Cholesterol Absorption and Hepatic Acyl-Coenzyme A:Cholesterol Acyltransferase Activity Play Major Roles in Lipemic Response to Dietary Cholesterol and Fat in Laboratory Opossums

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Partially inbred lines of laboratory opossums differ considerably in their low-density lipoprotein (LDL) cholesterol responses to dietary cholesterol and fat. Genetic analysis suggested that a single major gene is responsible for the variation in LDL cholesterol on the high cholesterol and high fat (HCHF) diet. We measured cholesterol absorption and acyl-coenzyme A:cholesterol acyltransferase (ACAT) activity in intestine and liver to narrow the search for the major gene. We measured plasma lipoproteins and percent cholesterol absorption by the fecal isotope ratio method in high and low responding lines of opossums on basal and HCHF diets. We also measured lipids in liver and ACAT activity in liver and intestine on the HCHF diet. High and low lines exhibited no differences in percent cholesterol absorption on the basal diet. However, high responding opossums had significantly higher percent cholesterol absorption, hepatic free and esterified cholesterol, and hepatic ACAT activity than low responding opossums on the HCHF diet. Hepatic ACAT activity but not the intestinal ACAT activity was associated with hepatic cholesterol concentration and percent cholesterol absorption. Cholesterol absorption is a major determinant of diet-induced hyperlipidemia in opossums. Hepatic ACAT activity but not the intestinal ACAT may also play a role in diet-induced hyperlipidemia in opossums.

THERE IS considerable variability in the responsiveness of plasma lipoprotein cholesterol to dietary lipids among animal species and among the individuals of any one species, including humans.[1,2] On the basis of their plasma cholesterol responses to dietary cholesterol and fat, high and low responding individuals within the same species have been identified. A number of animal models have been developed to determine the metabolic and molecular mechanisms of high and low plasma cholesterol response to dietary lipids. Selective breeding for high and low response, coupled with inbreeding, has produced partially inbred strains of laboratory opossums that show extreme variability in diet-induced hyperlipidemia.[3] However, these strains of laboratory opossums have quite uniform plasma and lipoprotein cholesterol levels on a low-cholesterol, low-fat basal diet.[3] Genetic analyses have indicated that the regulation of very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol concentration in laboratory opossums in response to dietary challenge is primarily determined by a single major gene.[4] This single major single gene is responsible for most (80%) of the variability in VLDL and LDL cholesterol on the high cholesterol, high fat (HCHF) challenge diet.[4] We conducted studies to determine the differences in hepatic activities of enzymes involved in lipid metabolism between high and low responding opossums.[5] The results of those previous studies suggested that sterol 27-hydroxylase and acyl-coenzyme A:cholesterol acyltransferase (ACAT) may affect diet-induced hyperlipidemia in laboratory opossums. The present studies were conducted to determine whether the high and low responding opossums differ in cholesterol absorption and whether ACAT activity is associated with the differences in cholesterol absorption between high and low responding opossums.

MATERIALS AND METHODS

Experimental Animals

At the Southwest Foundation for Biomedical Research (SFBR), an inbreeding program has produced 18 partially inbred strains of laboratory opossums (*Monodelphis domestica*) with inbreeding coefficients of greater than 0.6. Among these strains, 3 high responding and 3 low responding lines have been selectively bred for their LDL cholesterol response to dietary cholesterol and fat. Two of these strains have been designated ATHE (low responding) and ATHH (high responding). High and low responding laboratory opossums from these 2 stocks were used for these studies. The animals were maintained in polycarbonate rodent cages under laboratory conditions that have been standardized for this species.[6]

The protocol of these experiments was approved by the Institutional Animal Care and Use Committee of the SFBR. The SFBR is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care and is registered with the US Department of Agriculture.

Experimental Diets

The animals were fed a low cholesterol low fat basal diet or a HCHF experimental diet ad libitum. The basal diet is a commercial pelleted fox food (Reproduction Diet, Nutritionally Complete Fox Food Pellets, Milk Specialties Co, New Holstein, WI). As reported earlier, the fat content of the basal diet was 10% by weight, and the cholesterol content was relatively low (0.16% by dry weight basis).[5] The fat content of the HCHF experimental diet was 18.8% by dry weight and the cholesterol content was 0.71% by dry weight basis. The experimental diet was prepared from the commercial fox food by adding water, lard, and crystalline cholesterol as described earlier.[5] The pellets were stored at −20°C to prevent spoilage and oxidation.

