Dietary Cholesterol and Downregulation of Cholesterol 7α-Hydroxylase and Cholesterol Absorption in African Green Monkeys

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Abstract

In this study, hepatic production of bile acid was considered together with intestinal cholesterol absorption as potential regulatory sites responsive to dietary cholesterol. Sequential liver biopsies were taken from 45 feral African green monkeys studied during three different diet periods. Low-fat Monkey Chow was fed during the baseline period, a cholesterol and fat-enriched diet was then fed for 12 wk during period 2, and finally, after a washout period of 10 wk, three subgroups were fed low-, moderate-, and high-cholesterol diets for 12 mo during period 3. The percentage of cholesterol absorbed in the intestine was significantly lower when higher levels of cholesterol were fed; however, this percentage was significantly and positively correlated to plasma cholesterol concentration at each dietary cholesterol level. Hepatic free and esterified cholesterol content were significantly elevated by dietary cholesterol challenge and remained elevated even after 20 wk of low-cholesterol diets. Hepatic mRNA abundance for cholesterol 7α-hydroxylase (C7H) was significantly lower (~ 60%) when the high-cholesterol diet was fed, with the decrease being greater than that seen for low density lipoprotein (LDL) receptor mRNA. At the same time, hepatic mRNA abundance for apolipoprotein B and hepatic lipase were not diet sensitive. C7H activity was decreased to a similar extent by diet as was C7H mRNA, although the correlation between enzyme activity and mRNA abundance was only $r = 0.5$, suggesting that dietary regulation includes factors in addition to transcriptional regulation. Activity and mRNA abundance of C7H remained decreased when liver esterified cholesterol content was reduced to only a two- to three-fold elevation over baseline, at a time when plasma cholesterol and hepatic LDL receptor mRNA abundance had returned to baseline levels. These data on liver C7H, obtained in one of the few primate species predisposed to cholesterol gallstone formation, support the hypothesis that the liver may attempt to downregulate intestinal cholesterol absorption by decreasing bile acid production when increased amounts of absorbed dietary cholesterol reach the liver. Presumably this represents attempted downregulation of intestinal cholesterol absorption by limiting bile acid availability as a means to maintain hepatic cholesterol balance. (*J. Clin. Invest.* 1994. 93:2463–2472.) Key words: bile acids • low density lipoproteins • mRNA, low density lipoprotein receptors • hepatic lipase • apolipoprotein B

Introduction

Regulation of the disposition of dietary cholesterol after absorption is poorly understood. Cholesterol is normally present in the enterohepatic circulation of all mammalian species in micelles containing bile acids, phospholipids, and cholesterol. Dietary cholesterol mixes with bile in the intestinal lumen expanding the pool of cholesterol from which absorption will occur and increasing the ratio of cholesterol to bile acids. In some animal species, and variably among individuals within any one species, inclusion of cholesterol in the diet leads to an increase in plasma cholesterol concentrations, primarily within LDL (1). The tendency to become hypercholesterolemic through diet induction has been identified as a heritable trait in a number of species (2). In almost all cases, the trait has been shown to be polygenic, the implication being that a number of enzymes and/or other factors are involved in the response. Part of the response may be due to the downregulation of hepatic LDL receptors by cholesterol absorbed by the intestine and transported to the liver in chylomicron remnants (3).

Candidate enzymes that may be important in this regulation are those involved in cholesterol balance across the liver. The liver is a unique central clearing house for cholesterol in the body. The liver clears the bulk of lipoprotein cholesterol from plasma while, at the same time, secreting new lipoproteins containing cholesterol into plasma. All newly absorbed dietary cholesterol transported into the body in chylomicron particles is initially cleared from plasma by the liver during chylomicron remnant removal (4, 5). Accordingly, candidate sites in the liver for regulation of dietary cholesterol disposition include proteins involved in lipoprotein particle clearance from plasma such as the LDL receptor (3) and hepatic lipase (6). Apolipoprotein B100, the primary apolipoprotein of lipoproteins secreted with cholesterol from the liver, is also a candidate site for regulation (7, 8).

