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Effect of ezetimibe on plasma cholesterol levels, cholesterol absorption, and secretion of biliary cholesterol in laboratory opossums with high and low responses to dietary cholesterol

Jeannie Chan[a], Rampratap S. Kushwaha[b,*], Jane F. VandeBerg[a], John L. VandeBerg[a]

[a]*Department of Genetics and Southwest National Primate Research Center, Southwest Foundation for Biomedical Research, San Antonio, TX 78245-0549, USA*

[b]*Department of Physiology and Medicine, Southwest Foundation for Biomedical Research, San Antonio, TX 78245-0549, USA*

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Abstract

Partially inbred lines of laboratory opossums differ in plasma low-density lipoprotein cholesterol concentration and cholesterol absorption on a high-cholesterol diet. The aim of the present studies was to determine whether ezetimibe inhibits cholesterol absorption and eliminates the differences in plasma cholesterol and hepatic cholesterol metabolism between high and low responders on a high-cholesterol diet. Initially, we determined that the optimum dose of ezetimibe was 5 mg/(kg d) and treated 6 high- and 6 low-responding opossums with this dose (with equal numbers of controls) for 3 weeks while the opossums consumed a high-cholesterol and low-fat diet. Plasma and low-density lipoprotein cholesterol concentrations decreased significantly ($P < .05$) in treated but not in untreated high-responding opossums. Plasma cholesterol concentrations increased slightly ($P < .05$) in untreated low responders but not in treated low responders. The percentage of cholesterol absorption was significantly higher in untreated high responders than in other groups. Livers from high responders with or without treatment were significantly ($P < .01$) heavier than livers from low responders with or without treatment. Hepatic cholesterol concentrations in untreated high responders were significantly ($P < .05$) higher than those in low responders with or without treatment ($P < .001$). The gall bladder bile cholesterol concentrations in untreated high responders were significantly ($P < .05$) lower than those in other groups. A decrease in biliary cholesterol in low responders treated with ezetimibe was associated with a decrease in hepatic expression of *ABCG5* and *ABCG8*. These studies suggest that ezetimibe decreases plasma cholesterol levels in high responders mainly by decreasing cholesterol absorption and increasing biliary cholesterol concentrations. Because ezetimibe's target is NPC1L1 and *NPC1L1* is expressed in the intestine of opossums, its effect on cholesterol absorption may be mediated by inhibiting NPC1L1 function in the intestine.

1. Introduction

Responsiveness of plasma lipoprotein cholesterol to dietary lipids varies greatly among animal species and among the individuals of any one species, including humans [1,2]. High-responding individuals increase their plasma cholesterol levels to a considerable extent when challenged with a high-cholesterol and high-fat (HCHF) diet. However, low-responding individuals of the same species maintain or only moderately increase their plasma cholesterol levels when challenged with the same diet. High- and low-responding individuals of the same species have been developed by selective breeding for use in determining the metabolic and molecular mechanisms responsible for differences in plasma cholesterol response to dietary lipids. Selective breeding for high and low response, coupled with inbreeding, has produced partially inbred strains of laboratory opossums (*Monodelphis domestica*) that show extreme variability in diet-induced hyperlipidemia [3]. However, these strains of laboratory opossums have quite similar plasma and lipoprotein cholesterol levels on a basal diet that is lower in cholesterol and fat content [3]. Our studies have shown that diet-induced hyperlipidemia in opossums is mainly due to dietary cholesterol [4]. Genetic analyses have indicated that the regulation of very low-density lipoprotein

* Corresponding author. PO Box 760549, San Antonio, TX 78245-0549, USA. Tel.: +1 210 258 9615; fax: +1 210 670 3323.
E-mail address: kush@icarus.sfbr.org (R.S. Kushwaha).

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(VLDL) and low-density lipoprotein (LDL) cholesterol concentration in laboratory opossums in response to dietary challenge is largely determined by a single major gene [5]. This single major gene is responsible for most (80%) of the variability in VLDL and LDL cholesterol on the HCHF challenge diet [5]. Previously, we conducted studies to develop a better understanding of the metabolic and molecular mechanisms that are responsible for diet-induced hyperlipidemia in laboratory opossums. The results of those studies suggested that cholesterol absorption and hepatic acyl–coenzyme A:cholesterol acyltransferase play a major role in diet-induced hyperlipidemia in laboratory opossums [6]. The NPC1L1 protein plays an important role in cholesterol influx into enterocytes [7], and ezetimibe inhibits cholesterol absorption by inhibiting the activity of NPC1L1 [8]. The present studies were conducted to determine whether opossums express *NPC1L1* in their intestines and livers and whether ezetimibe would decrease cholesterol absorption, decrease plasma and VLDL + LDL cholesterol, and normalize hepatic cholesterol metabolism in the opossum model. Because NPC1L1 is the target protein for ezetimibe, these studies may provide evidence for a regulatory role of NPC1L1 in diet-induced hyperlipidemia in laboratory opossums.

2. Methods

2.1. Experimental animals

At the Southwest Foundation for Biomedical Research (SFBR), an inbreeding program has produced 18 partially inbred strains of laboratory opossums (*M domestica*) with inbreeding coefficients greater than 0.7. Among these strains, 3 high-responding and 3 low-responding lines have been selectively bred for their LDL cholesterol response to the HCHF diet. Two of these strains have been designated *ATHH* (high responding) and *ATHE* (low responding). Most but not all ATHH opossums are high responders, and most but not all ATHE opossums are low responders. High- and low-responding individuals 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 [9].

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.