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Experimental Design

We selected 20 high responding (ATHH stock) and 15 low responding (ATHE stock) opossums (5 to 7 months old) for this experiment. Initially, the animals were maintained on the basal diet and the cholesterol absorption was measured. Afterwards, the animals were bled to determine plasma lipoprotein cholesterol concentrations. After the animals were studied on the basal diet, they were switched to the HCHF diet. After consuming the HCHF diet for 5 weeks, the animals were subjected to the same procedures. After completion of the cholesterol absorption studies, the animals were bled and necropsies performed to collect intestine and liver. Only 12 animals in each group completed the study and, therefore, data for those animals are presented.

Blood Sampling

Blood (0.8 to 1.0 mL) was collected by cardiac puncture of animals after anesthetizing them with halothane.[6] The blood was collected in tubes containing EDTA. Plasma was obtained by centrifugation and plasma lipoprotein cholesterol was measured.

Tissue Collection

Animals were exsanguinated by cardiac puncture under halothane euthanasia, and the liver and intestine were removed and placed in plastic bags and frozen immediately in liquid nitrogen. Samples were stored at −80°C prior to use.

Plasma and Lipoprotein Cholesterol Analysis

Total plasma and high-density lipoprotein (HDL) cholesterol were measured by enzymatic methods with the Ciba-Corning Express Plus Analyzer (Infolab, Round Rock, TX). VLDL and LDL were precipitated by the Lipid Research Clinics procedure[7] and HDL cholesterol was measured in the supernatant. The VLDL + LDL cholesterol concentration was calculated as the difference between the total plasma cholesterol and HDL cholesterol concentrations. When the cholesterol level in a sample exceeded the value of the highest calibrator, 358 mg/dL, the sample was diluted with saline to bring it to the range of calibrators and analyzed again. For samples that were diluted for the total cholesterol assay, the same dilutions were used for the precipitation of VLDL + LDL. Plasma triglycerides in laboratory opossums are low and do not change upon consuming the HCHF diet and thus triglycerides do not interfere in the cholesterol assay.

Cholesterol Absorption Measurements

Cholesterol absorption was measured by the fecal isotope ratio method described by Turley et al[8] for hamsters. Opossums were given 1 μCi of [^{3}H]-β-sitosterol (American Radiolabeled Chemicals, St Louis, MO) and 0.5 μCi of [^{14}C]-cholesterol (Amersham Pharmacia, Piscataway, NJ) in corn oil (200 μL) intragastrically by syringe without anesthesia. This procedure is accomplished by holding the animal in the left hand and expelling the contents of the syringe on top of the tongue near the temporal mandibular joint at one side, and pointing down the throat, forcing the animal to swallow. Opossums were placed in individual cages for 4 days. They were allowed to have access to food and water ad libitum. Feces were collected daily for 4 days and pooled, and a small amount (2 g) was used to extract sterols as described by Turley et al.[8] The petroleum ether extracts were transferred into scintillation vials and evaporated to dryness under nitrogen. We added 10 mL of scintisol to each vial and counted radioactivity of both isotopes in a liquid scintillation counter (model LS-7500; Beckman, Palo Alto, CA). To correct for color-related quenching of ^{14}C and ^{3}H counts in fecal samples, quench curves were run using increasing amounts of fecal extracts (nonradioactive fecal samples from opossums) with standardized ^{14}C and ^{3}H-labeled toluene (Packard Instruments, Downers Grove, IL). Data were expressed as disintegrations per minute using the data reduction program of Beckman scintillation counter model LS-7500. The percent cholesterol absorption was calculated as follows: (^{14}C/^{3}H [dose] − ^{14}C/^{3}H [fecal sample])/(^{14}C/^{3}H [dose]) × 100 = percent cholesterol absorbed.