A further function of the liver as the clearing house for cholesterol is to rid the body of excess cholesterol. The only site for degradation of significant amounts of cholesterol is through conversion in the liver into bile acids. Bile micelles containing bile acids and cholesterol are then secreted into the intestine. Subsequent excretion or reabsorption of cholesterol and bile acid ensues. Bile acids are required for cholesterol absorption from the intestine and assist in the absorption of fatty acids (9).

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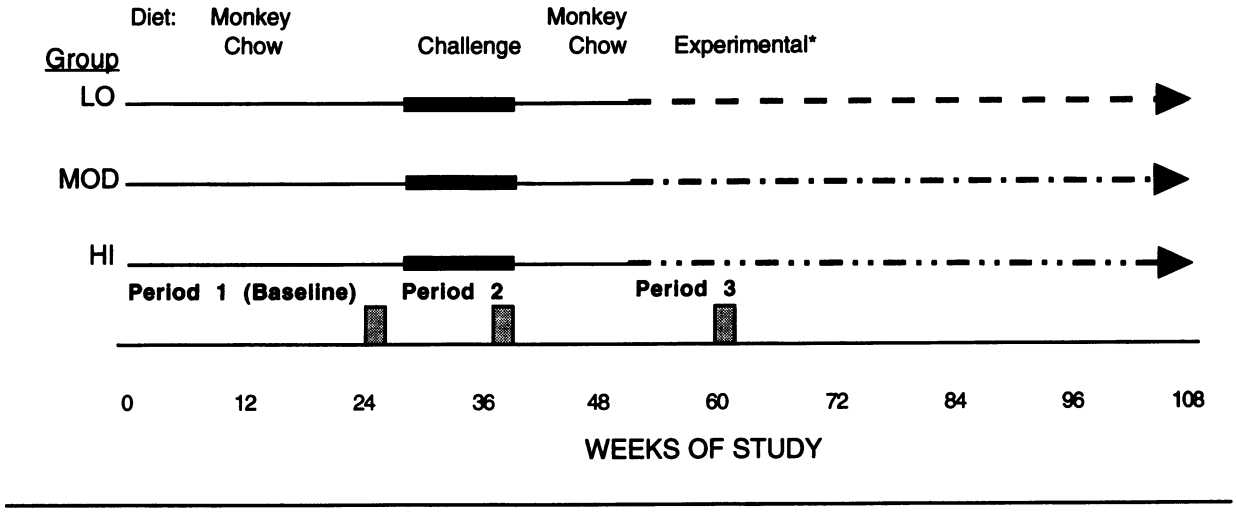

Figure 1. Experimental design for the study. 45 animals were entered into the study. Segments of the study are indicated by the designations of period 1 (baseline), period 2 (challenge with HI diet), and period 3 (experimental diets), and liver biopsies were taken in each of these segments. During periods 1 and 2, all animals were fed Monkey Chow and then the HI diet, respectively. Based on the responses to the challenge with the high cholesterol diet during period 2, animals were assigned to three experimental groups such that each group had equivalent TPC and HDL cholesterol (mean±SD, see Table II). The data of the study are displayed for each diet group even when this was done retrospectively, as for data of periods 1 and 2. In period 3, groups were fed the experimental diets. Group designation was HI, MOD, or LO if they were fed the HI, MOD, or LO diets during the experimental diet of period 3. The names are the abbreviated indication of the cholesterol level during period 3. It must be made clear that the HI, MOD, and LO groups were fed the same diets during periods 1 and 2.

Bile acid availability is regulated in the liver. Cholesterol 7α-hydroxylase (C7H)[1] is the rate-limiting enzyme in the conversion of cholesterol into bile acids (10). Diet-induced upregulation of bile acid formation via C7H would be expected to stimulate excretion of cholesterol into bile (10). Subsequently, increased availability of bile acids in the intestine would stimulate cholesterol absorption from the intestine in effect promoting the return to the liver of more cholesterol in chylomicron remnants. Thus, upregulation of bile acid production appears paradoxical in its effect on whole body cholesterol balance.