2.2. Experimental diets

For these experiments, the animals were fed a basal diet or a high-cholesterol and low-fat (HCLF) experimental diet ad libitum. However, before the experiments, the animals were challenged with the HCHF diet for 4 to 8 weeks to confirm each individual's dietary response as high for ATHH opossums and low for ATHE opossums. The basal diet is a commercial pelleted fox food (Reproduction Diet, Nutritionally Complete Fox Food Pellets; Milk Specialties, New Holstein, WI). The composition of these diets has been described previously [4]. The fat content of the basal diet was 10% of dry weight, and the cholesterol content was relatively low (0.16% by dry weight basis) [4]. The fat content of the HCLF experimental diet was 10.8% of dry weight, and the cholesterol content was 0.71% by dry weight basis. The fat content of the HCHF diet was 18.8% of dry weight, and the cholesterol content was 0.71% by dry weight basis. The experimental diets were prepared from the commercial fox food by adding water, lard, and crystalline cholesterol as described earlier [4]. The pellets were stored at −20°C to prevent spoilage and oxidation.

2.3. Experimental design

2.3.1. Experiment 1: dose response

To determine the optimal dose of ezetimibe, we selected 12 ATHH high-responding and 12 ATHE low-responding laboratory opossums (each weighing approximately 100 g). The animals were selected on the basis of response to the HCHF diet. The animals were then returned to the basal diet for 8 weeks so that their plasma cholesterol levels would return to baseline. The animals were divided into 4 groups of 6: (1) high responding, treated with ezetimibe; (2) high responding, untreated controls; (3) low responding, treated; and (4) low responding, untreated controls. The animals were fed the HCLF diet for 6 weeks; and at the end of the 6-week period, the animals were bled to determine the plasma lipoprotein cholesterol levels. During this time, 6 animals from the high-responding groups died; and thus, the experiment began with 4 animals in group 1, 2 animals in group 2, 6 animals in group 3, and 6 animals in group 4. At the start of ezetimibe treatment, 2 animals in group 1 and 2 animals in group 2 were high responders to HCLF diet; and the rest of the animals were low responders to that diet. Opossums in the treatment group were treated with a weekly escalating (in half-log increments) dose of ezetimibe starting at 0.625 mg/(kg d) for a week (week 1). Other doses were 1.344 (week 2), 2.216 (week 3), 3.654 (week 4), 5.025 (week 5), and 10.000 (weeks 6 and 7) mg/(kg d). The last dose (10 mg/[kg d]) was given for 2 weeks before blood was collected. The drug was mixed into the HCLF diet on the basis of food consumption. Each animal was given the food on the basis of its weight each week. At the end of each dose period, animals were weighed and bled to determine plasma lipoprotein cholesterol and triglyceride levels.

2.3.2. Experiment 2: cholesterol absorption

For cholesterol absorption measurements, 12 ATHH high-responding and 12 ATHE low-responding opossums to the HCHF diet were selected. The selected animals were then maintained on the basal diet for 8 weeks to bring their plasma cholesterol levels back to baseline levels. Animals were then started on the HCLF diet. After consumption of the HCLF diet for 4 weeks, plasma cholesterol and triglyceride concentrations

were measured. Afterward, the animals were divided into 4 groups as defined in the previous paragraph. Groups 1 and 3 were treated with ezetimibe at a dose of 5 mg/kg body weight per day, and groups 2 and 4 were used as the untreated controls. In this experiment, we maintained the animals for 4 rather than 6 weeks on the HCLF diet before starting them on ezetimibe because of the high mortality rate in the high-responding group in the initial experiment. The animals were treated for 3 weeks with ezetimibe, and plasma cholesterol and triglyceride concentrations were measured after the end of the second and third weeks. Intestinal cholesterol absorption was measured during the third week. After measurement of cholesterol absorption, the animals were necropsied; and livers were removed for measurement of gene expression and free and esterified cholesterol concentrations.

2.4. Blood and tissue collection

After an overnight fasting, the animals were exsanguinated by cardiac puncture under isoflurane anesthesia [9]. The blood was placed in tubes containing EDTA. The liver was removed, and bile was aspirated from the gall bladder by syringe and placed in a small vial. The liver was placed in a plastic bag and frozen immediately in liquid nitrogen. Samples were stored at −80°C.

2.5. Plasma and lipoprotein cholesterol analysis

Plasma was obtained by centrifugation, and total plasma cholesterol and high-density lipoprotein (HDL) cholesterol were measured by enzymatic methods with the Ciba-Corning Express Plus Analyzer (Infolab, Round Rock, TX). Very low-density lipoprotein and LDL were precipitated by the Lipid Research Clinics procedure [10], 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 within the range of the 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. Fasting plasma triglycerides in laboratory opossums are low and do not change upon consuming the HCHF diet; thus, triglycerides do not affect the results of cholesterol assay.

2.6. Cholesterol absorption measurements

Cholesterol absorption was measured by the fecal isotope ratio method described by Turley et al [11] for hamsters and by us for opossums [6]. Opossums were given 2 μCi of [^{3}H]-β-sitosterol (American Radiolabeled Chemicals, St Louis, MO) and 1 μCi of [^{14}C]-cholesterol (Amersham Pharmacia, Piscataway, NJ) in corn oil (200 μL) intragastrically by syringe without anesthesia [11]. 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 [11]. The petroleum ether extracts were transferred into scintillation vials and evaporated to dryness under nitrogen, and the radioactivity of both isotopes was counted 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). The percentage of cholesterol absorption was calculated as follows: [^{14}C/^{3}H (dose) − ^{14}C/^{3}H (fecal sample)]/[^{14}C/^{3}H (dose)] × 100 = percentage of cholesterol absorbed.

2.7. Measurement of hepatic cholesterol concentrations

Liver samples (200-500 mg) were homogenized and extracted with chloroform and methanol (2:1) by the method of Folch et al [12]. The chloroform extract was evaporated to dryness and dissolved in 200 μL isopropanol, and total and free cholesterol concentrations were measured by an enzymatic method using a kit (Wako Pure Chemicals USA, Richmond, VA). The cholesterol concentrations were expressed as milligram per gram of liver tissue.