Measurement of Hepatic and Intestinal Microsomal ACAT Activity

We used liver and intestine (jejunum) from high and low responding opossums to measure microsomal ACAT activity. Approximately 1 g tissue was chiseled from each frozen sample, and 10% homogenate was prepared with 10 mmol/L HEPES (*N*-[2-hydroethyl] piperazine-*N*′-[2-ethanesulfonic acid], pH 7.4 (Sigma Chemical Co, St Louis, MO) containing 0.25 mol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF; Sigma). The microsomes from each tissue were isolated via differential centrifugation as described by Griffith[9] and us.[5] The microsome pellet was immediately frozen at −80°C. For assay, the microsomal pellet was dissolved in potassium phosphate buffer, and protein concentration was measured using the Bio-Rad protein assay kit with bovine serum albumin as a standard (Bio-Rad, Richmond, CA). The ACAT assay reaction included 0.2 mg microsomal protein, 1.0 mg bovine serum albumin (Sigma), and 50 nmol cholesterol (Sigma) in a total volume of 0.3 mL. The reaction mixture was incubated at 37°C for 30 minutes. After the initial incubation, 30 nmol of ^{14}C-oleyl-coenzyme A (Amersham Pharmacia) was added and incubated further for 2 minutes at 37°C. The reaction was stopped by adding 6 mL chloroform:methanol (2:1) containing 15 μg cholesteryl oleate. Phases were separated after the addition of 1.2 mL saturated potassium chloride and the chloroform phase was aspirated and dried. The samples were resuspended with chloroform:methanol (2:1) by vortexing. A volume of 60 μL was applied to a silica gel plate and separated by thin-layer chromatography. The cholesteryl ester band was scraped off and transferred to a scintillation vial. To each vial, 10 mL of scintillation fluid was added and the samples were counted in a scintillation system (Beckman). The microsomal ACAT activity is expressed as picomoles per milligram protein per minute.

Measurement of Hepatic Cholesterol Concentration

Liver samples (200 to 500 mg) were homogenized and extracted with chloroform and methanol by the method of Folch et al.[10] The chloroform extract was evaporated to dryness and dissolved in 200 μL isopropanol and the cholesterol concentration measured by an enzymatic method using a kit (Wako Pure Chemicals USA, Richmond, VA). The cholesterol concentration was expressed as milligram per gram.

Data Analysis

Values in tables are expressed as the mean ± SD. Values for high and low responding groups were compared by a standard *t* test. Associations among the variables were determined by using Pearson's correlation. Significance was set at $P < .05$.

RESULTS

Lipoprotein Cholesterol Concentrations in High and Low Responding Laboratory Opossums

Table 1 presents data for lipoprotein cholesterol concentrations in high and low responding laboratory opossums. There was no difference in serum and lipoprotein cholesterol concentrations between high and low responding opossums on the low-cholesterol, low-fat basal diet. However, there was a significant difference in serum and LDL cholesterol concentrations between high and low responding opossums ($P < .001$) on the HCHF diet. However, there was no difference in HDL

Table 1. Plasma Lipoprotein Cholesterol Concentrations and Cholesterol Absorption in Low and High Responding Laboratory Opossums Consuming the Basal and HCHF Diets

	Basal Diet				HCHF Diet			
Phenotype	Plasma TC (mg/dL)	HDL-C (mg/dL)	VLDL-C + LDL-C (mg/dL)	Cholesterol Absorption (%)	Plasma TC (mg/dL)	HDL-C (mg/dL)	VLDL-C + LDL-C (mg/dL)	Cholesterol Absorption (%)
Low responding	91.3 ± 12.6	57.1 ± 6.93	34.2 ± 6.94	58.89 ± 8.22	92.5 ± 16.6*	68.8 ± 10.8	23.6 ± 10.1*	31.27 ± 6.54*†
High responding	80.1 ± 9.4	56.1 ± 6.64	24.0 ± 8.53	58.7 ± 9.73	1,164.7 ± 230.5†	71.8 ± 24.4	1092.9 ± 233.9†	61.30 ± 18.25

NOTE. Values are mean ± SD; N = 12 for each.

Abbreviations: TC, total cholesterol; VLDL-C, very-low-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

*Values are significantly different from high responding phenotype ($P < .001$).