The present studies were carried out to determine which components of cholesterol balance across the liver are responsive to dietary cholesterol. Male African green monkeys were used because of their many similarities to human beings in lipoprotein and cholesterol metabolism, including a predisposition to cholesterol gallstone formation (11, 12). The most dietary cholesterol-responsive liver protein identified was C7H; activity and mRNA abundance were both downregulated by dietary cholesterol by 60–70%. The degree and direction of response was surprising since, in rats, C7H has been shown to increase in response to dietary cholesterol (13). In contrast, our findings confirm earlier evidence in African green monkeys that hepatic C7H activity decreases in response to dietary cholesterol (14). In addition, the decreased efficiency of cholesterol absorption in response to increased diet cholesterol that we observed was consistent with the possibility that bile acid availability in these animals was limited.

1. *Abbreviations used in this paper:* C7H, cholesterol 7α-hydroylase; LO, MOD, and HI, low-, moderate-, and high-cholesterol diets, respectively; TPC, total plasma cholesterol.

Methods

Animals and experimental design. The monkeys used in these studies were feral adult male African green monkeys (*Cercopithecus aethiops*) purchased from Primate Imports, Port Arthur, NY. Animals were caged individually throughout the study in racks facing each other in rooms housing about 24 animals. After arrival, animals were maintained in quarantine for 10 wk and were further acclimated to the laboratory environment for an additional 20–25 wk. The diet fed during this time was Monkey Chow (Ralston Purina Co., St Louis, MO), a low-fat, low-cholesterol, high-fiber diet. This equilibration period is the baseline phase, also termed period 1 in the experimental design depicted in Fig. 1. All animals were evaluated during period 1 so that animals with clinical abnormalities could be excluded from the study. Near the end of the period 1, two sequential 10-ml blood samples were collected for lipid and lipoprotein analysis 1 wk apart and subsequently, each animal underwent a liver biopsy. About 2 g of liver was surgically removed through a midline laparotomy performed while the animals were anesthetized with ketamine HCl (Parke-Davis Co., Ann Arbor, MI). Immediately after removal, liver tissue was frozen in liquid nitrogen and was stored at − 80°C until analyzed.

After a period of at least 3 wk for recovery from surgery, the diet of all animals was changed to the high-cholesterol diet (see Table I) for the next 12 wk (period 2). Biweekly blood samples (5 ml) were collected and at 9 wk a 25-ml blood sample was taken for lipoprotein quantification (15). During weeks 10–12, a second liver biopsy was taken as before from each animal. After surgery, the animals were returned to the Monkey Chow diet for at least 10 wk. Blood samples were taken periodically to monitor the return of plasma cholesterol concentrations to their original lower levels. From the group of 45 animals, three groups of 15 animals each were selected. Group assignments were such that each group had equivalent total plasma cholesterol and HDL cholesterol (mean±SD) in response to the high-cholesterol diet challenge of period 2; body weights and plasma concentrations of apolipopro-

Table I. Experimental Diet Composition

Ingredient	Diet		
	HI	MOD	LO
	mg cholesterol/kcal		
Lard	16.4		
Wheat flour	35		
Dextrin	9.6		
Sucrose	10	Same for all diets	
Casein	9		
Lactalbumin	5		
Hegsted salts	4.8		
Vitamin mix	2.6		
Crystalline cholesterol	0.327	0.156	0
Alphacel	7.27	7.44	7.6

All diets contained a caloric distribution of 17% protein, 35% fat, and 48% carbohydrate. The HI, MOD, and LO diets contained high (0.8), moderate (0.4), and low (0.06 mg cholesterol/kcal), respectively, as determined by assay of over 50 separate diet preparations; the cholesterol (30 mg/100 g) in the LO diet all came from the lard added to the diet. The fatty acid composition (wt/wt) of the diets was determined by assay to be 14:0, 2%; 16:0, 25%; 16:1, 2%; 18:0, 15%; 18:1, 42%; 18:2, 12%; others, 2%.

