Effect of apolipoprotein A-I deficiency on lecithin:cholesterol acyltransferase activation in mouse plasma

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Abstract Plasma cholesteryl ester (CE) synthesis by lecithin cholesterol acyltransferase (LCAT) is activated by apolipoprotein (apo)A-I. We studied the effect of plasma apoA-I concentration on LCAT activation, using normal, heterozygous or homozygous apoA-I-deficient mice made by gene targeting. Plasma esterified cholesterol concentrations of mice fed chow diets were ordered (mean ± SEM): 105 ± 7 (normal) > 70 ± 5 (heterozygotes) > 26 ± 2 (homozygotes) mg/dl. Plasma free cholesterol concentrations were similar among the three genotypes. Endogenous LCAT activity, measured as the decrease in plasma free cholesterol after a 1 h incubation at 37°C, was ordered: 44 ± 3 (normal) > 21 ± 2 (heterozygotes) > 5 ± 1 (homozygotes) nmol CE formed/h per ml plasma. Using a recombinant exogenous substrate consisting of egg yolk phospholipid, [^{14}C]cholesterol, and apoA-I, CE formation of normals and heterozygotes was similar (27.4 ± 0.6 and 28.8 ± 1.3 nmol/h per ml plasma, respectively), but was significantly less for homozygotes (19.2 ± 1.7 nmol/h per ml plasma). However, using a small unilamellar vesicle substrate particle containing phospholipid and [^{14}C]cholesterol, CE formation was ordered: 1.6 ± 0.1 (normal) = 1.6 ± 0.1 (heterozygotes) > 0.6 ± 0.1 (homozygotes) nmol/h per ml plasma; addition of apoA-I to the plasma of homozygous animals restored CE formation to normal levels (1.6 ± 0.1). CE fatty acid analysis demonstrated that plasma from homozygous mice contained significantly more saturated and monounsaturated and fewer polyunsaturated fatty acids compared to normal and heterozygous mice. We conclude that mice with no detectable plasma apoA-I have marked reductions in plasma CE concentrations (70%) and CE synthesis (60%) and a more saturated plasma CE fatty acid profile, which likely reflects an increased hepatic contribution to the plasma CE pool. The decreased plasma CE synthesis in homozygous apoA-I-deficient mice is primarily due to a deficiency in apoA-I activator protein, but is also partly the result of a decrease in HDL substrate particles and LCAT enzyme activity. In addition, one half of the gene dosage of apoA-I in mice is sufficient for activation of plasma LCAT.—**Parks, J. S., H. Li, A. K. Gebre, T. L. Smith, and N. Maeda.** Effect of apolipoprotein A-I deficiency on lecithin:cholesterol acyltransferase activation in mouse plasma. *J. Lipid Res.* 1995. **36:** 349–355.

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Apolipoprotein A-I (apoA-I) is the major apolipoprotein of plasma high density lipoproteins (HDL). It is a 243 amino acid protein, encoded by a 1.9 kb gene containing 4 exons and 3 introns (1, 2). ApoA-I is secreted as a proprotein by the liver and intestine (3, 4). The proprotein (249 amino acids) is rapidly cleaved to the mature form (243 amino acids) after secretion into plasma (5, 6). The primary sequence of apoA-I consists of 6 tandem 22 amino acid repeats, which form amphipathic helixes (7, 8). The amphipathic alpha-helical repeats are hypothesized to play an important role in two of the major functions of apoA-I, providing structural stability to the HDL particle and activation of plasma lecithin:cholesterol acyltransferase (LCAT) (9–12).