†Values are significantly different from basal values ($P < .001$).

cholesterol concentrations between high and low responding opossums on the HCHF diet. The plasma lipoprotein cholesterol concentrations of low responding opossums did not change on the HCHF diet. However, high responding opossums exhibited a 14.5-fold increase in their total serum cholesterol concentrations ($P < .001$). Most of the increase in total serum cholesterol concentration in high responding opossums was due to the increase in the LDL cholesterol, which was increased by 54.5-fold ($P < .001$). However, there was no change in HDL cholesterol concentration in high responding opossums on the HCHF diet.

Cholesterol Absorption in High and Low Responding Opossums

Table 1 also presents cholesterol absorption data in high and low responding opossums on the low-cholesterol, low-fat basal diet and the HCHF diet. Percent cholesterol absorption did not differ between high and low responding opossums on the basal diet. However, there was a significant difference in percent cholesterol absorption between high and low responding opossums on the HCHF diet ($P = .001$). On the HCHF diet, there was a significant decrease in percent cholesterol absorption as compared to the basal diet in low responding opossums ($P < .001$). However, on the HCHF diet, cholesterol absorption stayed the same or increased slightly as compared with the basal diet in high responding opossums.

Cholesterol and Triglyceride Concentration in Liver of High and Low Responding Opossums

Table 2 presents hepatic cholesterol and triglyceride concentrations in high and low responding opossums on the HCHF diet. Hepatic total, free, and esterified cholesterol concentrations were approximately 3-fold higher in high responding opossums than in low responding opossums on the HCHF diet ($P < .003$). Approximately 80% of the total hepatic cholesterol was present in esterified form in both high and low responding opossums on the HCHF diet. Hepatic triglyceride concentrations varied considerably and were not different between high and low responding opossums.

Hepatic and Intestinal ACAT Activity From High and Low Responding Opossums

Table 3 presents data for hepatic and intestinal ACAT activity from high and low responding opossums on the HCHF diet. Hepatic microsomal ACAT activity was approximately 3-fold higher in high responding opossums than low responding opossums ($P < .001$) on the HCHF diet. However, intestinal microsomal ACAT activity did not differ between high and low responding opossums on the HCHF diet.

Relationship Between Hepatic Lipid Concentrations and Cholesterol Absorption

Figure 1 shows the relationship between hepatic cholesterol concentration and cholesterol absorption in high and low responding opossums on the HCHF diet. There was a significant association between cholesterol absorption and hepatic esterified ($r = 0.548$, $P = .007$) and free ($r = 0.503$, $P = .015$) cholesterol concentrations. However, there was no significant association between hepatic triglyceride concentration and cholesterol absorption ($r = 0.271$, $P = .212$, data not shown).

Relationship Between Hepatic and Intestinal ACAT Activity and Cholesterol Absorption

Figure 2 shows the relationship between hepatic ACAT activity and cholesterol absorption in high and low responding opossums on the HCHF diet. There was a significant association between hepatic ACAT activity and cholesterol absorption

Table 2. Cholesterol and Triglyceride Concentrations of Liver From Low and High Responding Laboratory Opossums Consuming the HCHF Diet

Phenotype	Total Cholesterol	Free Cholesterol	Esterified Cholesterol	Triglycerides
Low responding	3.09 ± 0.743*	0.61 ± 0.154†	2.48 ± 0.597‡	12.02 ± 5.988
High responding	10.64 ± 5.025	1.77 ± 1.062	9.14 ± 4.221	21.28 ± 16.370

NOTE. Values are mean ± SD (mg/g liver tissue); N = 12 for each.

*Value is significantly different from value of high responding phenotype ($P < .001$).

†Values are significantly different from values of high responding phenotype ($P < .003$).

‡Values are significantly different from values of high responding phenotype ($P < .001$).

Table 3. Hepatic and Intestinal ACAT Activity From Low and High Responding Laboratory Opossums Consuming the HCHF Diet

Phenotype	Hepatic	Intestinal
Low responding	86.33 ± 21.02*	325.17 ± 267.57
High responding	263.33 ± 82.76	305.83 ± 283.80

NOTE. Values are mean ± SD (pmol/min/mg protein); N = 12 for each.

*Value is significantly different from value of high responding phenotype ($P < .001$).