2.8. Measurements of cholesterol and total bile acids in gall bladder bile

Bile obtained from the gall bladder was diluted 10-fold with methanol. A small aliquot of the sample was used to measure cholesterol by the enzymatic method as described above for hepatic cholesterol. Total bile acids in bile samples were measured by the 3α-hydroxysteroid dehydrogenase method as described by Turley and Dietschy [13]. Each assay contained 1.5 mL of Tris-HCl buffer (0.133 mol/L Tris and 0.666 mmol/L EDTA), pH 9.5 (Sigma Chemical, St Louis, MO); 1 mL of hydrazine hydrate (1 mol/L), pH 9.5 (Sigma); 0.3 mL of NAD^+ (7 mmol/L), pH 7.0 (Sigma); 0.1 mL of methanolic extract of bile (1:10 dilution); and 0.1 mL of 3α-hydroxysteroid dehydrogenase (Worthington Biochemicals, Freehold, NJ) containing 2 U of enzyme activity per milliliter in Tris-HCl buffer (0.03 mol/L) containing 1 mmol/L EDTA, pH 7.2. For each assay, a reagent blank was prepared by adding 0.1 mL of methanol in place of a methanolic extract of bile. For each bile sample, an appropriate blank was prepared by adding 0.1 mL of buffer in place of the enzyme as described by Turley and Dietschy [13]. The reaction mixtures were incubated at 30°C for an hour. The standard curve was prepared by using an equal amount of sodium taurocholate and sodium taurochenodeoxycholate. The concentration of total bile acids was expressed as micromole per milliliter of bile.

2.9. Northern blot analysis

To measure levels of *NPC1L1* messenger RNA (mRNA) in the intestine, we used tissues collected from a previous

experiment. Total RNA was isolated from the small intestines of 3 high- and 3 low-responding animals fed the basal diet and from 3 high- and 3 low-responding animals fed the HCLF diet for 4 weeks. The small intestine was divided into 12 segments of equal lengths, and total RNA was isolated from the sixth segment (jejunum) from the proximal end. Total RNA was isolated from frozen, pulverized tissues using TRI Reagent (Molecular Research Center, Cincinnati, OH) and quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The *NPC1L1* complementary DNA (cDNA) (accession no. EU886296) was synthesized by reverse transcription–polymerase chain reaction (RT-PCR) using the forward (5′-cctctcacattcaaggatgg-3′) and reverse (5′-ctgtccagggagag-caaa-3′) primers and cloned into the pCR4-TOPO vector. The cDNA insert in the recombinant plasmid was amplified by PCR, and the PCR products were radiolabeled for use as the *NPC1L1* probe. Northern blotting was performed as described previously [14].

2.10. Real-time RT-PCR

Levels of *ABCG5*, *ABCG8*, and *NPC1L1* mRNA were measured by real-time PCR using SYBR Green chemistry on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Total RNA isolated using the TRI Reagent was treated with DNase from the TURBO DNA-free kit (Applied Biosystems) according to the instructions of the manufacturer. Single-stranded cDNA was synthesized from 1 μg of DNase-treated RNA in a 20-μL reaction using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). The reverse transcription reaction was diluted 30-fold (for hepatic genes), 300-fold (for intestinal *NPC1L1*), and 90,000-fold (for 18S ribosomal RNA [rRNA]); and then 3 μL of the diluted reaction was added to a mixture containing gene-specific primers and the Fast SYBR Green Master Mix (Applied Biosystems) for PCR amplification according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA (hepatic genes) or 18S rRNA (intestinal *NPC1L1*) was used as an internal control. Messenger RNA levels were determined by the standard curve method, and the results were expressed in arbitrary units. A standard curve was generated for each gene by serial dilutions (10-fold) of a reference sample. The reference sample was prepared by pooling cDNAs from high and low responders on the HCLF diet. After the PCR amplification step, a dissociation curve analysis was performed to ensure that only a single product was amplified.

The following oligonucleotides were used for real-time RT-PCR: (*a*) forward *ABCG5* primer, 5′-cagcagcgtgttg-tattgga-3′; reverse *ABCG5* primer, 5′-agccgcgcacagcaatacc-3′; (*b*) forward *ABCG8* primer, 5′-acttgaccgtctgggagactt-3′; reverse *ABCG8* primer, 5′-acactccccgcaggtactc-3′; (*c*) forward *NPC1L1* primer, 5′-gcttatgatggtgccgtgaa-3′; reverse *NPC1L1* primer, 5′-ccgaaggtcagctgtgatgt-3′; (*d*) forward *GAPDH* primer, 5′-ggagaaagctgccaaatacg-3′; reverse *GAPDH* primer, 5′-gaagagtgggtgtcgctgtt-3′; and (*e*) forward 18S rRNA primer, 5′-ccgtcgtagttccgaccata-3′; reverse 18S rRNA primer, 5′-aagtttcagctttgcaaccatact-3′. Primers for each gene (except 18S rRNA) were selected from sequences in different exons.