LCAT is the major source of cholesteryl esters (CE) in human plasma (13). The enzyme catalyzes the hydrolysis of fatty acyl groups from the *sn*-2 position of phospholipids and the transesterification of the fatty acyl group to the 3-β hydroxyl group of cholesterol to generate CE and lysophospholipid (14). The predominant lipoprotein substrate for LCAT in vivo is HDL, which also contain the cofactor apoA-I (13, 14). Several artificial substrate particles have been used to study the cofactor requirement for the LCAT reaction, including recombinant HDL (rHDL) and vesicles (10, 15, 16). rHDL consist of phospholipid, cholesterol, and apolipoprotein formed by cholate dialysis and contain both substrate lipids and apolipoprotein activator in a structurally stable discoidal particle. Small unilamellar vesicles consisting of phospholipid and cholesterol (but no apolipoprotein) contain substrate lipids but require the addition of activator

Abbreviations: CE, cholesteryl ester; LCAT, lecithin:cholesterol acyltransferase; apo, apolipoprotein; HDL, high density lipoprotein; rHDL, recombinant HDL; ACAT, acyl-CoA:cholesterol acyltransferase.

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to the incubation mixture. Using these artificial substrates, investigators have shown that while other apolipoproteins can activate the LCAT reaction in vitro, none is as effective as apoA-I (10, 17).

Several apoA-I deficiency states have been described (18, 19) and although all share very low levels of plasma apoA-I and high density lipoproteins, they differ in clinical manifestations (18, 19). In some cases such as Tangier disease, plasma esterification of cholesterol appears normal in most subjects (19). However, in other apoA-I deficiency states, endogenous cholesterol esterification in plasma is decreased (20, 21), as is plasma LCAT activity measured with an exogenous, apoA-I-containing substrate (rHDL) (21–23). These results have been extended by showing that plasma LCAT mass also is reduced in some apoA-I deficiencies (21–23). Since LCAT mass and cholesterol esterification rate both decrease with apoA-I deficiency in studies where direct measurements have been made, the interpretation of the data regarding the role of apoA-I on LCAT activation in plasma is confounded.

An alternative approach to studying LCAT activation by apoA-I in patients with apoA-I deficiency is to use animal models. However, no naturally occurring apoA-I deficiency state has been identified in an animal model. Using gene targeting methods, apoA-I-deficient mice have been created with low plasma HDL concentrations and no detectable apoA-I (24). The purpose of the present study was twofold: to determine plasma CE formation rate in vitro in apoA-I-deficient mice using defined substrate particles with or without apoA-I activator, and to assess the effect of apoA-I gene dosage on plasma CE formation by LCAT.

EXPERIMENTAL PROCEDURES

Animals

ApoA-I-deficient mice used for this study were generated by gene targeting in embryonic stem cells derived from a mouse of strain 129/Ola (24, 25). Mice with 129 genetic background were made by crossing chimeras from the modified embryonic stem cells to strain 129/J mice. Mice with C57BL/6 genetic background were made by crossing chimeras to strain C57BL/6J mice for seven generations prior to intercrossing heterozygotes. The mice were fed a chow diet and maintained in a temperature controlled room with a 14-h light and 10-h dark cycle.

After an overnight fast animals were anesthetized with methoxyflurane (Metofane®) for blood sampling. Blood was collected from the retro-orbital sinus into tubes containing 2 mM EDTA, 100 μg/ml gentamycin, and 0.046 trypsin inhibitor units of aprotinin/ml (final concentration). Plasma total, free, and esterified cholesterol concentrations were determined by enzymatic procedures as described previously (26, 27).

Cholesterol esterification assays

Endogenous CE formation. Endogenous CE formation was determined after incubation of plasma at 37°C for 1 h by measuring the decrease in free cholesterol using enzymatic procedures (26, 27).

rHDL substrate assay. CE formation also was measured using an exogenous substrate containing apoA-I. rHDL were prepared by cholate dialysis as described previously using egg yolk lecithin, [^{14}C]cholesterol (51 mCi/mmol), and human apoA-I at a molar ratio of 50:2:1, respectively (28). ApoA-I was isolated from human plasma (29). CE formation was assayed using 0.2 μg (0.518 nmol; i.e., initial velocity) or 1 μg (2.6 nmol; saturation substrate concentration) of rHDL [^{14}C]cholesterol, 2% bovine serum albumin (BSA), 10 mM β-mercaptoethanol in 0.5 ml of buffer (10 mM Tris, 140 mM NaCl, 0.01% EDTA, 0.01% NaN_3, pH 7.4). Incubations were performed for 1 h at 37°C after addition of 2–5 μl of mouse plasma as a source of LCAT enzyme. The reaction was terminated by addition of 2 ml $CHCl_3$–methanol 1:2 followed by a Bligh-Dyer extraction (30). The lower phase was subjected to thin-layer chromatography to separate free and esterified cholesterol. Liquid scintillation spectrometry was then used to quantify the amount of ^{14}C in free and esterified cholesterol. Percentage CE formed was converted to nmol CE formed/h per ml plasma.