($r = 0.508$, $P = .013$). However, there was no significant association between intestinal ACAT activity and cholesterol absorption ($r = 0.033$, $P = .882$, data not shown).

Relationship Between Hepatic Cholesterol Concentration and Hepatic ACAT Activity

Figure 3 shows the relationship between hepatic free and esterified cholesterol concentration and hepatic ACAT activity. There was a significant association between hepatic free ($r = 0.604$, $P = .002$) and esterified ($r = 0.704$, $P < .001$) cholesterol concentrations.

Relationship Between ACAT Activity and Plasma Lipoprotein Cholesterol

There was no association between plasma cholesterol concentrations and intestinal ACAT activity ($r = 0.030$, $P = .890$). However, there was a strong positive association between plasma cholesterol and hepatic ACAT activity ($r = 0.760$, $P < .0001$). The positive association between plasma cholesterol and hepatic ACAT activity was due to association of LDL cholesterol with hepatic ACAT activity ($r = 0.763$, $P < .0001$). There was no association between HDL cholesterol and hepatic ACAT activity ($r = 0.061$), $P = .777$).

DISCUSSION

The present results demonstrate that high responding opossums had a 2-fold higher percent cholesterol absorption than low responding opossums on the HCHF diet; therefore, cholesterol absorption is the major determinant of diet-induced hyperlipidemia in laboratory opossums. These studies further suggest that intestinal ACAT activity is not the basis for differences in cholesterol absorption between high and low responding opossums. Hepatic ACAT activity was associated with cholesterol absorption and hepatic cholesterol concentration and thus, may play a role in high and low responses to dietary lipids in opossums. Alternatively, the hepatic ACAT activity may be upregulated in high responding opossums due to increase in hepatic cholesterol concentration in response to increased cholesterol absorption.

High and low responding individuals of a number of species have been observed to differ in cholesterol absorption through the intestine.[1,2] However, studies for the investigations of molecular mechanisms for these differences in cholesterol absorption are limited.[2] Use of inhibitors of ACAT in experimental animals has lead to a decrease in cholesterol absorption and plasma cholesterol concentrations.[11-16] Differences in the expression of ACAT gene in duodenum and jejunum of baboons are also associated with cholesterol absorption through the intestine.[17] We measured intestinal ACAT activity in these studies to investigate whether the differences in intestinal ACAT activity may be the basis for the differences in cholesterol absorption in opossums. However, the results suggest that there are no differences in intestinal ACAT activity between high and low responding opossums and therefore, unlike in baboons, intestinal ACAT activity is not responsible for the differences in cholesterol absorption in opossums.

High responding opossums had only a 2-fold increase in their percent cholesterol absorption through the intestine compared with low responding opossums. However, high respond-