2.11. Data analysis

Values in figures and tables are expressed as mean ± SEM. Values for groups were compared by analysis of variance. If significant differences were found, pairwise comparisons were done using the Bonferroni test. The baseline values and the values obtained after the treatment for each group were compared using paired *t* test. Associations among the variables were determined by

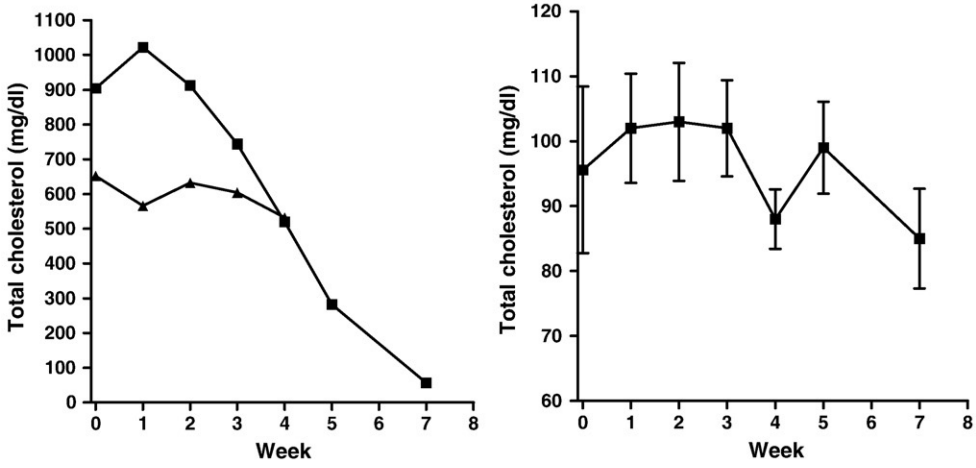

Fig. 1. Response of total plasma cholesterol concentration to ezetimibe treatment of 2 high- (individual values, left panel) and 8 low- (mean ± SEM, right panel) responding opossums on the HCLF diet (experiment 1: dose response). Before starting opossums on ezetimibe treatment, they consumed HCLF diet for 6 weeks. The dose of ezetimibe was escalated (in half-log increments) weekly beginning at 0.625 mg/(kg d). Other doses for weeks 2, 3, 4, 5, and 6 were 1.344, 2.216, 3.654, 5.025, and 10.000 mg/(kg d), respectively.

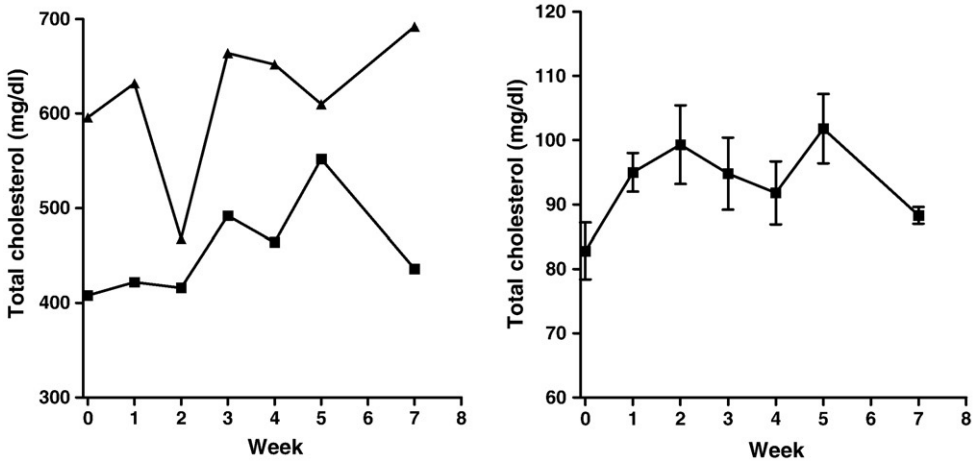

Fig. 2. Total plasma cholesterol concentration for untreated 2 high- (individual values, left panel) and 8 low- (mean ± SEM, right panel) responding opossums on the HCLF diet.

using the Pearson correlation. Significance was set at P less than .05.

3. Results

3.1. Optimum dose of ezetimibe in high- and low-responding laboratory opossums

Fig. 1 illustrates the effect of ezetimibe treatment on the total plasma cholesterol concentration of 2 high-responding and 8 low-responding opossums. One of the high responders had a linear decrease in plasma cholesterol concentrations in response to ezetimibe treatment after the first week, from 1022 mg/dL at week 1 to 57 mg/dL after 7 weeks of treatment. The other high responder also had a decrease (from 652 to 532 mg/dL) in response to ezetimibe treatment, but this opossum died after 4 weeks of treatment. There was a trend of decrease in plasma cholesterol levels of low-responding opossums treated with ezetimibe also, but these values were not significantly different from the baseline values (week 0) (P = .520).

Total plasma cholesterol values for 2 high- and 6 low-responding untreated opossums are shown in Fig. 2. The plasma cholesterol concentrations fluctuated considerably but did not decrease systematically from the baseline values during 7 weeks. Body weights of opossums treated with ezetimibe (body weights in grams: 92.0 ± 5.0, 91.9 ± 5.1, 91.4 ± 4.8, 91.0 ± 6.4, and 91.30 ± 6.60 at week 0, 1, 3, 5, and 7, respectively; n = 10) and untreated controls (96.8 ± 8.6, 96.7 ± 8.6, 94.6 ± 8.8, 97.2 ± 8.0, and 100.2 ± 8.0 at week 0, 1, 3, 5, and 7, respectively; n = 8) did not change with time on the HCLF diet. On the basis of these results, we decided to use dose of ezetimibe at 5 mg/(kg d) for laboratory opossums and decided to start the treatment after 4 weeks of feeding the HCLF diet.