Vesicle substrate assay

CE formation also was measured using vesicles containing phosphatidylcholine and [^{14}C]cholesterol with no exogenous apoA-I. The vesicle assay tested the ability of mouse plasma to provide both LCAT enzyme as well as activator proteins (i.e., apoA-I, E) for CE formation. Aliquots of egg yolk lecithin (1500 μg) in $CHCl_3$ and [^{14}C]cholesterol (35.5 μg; 5 μCi) in ethanol were dried under N_2 and lyophilized for 1 h to remove residual solvent. Two ml of buffer was added and the sample was sonicated in an ice water bath for 15 min. After sonication the vesicle preparation was size-fractionated on a 1.6 × 30 cm Sepharose CL-4B column equilibrated with buffer and running at a flow rate of 42 ml/h. Two-minute fractions were collected and elution of [^{14}C]cholesterol was monitored by liquid scintillation spectrometry. The descending part of the included peak eluting from the column that contained small unilamellar vesicles was pooled, chemically analyzed (31) and used as substrate. The pooled vesicle fraction had a phospholipid-to-[^{14}C]cholesterol molar ratio of 38:1. An aliquot of the vesicle preparation equivalent to 0.2 μg [^{14}C]cholesterol was used in a LCAT assay identical to that described for rHDL. In some incubations, 5 μg of purified human

apoA-I was added to the incubation mixture as an exogenous activator of LCAT. The apoA-I was added to the incubation tubes containing substrate vesicles, BSA, and buffer, and the tubes were incubated for 5 min at 37°C. Previous studies have shown that apoA-I binding to vesicles is very rapid and occurs within minutes after mixing at 25°C (32). β-Mercaptoethanol and plasma were added to initiate the cholesterol esterification reaction. Using the vesicle substrate, CE formation was linear when 1–10 μl of plasma was used as a source of LCAT. In addition, CE formation was linear from 0.5–2 h when 5 μl plasma was used as a source of LCAT, and the vesicle substrate cholesterol amount was 0.2 μg.

Cholesteryl ester fatty acid analysis

Plasma CE fatty acids were quantified by gas–liquid chromatography (33). Briefly, 100 μl of mouse plasma was extracted by the method of Bligh and Dyer (30). The lower phase was subjected to thin-layer chromatography using hexane–ether–acetic acid 70:30:1 to separate CE. The position of the CE band was identified by spraying a standard lane with rhodamine B. The CE band was scraped and CE were eluted with 10 ml of $CHCl_3$–methanol 2:1. The CE were then saponified with KOH, the free cholesterol was extracted with hexane and discarded, the aqueous phase was acidified with 8–9 drops of concentrated H_2SO_4, and the protonated free fatty acids were extracted into hexane. The fatty acids were then methylated with boron trifluoride and subjected to gas–liquid chromatography as described previously (33).

Data analysis

Data are presented as mean ± standard error of the mean. Analysis of variance and Fisher's least significant difference tests were used to identify significant differences among genotypes.

TABLE 1. Plasma cholesterol concentration of apolipoprotein A-I-deficient mice

ApoA-I Genotype	Plasma Cholesterol			
	Total	Free	Esterified	EC/TC
	mg/dl			
Normal (n = 13)	126 ± 7^a	22 ± 2	105 ± 7^a	0.82 ± 0.02^a
Heterozygote (n = 14)	89 ± 6^b	19 ± 2	70 ± 5^b	0.78 ± 0.03^a
Homozygote (n = 11)	45 ± 2^c	19 ± 2	26 ± 2^c	0.58 ± 0.03^b
P Value	0.0001	0.579	0.0001	0.0001

Values are mean ± SEM. *P* values are derived by analysis of variance; values with unlike superscripts are significantly different ($P < 0.05$) by Fisher's least significant difference post-hoc analysis. EC, esterified cholesterol; TC, total cholesterol.