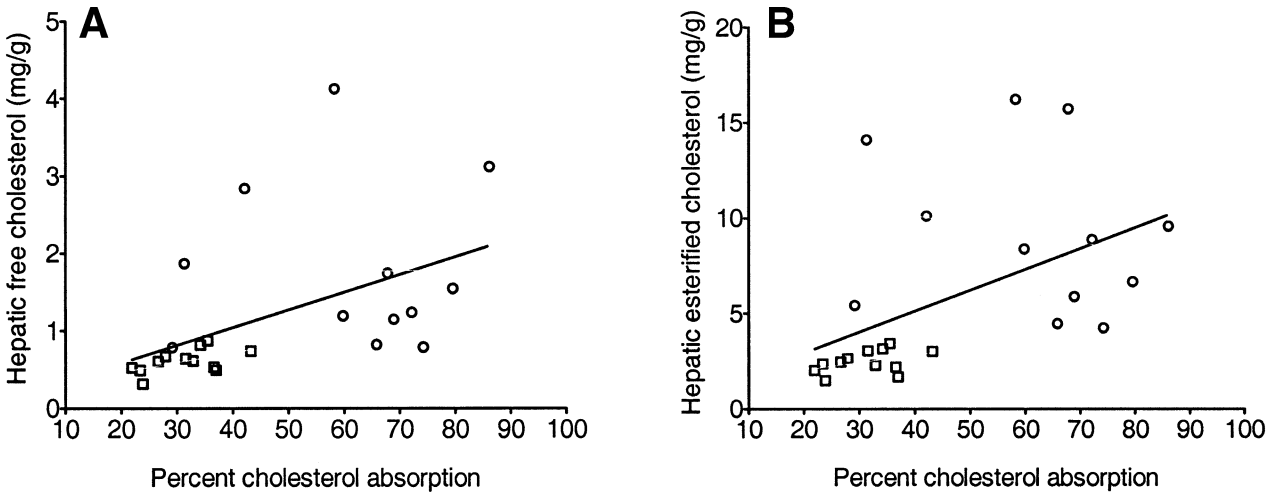

Fig 1. Association of percent cholesterol absorption with (A) free and (B) esterified cholesterol concentration in the liver of high (○) and low responding (□) opossums on the HCHF diet. There were significant associations between cholesterol absorption and hepatic free ($P = .015$) and esterified ($P = .007$) cholesterol concentrations.

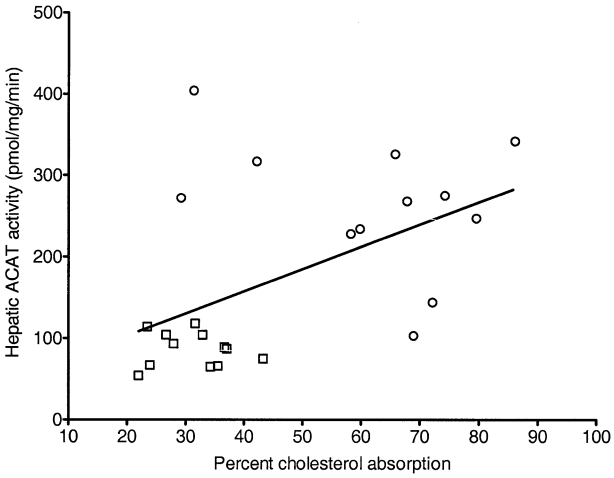

Fig 2. Association of hepatic ACAT activity with intestinal cholesterol absorption in high (○) and low (□) responding opossums on the HCHF diet. There was a significant association between hepatic ACAT activity and intestinal cholesterol absorption ($P = .013$).

ing opossums had a 45.5-fold increase in plasma VLDL + LDL cholesterol concentrations and a 3-fold increase in hepatic cholesterol concentrations. Low responding opossums did not change their fractional cholesterol absorption, but their cholesterol intake increased due to the increase in dietary cholesterol. However, their plasma lipoprotein cholesterol concentration did not change on the HCHF diet as compared with that on the basal diet. Therefore, low responding opossums must have another mechanism operating that leads to maintenance of the basal cholesterol concentration in their liver. Primates, which are high responders to dietary lipids, upregulate their hepatic ACAT activity.[18] We also observed higher hepatic ACAT activity in high responding opossums than in low responding opossums. It is likely that the increased hepatic ACAT activity of high responding opossums enables them to store more esterified cholesterol in hepatocytes, as seen in these studies, and to secrete more cholesterol in VLDL and LDL, as observed in baboons.[19] ACAT2 has been shown to participate in lipoprotein secretion, and thus it is likely that opossums ACAT2 is polymorphic. Alternatively, opossum ACAT2 may be regulated post-trancriptionally as in primates,[18] and the regulation may differ between high and low responding opossums. Further studies are needed to determine the mechanism by which ACAT activity differs between high and low responding opossums.

By complex segregation analysis, Rainwater et al[4] have shown that most (80%) of the variability in VLDL + LDL cholesterol concentrations in laboratory opossums on the HCHF diet is determined by a single major gene (single gene locus). However, this major gene has not been mapped due to the unavailability of linkage map for this species. We conducted these studies to identify candidate genes, which have major influence on the responsiveness to diet by affecting cholesterol absorption or hepatic cholesterol metabolism in laboratory opossums. ACAT may be one such candidate gene that may regulate the lipemic response to diet, but we cannot suggest that this is the major gene detected by complex segregation analysis.

