these preparations either by feeding cholesterol or FA 18:1 was usually associated with an increase in the rate of sterol secretion in VLDL (59–62). Presumably, this cholesteryl ester, along with triacylglycerol, is incorporated into the nascent VLDL particle under the influence of microsomal triacylglycerol transfer protein (59, 60, 62–65). This possibility is supported by the observations that VLDL-C secretion from the liver is markedly suppressed by inhibitors of either ACAT or microsomal triacylglycerol transfer activity (61, 66).

There were, however, subtle differences in the effect of these various FAs with respect to the composition and rates of secretion of the VLDL particle. In Fig. 8, the intercepts of the linear regression curves give the rates of cholesterol secretion when no sterol was added to the diets. As is apparent, these secretion rates were significantly lower in the animals fed with the FA 8:0 and 14:0 (135 and 126 mg/dl, respectively) than in those fed the FA 18:1 and 18:2 (478 and 212 mg/dl, respectively), a finding that probably reflected the lower triacylglycerol levels in the livers of the former groups (Fig. 5B). However, once challenged with increasing amounts of dietary sterol, the predominant determinant of cholesterol output from the liver was the steady-state concentration of hepatic cholesteryl ester, although the coupling of these two events was significantly different for the FA 18:1 and 18:2. The rate of cholesterol secretion from the liver enriched with FA 18:1 increased by 49 mg/dl per mg/g increase in ester (Fig. 8C) but by only 36 mg/dl per mg/g in mice fed FA 18:2 (Fig. 8D). Nevertheless, in both cases the increase in cholesterol output from the liver primarily reflected an increase in the amount of esterified sterol present in each lipoprotein particle (Fig. 7A).

Previous studies have reported the effect of these same FAs on hepatic LDLR activity (30). The ability of these specific FAs to enrich the liver and increase LDLR activity followed a similar sequence, i.e., FA 18:1>18:2>8:0>14:0 (30, 31). Thus, these two sets of data are consistent with

the hypothesis that under conditions where net cholesterol balance across the liver is constant, the distribution of this sterol between an unesterified pool in the endoplasmic reticulum and a pool of cholesteryl ester is dictated by enrichment of the hepatocyte with specific FAs that either promote or inhibit the ACAT reaction. At one extreme, feeding the FA 18:1 shifts unesterified cholesterol out of the regulatory pool in the endoplasmic reticulum and into the cholesteryl ester pool. As a consequence, LDLR activity is increased as is the rate of sterol secretion from the liver. At the other extreme, enrichment of the liver with FA 14:0 inhibits esterification and leads to a reduction in both LDLR activity and hepatic sterol secretion. Presumably, cholesterol is the regulatory molecule, but the FA dictates the distribution of this regulator between the unesterified sterol pool that controls LDLR activity in the liver and the pool of cholesteryl ester that dictates, in part, the rate of VLDL-C secretion from this organ.

Clearly, in animals with normal LDLR activity fed diets with small amounts of cholesterol and large amounts of triacylglycerol, all three of these factors interact to determine the steady-state LDL-C concentration. This interaction is seen, for example, in studies reported in the African green monkey (67). Feeding triacylglycerol rich in monounsaturated FAs enriched the liver with FA 18:1, elevated the hepatic cholesteryl ester level, and increased the rate of VLDL-C secretion, as measured in the perfused liver. Under these conditions, the steady-state plasma LDL-C concentration was elevated in the intact monkey, presumably because of the marked increase in VLDL-C secretion and, hence, in LDL-C production. When the dietary triacylglycerol contained more saturated FAs, the plasma LDL-C concentration was significantly higher than that seen with the monounsaturated FA at any level of hepatic cholesteryl esters and VLDL-C secretion. This finding undoubtedly reflected the partial suppression of LDLR activity that is brought about by these more saturated FAs. Thus, it is the interplay of the small amounts of dietary cholesterol and the large amounts of dietary FAs that dictate the plasma LDL-C level through effects on hepatic VLDL-C secretion and LDLR activity.

Finally, these studies in the mouse, as well as those in the monkey (67), are consistent with similar, albeit limited, observations in humans. As would be predicted, the plasma LDL-C concentration should be lowest in individuals on diets with little cholesterol and triacylglycerol where LDLR activity would be expected to be relatively high and VLDL-C secretion relatively low. Indeed, in such populations where carbohydrate is the major caloric source in the diet, TC concentrations range from 110–160 mg/dl and LDL-C levels vary from 50–80 mg/dl (68–71). The addition of cholesterol alone or triacylglycerol alone to such diets would be expected to have relatively little effect on plasma lipid concentrations, a prediction also supported by limited observations in humans (71, 72). The largest increase in plasma LDL-C levels would be anticipated with diets that combine small amounts of cholesterol and large amounts of triacylglycerol containing mixtures of FAs where there would be a marked increase in VLDL-C secretion coupled with partial suppression of LDLR activity. Indeed, in humans the plasma LDL-C concentration increases nearly 40% within 2 weeks of beginning such diets (73). Consumption of these diets over a lifetime, as is common in Western societies, probably accounts for the elevated cholesterol levels typically found in the liver and the near doubling of values for both the TC and LDL-C concentrations characteristic of these groups (74, 75).

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