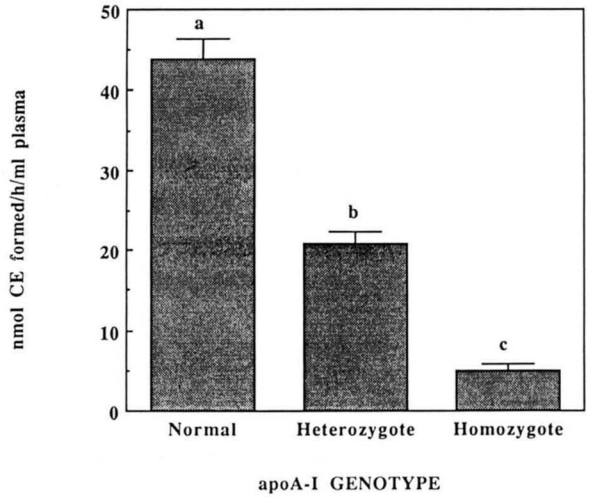

Fig. 1. Endogenous cholesterol esterification rate in apoA-I-deficient mouse plasma. Cholesterol esterification was calculated from the decrease in free cholesterol during a 1-h incubation of plasma at 37°C. Values are mean ± SEM (normal = 8, heterozygote = 19, homozygote = 15). Values with unlike letters are significantly different ($P < 0.05$) by analysis of variance and Fisher's least significant difference post-hoc analysis.

RESULTS

Previous studies have shown that total plasma and HDL cholesterol concentrations were significantly lower in heterozygous and homozygous apoA-I deficient mice compared to normal mice (24, 25). **Table 1** shows the total, free, and esterified cholesterol concentrations in plasma as a function of apoA-I genotype. Total plasma cholesterol concentrations were significantly different among the three genotypes, with normal mice having the highest values and homozygous mice having the lowest values. The differences in total plasma cholesterol among genotypes were due entirely to differences in esterified cholesterol concentrations; plasma free cholesterol values were similar. Thus, esterified cholesterol concentrations were 67% and 25% of normal for heterozygotes and homozygotes, respectively.

To determine the effect of apoA-I genotype on endogenous CE production, mouse plasma was incubated for 1 h at 37°C and the decrease in plasma free cholesterol was measured and used to calculate an esterification rate. **Figure 1** shows the results of the study. Heterozygotes had one half the endogenous CE formation rate (20.9 ± 1.5 nmol/h per ml) of normal mice (43.9 ± 2.5 nmol/h per ml). Homozygous mice had endogenous cholesterol esterification rates that were significantly lower (4.9 ± 0.9 nmol/h per ml) than those of heterozygotes or normals. However, from these data it is not possible to know whether the decrease in cholesterol esterification was due to decreased plasma apoA-I (activator), LCAT (enzyme), or HDL (substrate).

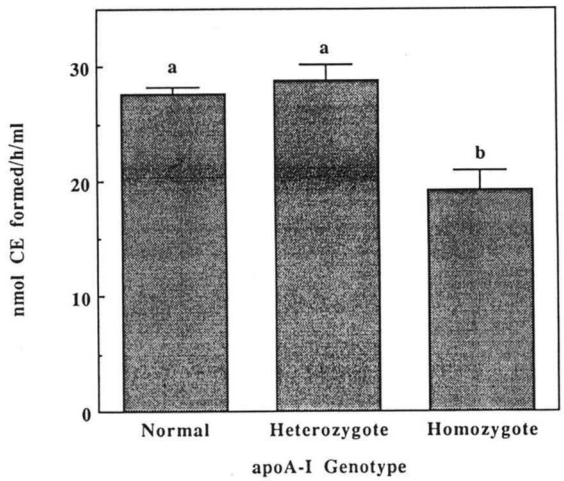

Fig. 2. Measurement of plasma cholesterol esterification rate using exogenous rHDL substrate particles containing egg yolk phosphatidylcholine, [^{14}C]cholesterol, and apoA-I (50:2:1 molar ratio). Details of assay conditions are given in Methods. Values are mean ± SEM (n = 5 for each group). Values with unlike letters are significantly different ($P < 0.05$) by analysis of variance and Fisher's least significant difference post-hoc analysis.