3.2. Plasma and lipoprotein cholesterol response to ezetimibe in high- and low-responding opossums

Table 1 presents plasma and lipoprotein cholesterol concentrations of high- and low-responding opossums assigned to the cholesterol absorption experiment after they had consumed the HCLF diet for 4 weeks. Plasma cholesterol and LDL cholesterol concentrations of low-responding opossums differed significantly ($P < .001$) from those of high-responding opossums. Ezetimibe treatment was started after 4 weeks of consuming the HCLF diet, at a dose of 5 mg/(kg d) for 3 weeks; the animals continued to be fed the HCLF diet during these 3 weeks. Fig. 3 illustrates plasma cholesterol values of these groups on the ezetimibe treatment. There was a significant (P = .004) decrease in plasma cholesterol concentrations of high-responding opossums treated with ezetimibe for 3 weeks, whereas there was no significant ($P > .05$) change in plasma cholesterol

Table 1
Plasma lipoprotein cholesterol concentrations of low- and high-responding laboratory opossums selected for ezetimibe treatment after consuming the HCLF diet for 4 weeks (experiment 2: cholesterol absorption)

Group assignment	Phenotype	Plasma cholesterol (mg/dL)	LDL cholesterol (mg/dL)	HDL cholesterol (mg/dL)
1. ATHH treated	High responding	489.20 ± 92.33*	419.20 ± 100.88	70.00 ± 12.35
2. ATHH untreated	High responding	443.60 ± 76.49	390.60 ± 77.50	53.00 ± 3.08
3. ATHE treated	Low responding	83.00 ± 4.58†	29.50 ± 2.38†	53.50 ± 2.63
4. ATHE untreated	Low responding	83.83 ± 3.73†	28.33 ± 5.77†	59.5 ± 6.12

* Mean ± SEM (n = 5 for high-responding groups, and n = 6 for low-responding groups).
† Values are significantly different from values of groups 1 and 2 ($P < .001$).

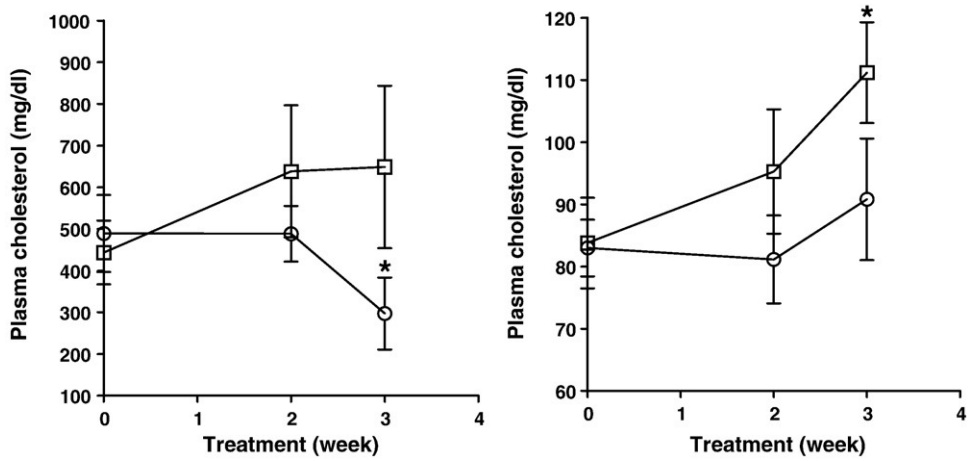

Fig. 3. Effect of ezetimibe treatment (5 mg/[kg d]) on plasma cholesterol concentrations of high- (mean ± SEM, n = 5, left panel) and low- (n = 6, right panel) responding opossums (experiment 2: cholesterol absorption). There was a significant (P = .004) decrease in plasma cholesterol concentrations of high-responding ATHH opossums treated with ezetimibe (○) (marked with an asterisk) but not in low-responding ATHE opossums treated with ezetimibe (○). There was no change in plasma cholesterol concentrations of untreated high-responding opossums (□), but there was a significant (P = .008) increase in plasma cholesterol concentrations of untreated low-responding opossums (□) (marked with an asterisk).

concentrations of untreated high-responding opossums. On the other hand, there was no significant (P > .05) change in plasma cholesterol concentrations of low-responding opossums treated with ezetimibe, whereas there was a significant (P = .008) increase in plasma cholesterol concentrations of untreated low-responding opossums. The change in plasma cholesterol concentrations was due to the change in plasma LDL cholesterol concentrations; there was no change in HDL cholesterol concentrations in these groups (HDL cholesterol: high responders treated with ezetimibe, 70.0 ± 12.4 mg/dL at baseline and 60.0 ± 6.8 mg/dL after 3 weeks of treatment; low responders treated with ezetimibe, 53.5 ± 2.6 mg/dL at baseline and 60.3 ± 5.1 mg/dL after 3 weeks of treatment; high-responder controls, 59.5 ± 6.1 mg/dL at baseline and 60.3 ± 5.1 mg/dL after 3 weeks with no treatment; low-responder controls, 59.8 ± 5.8 mg/dL at baseline and 64.3 ± 8.5 mg/dL after 3 weeks with no treatment).

3.3. Effect of ezetimibe treatment on cholesterol absorption and liver weights in high- and low-responding opossums

Fig. 4 presents the cholesterol absorption data from high- and low-responding opossums on the HCLF diet in response to the ezetimibe treatment. The percentage of cholesterol absorption was highest in untreated high-responding opossums (62.66 ± 3.29) and was significantly higher than high-responding opossums treated with ezetimibe (32.79 ± 4.54, P < .001) and low-responding opossums treated with (24.33 ± 4.13, P < .001) or without (33.35 ± 1.68) ezetimibe. However, there was no effect of ezetimibe on liver weights (Table 2). Livers from high-responding opossums with (6.74 ± 1.11 g) or without (6.78 ± 0.99) treatment with ezetimibe had significantly (P < .01) higher weights than low-responding opossums with (3.05 ± 0.24) or without (2.97 ± 0.25) treatment with ezetimibe.