teins A-I, B, and E were also equivalent among groups. Groups were then fed their respective experimental diets (period 3). The experimental diets were identical except for the amount of cholesterol, which was 0.06, 0.4, and 0.8 mg of cholesterol/kcal in the low cholesterol (LO), moderate cholesterol (MOD), and high cholesterol (HI) diets, respectively. Diet was weighed out daily for two meals (morning and afternoon) and fed so that each animal received 90 kcal/kg body weight, an amount that allowed each animal to maintain constant body weight throughout the study. Diet compositions are given in Table I. The blood sampling schedule was the same during the first 10 wk of experimental diet feeding as it was during the high cholesterol diet challenge period (period 2). During the 10th–12th wk of feeding the experimental diets, a third liver biopsy was taken from each animal. As a result, three sequential liver biopsies taken during three different diet periods from each animal were available for analysis.

Blood samples were drawn from the femoral vein while the animals were restrained with ketamine (10 mg/kg); all animals were fasted overnight before blood samples were drawn. Body weights were measured bimonthly and all animals were found to maintain a constant weight throughout the study.

Intestinal cholesterol absorption. Intestinal cholesterol absorption was measured by the fecal isotope ratio method (16, 17). The animals were fed an apple slice that contained a dose with a known amount ($\sim 1 \times 10^6$ dpm) and ratio (~ 5) of radioactive cholesterol and β-sitosterol. The [^{3}H]cholesterol and [β-4-^{14}C]sitosterol were purified by HPLC to be > 99% pure. The regular afternoon meal was fed immediately after the apple slice, so that the dose was effectively mixed with a typical meal in the gut. Quantitative fecal collections were made for 4 d after the dose was given. Feces were homogenized in a blender with an equal volume of water, then an aliquot was removed and saponified in alcoholic KOH. The nonsaponifiable sterols were extracted with hexane. To prevent color quenching, hexane extracts were bleached under an ultraviolet lamp until no color was apparent. The hexane was then evaporated under nitrogen, scintillation cocktail (BioSafe II; RPI, Mt. Prospect, IL) was added and the radioactivity was determined in a liquid scintillation spectrometer. The percentage of cholesterol absorbed was calculated as the ratio of ^{3}H to ^{14}C radioactivity in the fecal extracts divided by the ratio in the dose times 100. Repeatability of this measurement in several groups of animals ($n \geq 35$) retested without diet change was $r \geq 0.85$ with mean values±5%, indicating that the estimate of percentage cholesterol absorption by this method was reproducible. The fact that β-sitosterol has limited absorbability in this species was documented earlier (18).

Bile acid secretion in vivo. In four animals purchased separately, in vivo bile acid production measurements were made after cannulation of the common bile duct, after the procedures of Dowling et al. (19) with modifications. Although all animals had consumed the HI diet previously, for these studies, the animals were fed the LO diet and one animal was subsequently studied while being fed the HI diet. Animals were trained to wear jackets outfitted with tethers before study. The common bile duct was cannulated with a silicone rubber cannula and tied off just past the cannula. For bile return, a separate cannula was inserted through the wall of stomach just above the pyloric valve. The cannulae were then exteriorized through a stab wound in the abdominal wall, tunneled under the skin to the mid-back, and then exteriorized through a stab wound in the skin. Cannulae were then passed through a tether connected to the jacket, and attached so that a loop in bile flow from the common duct back into the stomach was accessible from outside the monkey's cage. The animals were allowed 2–3 d to recover from the surgery. Bile collections were then initiated. Bile samples representing < 5% of bile flow were taken periodically. Bile flow rates were measured by collection in a graduated cylinder and bile was then returned through a peristaltic pump to the animal via the stomach cannula. At the end of the period of sampling from an intact enterohepatic circulation, return of bile to the animal was stopped and quantitative bile collections were made. Aliquots of bile were assayed for bile acid content after extraction of bile samples with chloroform/methanol, 2:1, by the method of Folch et al. (20). Bile acid content in the aqueous phase was determined using the enzymatic steroid dehydrogenase procedure (21).