An exogenous substrate particle (rHDL) containing phospholipid, [^{14}C]cholesterol, and apoA-I also was used to measure cholesterol esterification. The results are shown in **Fig. 2.** At excess substrate concentrations (i.e., zero order kinetics), CE formation was significantly less for the homozygous mice (19.2 ± 1.7 nmol/h per ml; n = 5) compared to the normal (27.4 ± 0.6 nmol/h per ml; n = 5) or heterozygous (28.8 ± 1.3 nmol/h per ml; n = 5) mice ($P < 0.001$). Similar results were obtained when initial CE formation rates were measured at lower substrate concentrations. Thus, even when exogenous human apoA-I was present in the incubation on substrate particles, cholesterol esterification was 30% lower for the homozygous mice.

To determine the plasma cholesterol esterification rate with exogenous substrate but no exogenous apoA-I to serve as an activator for LCAT, vesicles containing phospholipid and [^{14}C]cholesterol were incubated with plasma and cholesterol esterification was measured (**Fig. 3**). The esterification rate was identical for normal (1.63 ± 0.09 nmol/h per ml; n = 16) and heterozygous mice (1.60 ± 0.10 nmol/h per ml; n = 17), but was reduced by 60% ($P < 0.0001$) for the homozygous mice (0.64 ± 0.07 nmol/h per ml; n = 17). Addition of 5 μg of human apoA-I to the incubation mixture resulted in a cholesterol esterification rate (1.64 ± 0.09 nmol/h per ml; n = 17) indistinguishable from that of normal and heterozygous mice.

To determine whether the decreased rate of endogenous cholesterol esterification in heterozygous and homozygous mice influenced the distribution of plasma CE species, plasma CE fatty acid percentage composition among genotypes was quantified (**Table 2**). Compared to normal and heterozygous mice, homozygous apoA-I-deficient mice had a significantly greater proportion of plasma CE fatty acids as 14:0, 16:0, 18:0, and 18:1, while the proportion of CE containing 18:2 and 20:5 n-3 was significantly less. CE species containing 20:4 and 22:6 n-3 averaged less in the homozygous mice also, but the values were not statistically different from those of the normal and heterozygous mice. Thus, the homozygous apoA-I-deficient mice had fewer CE in plasma compared to normal mice (Table 1), and the CE species present were relatively more saturated.

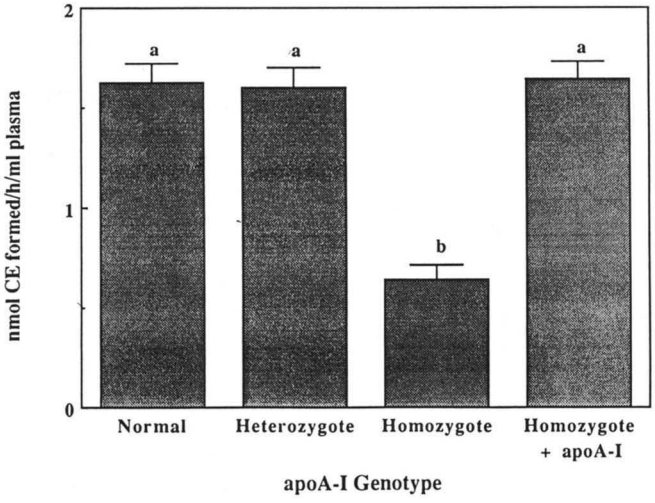

Fig. 3. Measurement of plasma cholesterol esterification rate using exogenous vesicle substrate particles, containing egg yolk phosphatidylcholine and [^{14}C]cholesterol (38:1 molar ratio). In some incubations 5 μg of human apoA-I was added to the incubation mixture as a source of exogenous activator protein. Details of incubation are given in Methods. Values are mean ± SEM. Values with unlike letters are significantly different ($P < 0.05$) by analysis of variance and Fisher's least significant difference post-hoc analysis.