3.4. Effect of ezetimibe on liver cholesterol and gall bladder cholesterol and total bile acids in high- and low-responding opossums

Table 2 presents hepatic cholesterol concentrations in high- and low-responding opossums with or without the treatment with ezetimibe. Hepatic total cholesterol concentrations in high-responding opossums treated with ezetimibe were highly variable and were not different from other groups. However, hepatic total cholesterol concentrations were approximately 3-fold higher in untreated high- responding opossums than in low-responding opossums with or without treatment with ezetimibe (P < .001). Approximately 70% to 80% of the

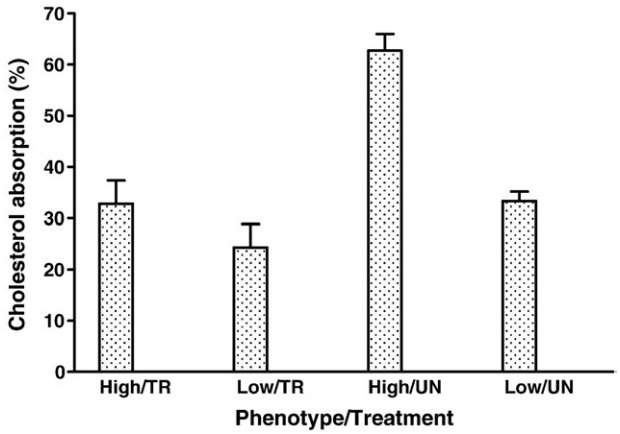

Fig. 4. The percentage of cholesterol absorption (mean ± SEM) in high-responding ATHH and low-responding ATHE opossums maintained on the HCLF diet and treated with ezetimibe (5 mg/[kg d]) for 3 weeks or untreated controls (experiment 2: cholesterol absorption). n = 5 for the high groups, and n = 6 for the low groups. TR indicates treated with ezetimibe; UN, untreated.

Table 2
Liver weights and hepatic cholesterol concentrations in low- and high-responding laboratory opossums after treatment with or without ezetimibe while consuming the HCLF diet (experiment 2: cholesterol absorption)

Group assignment	Phenotype	Liver weight (g)	Total cholesterol (mg/g liver tissue)	Free cholesterol (mg/g liver tissue)	Esterified cholesterol (mg/g liver tissue)
1. ATHH treated	High responding	6.74 ± 1.11*	7.52 ± 2.219	2.12 ± 0.929	5.40 ± 1.611
2. ATHH untreated	High responding	6.78 ± 0.99	8.55 ± 1.142	2.61 ± 0.71	5.95 ± 1.021
3. ATHE treated	Low responding	3.05 ± 0.24†	3.15 ± 0.183†	0.78 ± 0.059†	2.35 ± 0.198†
4. ATHE untreated	Low responding	2.97 ± 0.25†	3.71 ± 0.253†	0.68 ± 0.253†	3.03 ± 0.312†

* Mean ± SEM (n = 5 for high-responding groups, and n = 6 for low-responding groups).
† Value is significantly different from value of groups 1 and 2 ($P < .05$).

total hepatic cholesterol was present in esterified form in both high- and low-responding opossums, and there was no effect of treatment with ezetimibe (Table 2). Fig. 5 presents the gall bladder total cholesterol concentrations in high- and low-responding opossums with or without treatment with ezetimibe while consuming the high-cholesterol diet. The gall bladder cholesterol concentration was lowest (0.798 ± 0.185 mg/mL) in untreated high-responding opossums and significantly different ($P < .03$) from low- (2.342 ± 0.238 mg/mL) and high- (2.564 ± 0.450 mg/mL) responding opossums treated with ezetimibe. The gall bladder cholesterol concentration of untreated low-responding opossums (4.973 ± 0.447) was the highest and was significantly ($P < .002$) different from the other 3 groups. There was no difference in gall bladder cholesterol concentrations between high- and low-responding opossums treated with ezetimibe. There was also no difference in total bile acid concentration of high- and low-responding opossums treated with or without ezetimibe (bile acid concentration: high responders treated with ezetimibe, 8.58 ± 1.87 μmol/mL; high-responder controls, 9.31 ± 3.00 μmol/mL; low responders treated with ezetimibe, 10.22 ± 2.99 μmol/mL; low-responder controls, 9.47 ± 1.09 μmol/mL).

3.5. Relationship between cholesterol absorption and liver and plasma variables

There was a significant association between cholesterol absorption and liver weight ($r = 0.468$, $P = .028$). There was also a significant association between cholesterol absorption and plasma ($r = 0.567$, $P = .006$) and VLDL + LDL ($r = 0.572$, $P = .005$) cholesterol concentrations at the time of cholesterol absorption measurement (7 weeks on the HCLF diet). However, there was no association between cholesterol absorption and HDL cholesterol concentrations.

3.6. Variables affecting liver metabolism

There was a strong and significant association between liver weight and hepatic total ($r = 0.758$, $P < .001$), esterified ($r = 0.620$, $P = .002$), and free ($r = 0.738$, $P < .001$) cholesterol concentrations. However, there was a significant and negative association between liver weight and gall bladder cholesterol concentration ($r = -0.537$, $P = .012$). There was also a strong and negative association between gall bladder cholesterol and hepatic total ($r = -0.604$, $P = .004$), esterified ($r = -0.0547$, $P = .010$), and free ($r = -0.498$, $P < .022$) cholesterol concentrations. There was a strong and significant correlation between liver weight and plasma ($r = 0.899$, $P < .0001$) and LDL ($r = 0.903$, $P < .0001$) cholesterol concentrations before the treatment was started with ezetimibe. After the treatment with ezetimibe, the association between liver weight and plasma and LDL cholesterol concentrations stayed significant, but was reduced as indicated by r values (from 0.899 to 0.484 for plasma and from 0.903 to 0.479 for LDL cholesterol concentrations).