Lipid and lipoprotein analyses. The measurement of plasma cholesterol and HDL cholesterol was done in the Lipid Analytic Laboratory using procedures standardized through the Centers for Disease Control (22, 23). Plasma lipoprotein classes were separated from plasma by ultracentrifugation and isolated by gel filtration chromatography (15), and the cholesterol content of each fraction was measured enzymatically (Boehringer Mannheim Diagnostics, Indianapolis, IN). Apolipoprotein concentrations in plasma were measured with ELISAs standardized for monkey apolipoproteins for apoA-I (24), apoB (8), and apoE (25). For all ELISAs, coefficients of variation were < 9%.

Hepatic mRNA abundance. Total RNA from monkey liver was isolated by the guanidine isothiocyanide-cesium chloride procedure (26). Integrity was evaluated by glyoxal/agarose gel electrophoresis and concentration was estimated by measurement of A_{260} with a light-scattering correction with measurement of A_{310}. The abundance of mRNA was quantitated using a cRNA excess solution hybridization/S1 nuclease protection assay (27, 28). Portions of human cDNAs for the LDL receptor (29) representing amino acids 219–547, apoB (30) representing amino acids 270–570, hepatic lipase (31) representing amino acids 14–236, and C7H (32) representing amino acids 34–234 were subcloned into the pBSSK$^-$ plasmid (Stratagene Inc., LaJolla, CA) to facilitate the production of discrete length, single-stranded, antisense riboprobes which have been shown to specifically hybridize to monkey RNA sequences. For technical reasons, the final lengths of the antisense probes generally covered only a portion of the cDNA insert and included some polylinker sequence. The actual probe lengths are: LDL receptor, 479 nucleotides; apoB, 537 nucleotides; hepatic lipase, 703 nucleotides; and C7H, 357 nucleotides. The antisense probes were transcribed with bacteriophage T3 or T7 polymerase in the presence of [^{32}P]UTP (Amersham Corp., Arlington Heights, IL) based on the protocol supplied by the manufacturer of the polymerases (Stratagene Inc.). These probes each hybridized to a band of the expected size on Northern blots of monkey liver RNA.

For the solution assay, 5–50 μg of total RNA was hybridized to 2 $\times 10^5$ cpm of probe at 65°C for 12–24 h (length of time determined empirically for each probe) in 300 μl of buffer containing 30 μg of

tRNA, 50% formamide, 40 mM MOPS, 1 mM EDTA, and 400 mM NaCl. Samples were then digested at 37°C for 60 min with the addition of 200 μl of S1 buffer at pH 4.6 containing 2,000 U of nuclease S1 (Sigma Chemical Co., St Louis, MO), 60 mM NaOAc, 200 mM NaCl, 4 mM ZnCl, and 10% glycerol. 100 μg of carrier DNA was added to each sample which was then maintained at 37°C for 30 min. 50 μl of 100% TCA was then added to each sample and precipitation proceeded at 4°C for 10 min. The solution was filtered and the amount of protected material was then determined by scintillation counting. Background values were < 5% of total added radioactivity. Each reaction also contained a ^{3}H-labeled actin riboprobe to monitor the amounts of sample RNA added to each assay.

Digested and undigested aliquots of the probes and subsets of the reactions from the liver RNAs and the standards were also analyzed on 6% polyacrylamide gels electrophoresed under denaturing conditions. Autoradiography of these gels confirmed both integrity of the labeled cRNA and the expected size of the fragment protected by hybridization. The mRNA values were determined by reference to standard curves generated using overlapping single-stranded sense RNAs synthesized off the complementary strand of the same vectors used for probe synthesis. Large amounts of the "sense" controls were produced with the megascript protocol (Ambion Inc., Austin, TX) and were stored as frozen aliquots to permit comparison of assays done at different points in time. Adjustment for the size differences between the probe and full-length message indicated the amount of the specific message in each sample expressed as pg mRNA/μg total RNA.