TABLE 2. Percentage plasma cholesteryl ester fatty acid composition of apoA-I-deficient mice

CE Fatty Acid	ApoA-I Genotype			*P* Value
	Normal n = 5	Heterozygote n = 5	Homozygote n = 5	
	%			
14:0	0.4 ± 0.1^a	0.7 ± 0.2^a	2.1 ± 0.5^b	0.008
16:0	6.4 ± 0.7^a	6.9 ± 0.7^a	15.0 ± 2.0^b	0.0009
16:1	1.6 ± 0.2	2.1 ± 0.3	3.0 ± 0.7	NS
18:0	3.9 ± 0.6^a	3.8 ± 0.6^a	12.0 ± 0.8^b	0.0001
18:1	7.1 ± 0.2^a	6.5 ± 0.2^a	14.3 ± 1.5^b	0.0001
18:2	51.1 ± 1.0^a	50.3 ± 1.7^a	27.1 ± 1.8^b	0.0001
20:4	15.6 ± 0.5	15.4 ± 1.2	11.7 ± 2.0	NS
20:5 n-3	4.5 ± 0.2^a	4.3 ± 0.3^a	3.1 ± 0.6^b	0.05
22:6 n-3	5.7 ± 0.4	5.4 ± 0.9	3.4 ± 0.8	NS
Other	3.7 ± 0.1^a	4.5 ± 0.3^a	8.3 ± 1.5^b	0.006

Mean ± SEM. Values with unlike letters are significantly different ($P > 0.05$) by analysis of variance and Fisher's least significant difference post-hoc analysis.

DISCUSSION

The purpose of this study was to determine the effect of apoA-I gene dosage on plasma CE formation. Mice made apoA-I deficient by gene targeting had plasma CE mass and endogenous CE formation rates that were proportional to apoA-I gene dosage (Table 1 and Fig. 1). Studies were designed to test whether the decrease in plasma CE mass and endogenous cholesterol esterification was the result of a decrease in apoA-I activator. Vesicle substrate particles that lacked apoA-I activator had cholesterol esterification rates in the plasma of homozygous mice that were 30% that of normal or heterozygous mice (Fig. 3). However, addition of exogenous apoA-I resulted in full restoration of LCAT activity. This was in contrast to results with apoA-I-containing rHDL, in which the esterification rate for homozygote plasma was 70% of normal (Fig. 1). Taken together, these data support the conclusion that the decreased plasma CE mass and cholesterol esterification rate in apoA-I-deficient mice was, in part, attributable to a decrease in plasma apoA-I mass.

Heterozygous apoA-I-deficient mice have a sufficient mass of apoA-I in plasma to fully activate LCAT. CE production using vesicular substrate particles was the same for normal mice and heterozygotes, demonstrating that apoA-I was not limiting for the LCAT reaction in the plasma of heterozygous mice (Fig. 3). However, in spite of a normal cholesterol esterification rate with vesicle substrate particles, heterozygote mice had decreased (~30%) CE mass in plasma (Table 1) and decreased (~50%) endogenous cholesterol esterification rates (Fig. 1). The most likely explanation for the data is that half the gene dosage of apoA-I results in a decrease of HDL substrate pool in plasma, leading to a decrease in plasma CE production by LCAT. Thus, in heterozygous mouse plasma, LCAT activity and apoA-I activator protein are not limiting, but HDL substrate particles likely are. These data suggest that half of the gene dosage of apoA-I leads to plasma HDL substrate depletion relative to normal mice, which results in decreased net plasma CE production by LCAT.