3.7. Expression of NPC1L1 in intestine of opossums

Fig. 6 reflects *NPC1L1* mRNA levels in the intestines (jejunum) of high- and low-responding opossums consuming the basal diet (left panel) or the HCLF diet (right panel). The levels in intestines did not differ between high- and low-responding opossums on the basal diet, but the

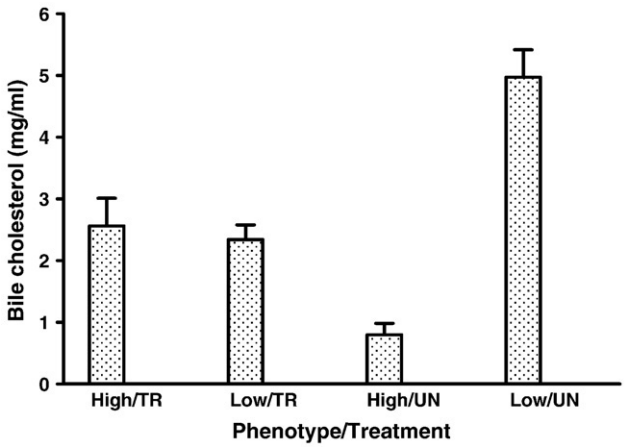

Fig. 5. Gall bladder bile cholesterol concentrations (mean ± SEM) of high-responding ATHH and low-responding ATHE opossums maintained on the HCLF diet and treated with or without ezetimibe (5 mg/[kg d]) for 3 weeks (experiment 2: cholesterol absorption). Gall bladder bile cholesterol concentrations in untreated low-responding opossums were significantly ($P < .002$) higher than those in the other groups. Gall bladder cholesterol concentrations in the untreated high-responding group were significantly ($P < .001$) lower than those in high- and low-responding opossums treated with ezetimibe. n = 5 for the high groups, and n = 6 for the low groups.

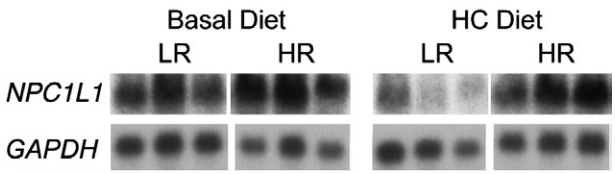

Fig. 6. Northern blot of *NPC1L1* in the intestines (jejunum) of 3 low-responding and 3 high-responding opossums on the basal diet (left panel) and 3 low-responding and 3 high-responding opossums on the high-cholesterol diet (right panel). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. LR indicates low-responding opossums; HR, high-responding opossums; HC, high-cholesterol diet.

levels in the intestines of 2 of the 3 low responders were lower than those in the intestines of high-responding opossums on the HCLF diet. These observations suggest that the expression of *NPC1L1* in the intestine is variable. We quantified the levels of *NPC1L1* mRNA in these high- and low-responding opossums on the HCLF diet by quantitative RT-PCR. The levels were normalized to 18S rRNA and expressed in arbitrary units. The values for high responders were higher (534 ± 29, n = 3) than those for low responders (430 ± 66, n = 3), but these were not significantly different (P = .195).

3.8. Expression of *ABCG5*, *ABCG8*, and *NPC1L1* in livers of opossums

Table 3 presents the values for mRNA levels of *ABCG5*, *ABCG8*, and *NPC1L1* in livers of opossums treated with or without ezetimibe while they consumed the HCLF diet. Expression of *ABCG8* in the livers of untreated low-responding opossums was significantly higher than that in the livers of untreated high-responding opossums. Ezetimibe treatment significantly decreased the expression of both *ABCG5* and *ABCG8* in the livers of low-responding opossums but not in the livers of high-responding opossums. On the other hand, ezetimibe treatment significantly increased the expression of *NPC1L1* in the livers of high-responding opossums but not in the livers of low-responding opossums.

4. Discussion

The results demonstrate that ezetimibe treatment of high-responding opossums rapidly decreases plasma and VLDL + LDL cholesterol. This reduction in plasma and VLDL + LDL cholesterol in high-responding opossums is due in part to a 50% decrease in intestinal cholesterol absorption (Fig. 4). Although treated low-responding opossums did not differ significantly from untreated controls in the percentage of cholesterol absorption, they had the lowest level of cholesterol absorption and also the lowest plasma and VLDL + LDL cholesterol concentrations. Thus, a small decrease in the percentage of cholesterol absorption due to ezetimibe treatment in low-responding opossums may also cause a reduction in their plasma and VLDL + LDL cholesterol levels. Our observations also showed that high- and low-responding opossums express *NPC1L1* in their intestines on the HCLF diet, but the expression is variable in low-responding opossums (Fig. 6). Because NPC1L1 protein is required for cholesterol absorption through the intestine and is the target of ezetimibe [7,8], the decrease in the percentage of cholesterol absorption in high-responding opossums treated with ezetimibe may be due to the inhibition of NPC1L1 function in the intestine [15].

Low-responding opossums also express NPC1L1 in their intestine; however, ezetimibe does not have a significant effect on the percentage of cholesterol absorption in low-responding opossums. Our previous studies have shown that low-responding opossums decrease their percentage of cholesterol absorption by 50% on the HCHF diet, whereas high-responding opossums cannot down-regulate their percentage of cholesterol absorption on the same HCHF diet [6]. It is therefore likely that low-responding opossums have another mechanism that down-regulates cholesterol absorption on a high-cholesterol diet with or without high fat, which may involve other proteins such as cholesterol esterase [16], phospholipase A2 [17], acyl–coenzyme A:cholesterol acyltransferases [18], 3-hydroxy-3-methylglutaryl coenzyme A [19], scavenger receptor B1, and ABC transporters (ABCG5, ABCG8, ABCB4, and ABCB11) [20-22].