It has been suggested that the activity of cholesterol esterase and adenosine triphosphate (ATP)-binding cassette transporters also play important roles in cholesterol absorption.[20-25] Cholesterol esterase hydrolyzes phospholipids in the bile salt micelles, thereby releasing cholesterol from the micelles and increasing the availability of cholesterol for diffusion through the enterocyte membrane.[26] Overexpression of ABCG5 and ABCG8 half-transporters in transgenic mice has been shown to decrease cholesterol absorption.[23] Further studies are needed to determine whether cholesterol esterase or ABCG5 and ABCG8 play a role in affecting cholesterol absorption in opossums.

Our previous studies have shown that low responding opossums induce hepatic and extrahepatic activity of sterol 27-

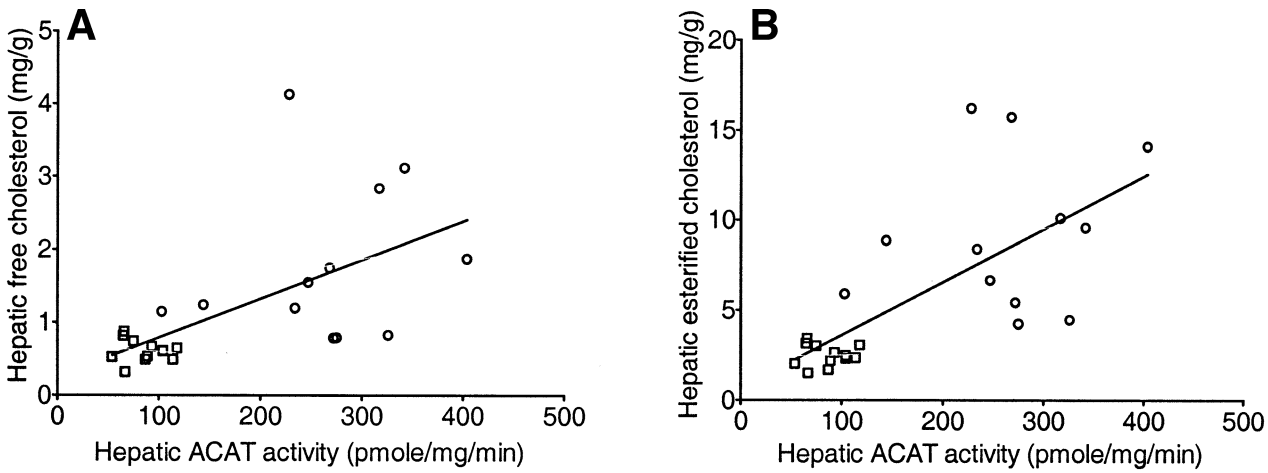

Fig 3. Association of hepatic ACAT activity with hepatic (A) free and (B) esterified cholesterol concentrations in high (○) and low (□) responding opossums on the HCHF diet. There were significant associations between hepatic ACAT activity and hepatic free ($P = .002$) and esterified ($P = .000$) cholesterol concentrations.

hydroxylase in response to dietary cholesterol and fat.[5] The increased activity of sterol 27-hydroxylase in low responding opossums would lead to increased production of 27-hydroxycholesterol as compared with high responding opossums. The 27-hydroxycholesterol produced in extrahepatic tissues is brought to the liver by lipoproteins and there is considerable more increase in 27-hydroxycholesterol in the liver of low responding opossums than in high responding opossums on the HCHF diet.[5] Cholestenoic acid, a metabolite of 27-hydroxycholesterol, has been suggested to be the naturally occurring ligand for liver X receptor α, which has been shown to regulate the expression of ABCG5 and ABCG8.[27] Treatment of mice expressing ABCG5 and ABCG8 with liver X receptor agonist produced a 3-fold increase in biliary cholesterol concentration and a decrease in cholesterol absorption.[28] However, treatment of transgenic mice expressing no ABCG5 and ABCG8 with liver X receptor agonist did not result in such increase in biliary cholesterol secretion. Thus, the increase in hepatic and extrahepatic sterol 27-hydroxylase may upregulate the expression of ABCG5 and ABCG8 in low responding opossums as compared with high responding opossums and may decrease cholesterol absorption and increase hepatic biliary cholesterol secretion. Further studies are needed to determine whether sterol 27-hydroxylase mediates changes in the expression of ABCG5 and ABCG8 and regulates cholesterol absorption in low responding opossums.

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