Homozygous apoA-I-deficient mice have 25% of normal CE mass plasma (Table 1) and endogenous CE production rates that are 10% of normal (Fig. 1). This is due primarily to a deficiency of apoA-I activator. Addition of exogenous rHDL substrate restored plasma CE production in homozygote mouse plasma to 70% of that in normal plasma. Using vesicular substrate particles, CE production rate in homozygote mouse plasma could be fully restored in the presence of exogenous apoA-I, but was only 30% of normal in the absence of added apoA-I (Fig. 3). It is not clear why normal CE production rates were observed in the plasma of homozygous mice using vesicle substrate particles with added apoA-I, but not with rHDL particles. The homozygote mice may have a slight deficiency in plasma LCAT activity relative to normal mice that was detected by the rHDL substrate, but not the vesicular substrate particles, because of the higher intrinsic reactivity of rHDL with LCAT (compare rates in Figs. 2 and 3; ref. 16). In some human apoA-I-deficient states LCAT activity is decreased, even when assayed with rHDL containing apoA-I. In these same studies plasma LCAT mass also was decreased (21, 22). LCAT may have a greater turnover rate when plasma HDL pools are decreased in apoA-I deficiency, perhaps because lipoprotein-free LCAT is catabolized more rapidly than LCAT bound to HDL particles. Nonetheless, it is clear that the marked decrease in CE mass and esterification rate of cholesterol in the plasma of homozygous mice is primarily the result of decreased apoA-I activator protein.

A deficiency in HDL substrate particles also is likely to contribute to the very low endogenous CE production rate (10% of normal) in the plasma of homozygous apoA-I-deficient mice, as addition of adequate substrate in the form of apoA-I-free vesicle particles resulted in CE production rates that were 30% of normal and addition of apoA-I in the presence of vesicle substrate particles restored cholesterol esterification rates to normal values. Therefore, the deficiency of HDL substrate particles in homozygous apoA-I-deficient mice seems to have less impact on endogenous CE production rates than the deficiency in apoA-I activator protein.

The small amount of CE in the plasma of homozygous apoA-I-deficient mice was likely the result of liver acyl CoA:cholesterol acyltransferase (ACAT) activity and/or residual plasma LCAT activity. LCAT prefers to use polyunsaturated fatty acids to generate CE, while ACAT, the intracellular cholesterol esterification enzyme, prefers monounsaturated fatty acyl groups (34–37). The plasma CE fatty acid profiles of homozygous mice were relatively enriched in saturated and monounsaturated fatty acids and depleted of polyunsaturated fatty acids, suggesting a greater contribution of hepatic ACAT to the plasma CE pool. The contribution of hepatic ACAT to the plasma CE pool increases with cholesterol feeding as the liver begins to accumulate CE (38, 39). However, our data suggest that liver ACAT may contribute to the plasma CE pool even in chow-fed animals. Residual LCAT activity in the plasma of homozygous mice also may contribute to the plasma CE pool, as LCAT activity was 30% of normal for the homozygotes in the absence of apoA-I. The residual LCAT activity likely results from the ability of other apolipoproteins to activate LCAT, albeit not as efficiently as apoA-I (10, 17). We have recently shown that plasma HDL apoE content is greater in homozygous mice compared to heterozygous or normal mice (25). The increased apoE in homozygous mice may function to maintain a low amount of LCAT activity in plasma by activation of the enzyme in

the absence of apoA-I (40).

It is important to emphasize the distinctions in CE production between the apoA-I-deficient mouse compared to human subjects deficient in apoA-I. The plasma esterified to total cholesterol ratio in apoA-I-difficient human subjects is close to normal values (i.e., 0.75; refs. 22, 41, 42), whereas homozygous apoA-I-deficient mice have lower values (0.587; Table 1). In addition, the endogenous cholesteryl ester formation rate of homozygous apoA-I-deficient mice is 10% of normal whereas that of apoA-I-deficient human subjects is 20–50% of normal (20–22). The reason for these differences is not clear. Notwithstanding these differences, the apoA-I-deficient mouse should prove to be a valuable animal model to pursue metabolic questions regarding lipoprotein metabolism in the absence of apoA-I.

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