Another aim of our studies was to investigate whether the reduction in cholesterol absorption caused by the ezetimibe treatment would normalize plasma cholesterol concentrations and modulate hepatic cholesterol metabolism. The results demonstrate that treatment of high-responding opossums with ezetimibe for 3 weeks decreased plasma cholesterol concentration and altered the hepatic cholesterol metabolism. The major change of hepatic cholesterol metabolism was in cholesterol concentration

Table 3
Messenger RNA levels of *ABCG5*, *ABCG8*, and *NPC1L1* in livers of low- and high-responding laboratory opossums after treatment with or without ezetimibe while consuming the HCLF diet (experiment 2: cholesterol absorption)

Group assignment	Phenotype	*ABCG5*	*ABCG8*	*NPC1L1*
1. ATHH treated	High responding	1.16 ± 0.08*	0.94 ± 0.05	1.43 ± 0.26
2. ATHH untreated	High responding	1.22 ± 0.18	0.80 ± 0.15	0.74 ± 0.12§
3. ATHE treated	Low responding	1.10 ± 0.06	1.13 ± 0.06	1.86 ± 0.26
4. ATHE untreated	Low responding	1.61 ± 0.19†	1.73 ± 0.19†,‡	1.85 ± 0.15

* Mean ± SEM (normalized to *GAPDH* mRNA levels) (n = 5 for high-responding groups, and n = 6 for low-responding groups).
† Value is significantly different from value of group 3 (P < .05).
‡ Value is significantly different from values of groups 1, 2, and 3 (P < .05).
§ Value is significantly different from values of groups 1, 3, and 4 (P < .05).

of gall bladder bile in high-responding opossums treated with ezetimibe. High-responding opossums treated with ezetimibe had higher cholesterol concentrations in their bile than untreated high-responding opossums. Another change in hepatic cholesterol metabolism in high-responding opossums treated with ezetimibe was observed in hepatic cholesterol concentration. Untreated high-responding opossums had significantly higher hepatic cholesterol concentrations than low-responding opossums with or without ezetimibe treatment (Table 2). However, high-responding opossums treated with ezetimibe did not differ significantly in hepatic cholesterol concentrations from low-responding opossums with or without the ezetimibe treatment. Gall bladder biliary cholesterol concentration was strongly and negatively associated with hepatic cholesterol concentration and liver weight. Increase in biliary cholesterol concentration after ezetimibe treatment in high-responding opossums modulated hepatic cholesterol concentration. These studies, therefore, demonstrate that the main defect of hepatic cholesterol metabolism in high-responding opossums is an inability to increase the output of biliary cholesterol when fed the HCLF diet.

Because high-responding opossums as compared with low-responding opossums have higher intestinal cholesterol absorption and lower secretion of cholesterol in their bile, most of the dietary cholesterol has to be secreted in plasma lipoproteins or has to be stored in hepatocytes. On a high-cholesterol diet, plasma cholesterol concentration increases rapidly, probably saturating the system. Consequently, a large amount of cholesterol has to be stored in the liver. Because a limited amount of cholesterol can be stored in hepatocytes, liver size (weight) of high-responding opossums increases as excess dietary cholesterol accumulates there. Ezetimibe treatment decreases cholesterol absorption and increases secretion of cholesterol into bile; and thus, it decreases cholesterol accumulation in the liver.

The effect of ezetimibe on biliary cholesterol concentration in low-responding opossums was different from that in high-responding opossums. The ezetimibe treatment of low-responding opossums decreased biliary cholesterol concentration, whereas the ezetimibe treatment of high-responding opossums increased biliary cholesterol concentration. ABCG5 and ABCG8 have been shown to play a major role in the secretion of cholesterol into bile [23,24]. To determine whether ezetimibe treatment affects the expression of *ABCG5* and *ABCG8* in the liver, we measured the expression of these genes in the livers of high- and low-responding opossums. These results clearly demonstrated that ezetimibe treatment of low-responding opossums causes a decrease in *ABCG5* and *ABCG8* mRNA levels. Thus, the decrease in biliary cholesterol secretion in low-responding opossums may be due to decreased expression of *ABCG5* and *ABCG8* in their livers. The increase in biliary cholesterol secretion in ezetimibe-treated high-responding opossums may be due to increased excretion of cholesterol and bile acids from the body caused by a decrease in cholesterol absorption.

The human liver has been shown to express *NPC1L1* in large amounts [5,15,25]. A recent report has further shown that hepatic NPC1L1 regulates biliary cholesterol concentration and is a target of ezetimibe [26]. We quantified *NPC1L1* mRNA levels in the livers of high- and low-responding opossums treated with or without ezetimibe. The livers of low-responding opossums with or without the treatment with ezetimibe had significantly higher *NPC1L1* mRNA levels than the livers of untreated high-responding opossums. The ezetimibe treatment significantly increased the *NPC1L1* mRNA levels in the livers of high-responding opossums but not in the livers of low-responding opossums. The relevance of increased *NPC1L1* mRNA levels in the secretion of biliary cholesterol in high-responding opossums is not clear from these results. Further studies are needed to establish the role of NPC1L1 in the secretion of cholesterol in the bile of high- and low-responding opossums.

Untreated low-responding opossums had a much higher concentration of cholesterol in their gall bladder bile than any other group. Our previous studies have shown that low-responding opossums have higher hepatic activity of sterol 27-hydroxylase and higher concentration of 27-hydroxycholesterol in plasma and liver in response to the HCHF diet [27]. Our previous studies have also shown that hepatic expression of sterol 27-hydroxylase, *ABCG5*, and *ABCG8* in low-responding opossums was higher than hepatic expression of these genes in high-responding opossums fed the HCLF diet [14]. 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* [28,29]. Treatment of mice expressing *ABCG5* and *ABCG8* with liver X receptor agonist produced a considerable increase in biliary cholesterol concentration and a decrease in cholesterol absorption but not in mice lacking ABCG5 and ABCG8 [30]. Thus, the increase in hepatic sterol 27-hydroxylase may up-regulate the expression of *ABCG5* and *ABCG8* in low-responding opossums as compared with high-responding opossums and may increase hepatic biliary cholesterol secretion.

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