Cholesterol 7α-hydroxylase assay. Samples of frozen liver (100–300 mg) were minced in 1 ml of ice cold isolation buffer (0.25 M sucrose, 1 mM Na_2EDTA, 50 mM NaF, 0.1 M K_2HPO_4, pH 7.4) and then disrupted in a 2-ml Potter-Elvejem homogenizer. Homogenates were centrifuged for 15 min at 13,000 rpm in a SW-55 rotor (Beckman Instruments, Palo Alto, CA) at 4°C, and the supernate was recentrifuged for 60 min at 38,000 rpm. The microsomal pellet from the second spin was resuspended in 1 ml of assay buffer (1 mM Na_2EDTA, 50 mM NaF, 5 mM dithiothreitol, 0.015% CHAPS, 0.1 K_2HPO_4, pH 7.4). Aliquots of liver microsomes were then quick frozen in liquid nitrogen and stored at − 80°C until assayed.

A reversed-phase HPLC method, with modifications to adapt it for monkey tissue, was used for C7H activity assay (33). This assay uses as substrate the endogenous cholesterol in the microsomes. Incubation mixtures containing 0.062 nmol of 20α-hydroxycholesterol in 25 μl of methanol (internal standard), 800 μl of assay buffer, and 200 μl of microsomal preparation containing about 1 mg of protein were preincubated for 5 min. Then 100 μl of freshly prepared 10 mM NADPH or distilled water (controls) was added. Incubation for 30 min at 37°C in a shaking water bath was followed by the addition of 25 μl of 20% sodium cholate to stop the reaction. Cholesterol oxidase (from *Nocardia erthropolis,* 45 U/mg; Boehringer Mannheim) was then added to oxidize the products of the reaction, including 7α-hydroxycholesterol. 15 min later, assay mixtures were extracted with 5 ml of petroleum ether, and extracts were stored under nitrogen until analysis.

The petroleum ether extract was evaporated under nitrogen and then dissolved in the HPLC solvent of acetonitrile/methanol, 70:30, vol/vol. Injection was onto a Spherex 5 ODS-2 column, 250 × 3.2 mm with a 30 × 3.2 mm precolumn (Phenomex, Torrance, CA) connected to a model 2350 pump and model 2360 gradient programmer (Isco, Lincoln, NE). The oxidation products (from cholesterol oxidase) of 7α-hydroxycholesterol and 20α-hydroxycholesterol were eluted at about 11 and 9 min, respectively, at a flow rate of 0.4 ml/min. The column was washed with running solvent at 0.8 ml/min between injections. Detection was at 240 nm with a model V4 variable wavelength detector (Isco) connected to an HP3396 Series II integrator (Hewlett Packard Co., Palo Alto, CA) interfaced with an IBM model 50Z PS/2 personal computer. Chrom Perfect software (Justice Innovations, Palo Alto, CA) was used for final signal analysis, baseline construction, and integration. Amounts of metabolites generated were calculated from standard curves referencing to the internal standard.

Samples of monkey livers taken fresh for microsomal isolation were compared with samples from the same livers that were quick frozen in liquid nitrogen before microsomal isolation. Freezing before microsomal isolation did not affect the measured activity of C7H.

Liver cholesterol concentration. Small samples (~ 100 mg) of the liver biopsies were taken for lipid extraction using chloroform/methanol, 2:1 (20). After separation of the phases with 0.05% H_2SO_4, the lower layer was filtered and cholesterol and unesterified cholesterol were measured enzymatically according to the procedure of Carr et al. (34). The protein pellets were dissolved in 1 N NaOH, and aliquots were used for measurement of protein (35).

Statistical analysis. The data were expressed as mean±SEM, and depending on the endpoint in question, group and/or diet comparisons were done as paired *t* tests, analysis of variance, or analysis of covariance. Correlation and regression analyses were also done. The Statview and SuperANOVA software (Abacus Concepts, Inc., Berkeley, CA) for the Macintosh was used for statistical evaluations.

Results

Placement of individual animals in experimental groups was done so that three groups were established (Fig. 1) with equal means and standard deviations for plasma cholesterol based on the response to the challenge during period 2 with the HI diet (Table I). Once group assignments were made, the data for periods 1 and 2 were retrospectively analyzed by group. The data taken at baseline (period 1) and 9 wk into periods 2 and 3 are shown in Table II. All three groups had comparable means and standard deviations for total, LDL, and HDL cholesterol concentrations in periods 1 and 2. The effect of increasing dietary cholesterol was to significantly increase total and LDL cholesterol and to decrease HDL cholesterol. The magnitude of the effects depended on the amount of cholesterol in the diet, with the amounts of dietary cholesterol having been selected to put animals at low, moderate, and high risk for premature coronary artery atherosclerosis. It can be noticed that the plasma cholesterol concentrations during period 3 in the LO group are

Table II. Plasma Lipoprotein Cholesterol Response to Diet

			Cholesterol (mg/dl)		
Group	Period	*n*	Total	LDL	HDL
LO	1	15	141±6	54±4	79±3
MOD	1	15	142±6	53±3	80±4
HI	1	15	136±6	46±2	86±6
LO	2	15	383±37	278±33	64±4
MOD	2	15	402±33	296±31	64±7
HI	2	15	386±36	276±31	64±6
LO	3	15	146±7*	48±5*	78±4*
MOD	3	15	255±21‡	154±19‡	62±5‡
HI	3	15	386±36§	240±29§	56±6§

All values are mean±SEM. LO, MOD, and HI are the abbreviated names for the diet groups for the diets fed for the experimental diet in period 3. Individual animals were assigned to groups after the data for the period 2 challenge was evaluated so that three groups with equivalent total and HDL cholesterol responses (mean±SEM) to dietary cholesterol could be established. Therefore, values for periods 1 and 2 were calculated retrospectively after the grouping was determined. Different superscript symbols indicate a statistically significant difference ($P < 0.01$) within a period, as determined by ANOVA with post hoc analysis.

essentially the same as when the animals were fed Monkey Chow in the baseline period 1.

In representative subgroups of four animals from each of the diet groups, a portion of the liver biopsies was used for measurement of liver cholesterol concentrations. The data are shown in Table III. In the liver biopsies taken at the end of baseline (period 1), the liver free and esterified cholesterol concentrations were about 13 and 2 mg/g protein. At the end of period 2, after 10–12 wk of ingestion of the HI diet, liver concentrations of free cholesterol had almost doubled in all groups to between 21 and 25 mg/g protein while esterified cholesterol had increased nearly 10-fold to between 14 and 24 mg/g protein. In period 3, liver free cholesterol concentrations varied according to the amount of cholesterol in the diet, being the lowest (16.4 mg/g protein) in the samples from the LO group and highest in the samples from the HI group (22 mg/g protein). In the LO group, the free cholesterol concentration in the liver biopsies from period 3 remained significantly ($P < 0.01$) higher than in the biopsies taken at baseline (16 vs. 12 mg/g protein).

The esterified cholesterol concentrations also varied in proportion to the dietary cholesterol level in period 3 liver biopsies (Table III). The highest level was in the biopsies from the HI diet group (19 mg/g protein) and was comparable to that seen when the same diet was fed during period 2. Significantly lower esterified cholesterol concentrations were found in the period 3 liver biopsies in the MOD (12 mg/g protein) and LO (5.8 mg/g protein) diet groups. In the period 3 liver biopsy of the LO diet group, the esterified cholesterol concentration was significantly higher than in the period 1 biopsy (5.8 vs. 1.8 mg/g protein), even though the animals had been fed low-cholesterol diets for over 20 wk after being exposed to the high-cholesterol diet for only 12 wk.

Cholesterol absorption measurements were made in each of the animals near the end of the Monkey Chow wash out after period 2 (Fig. 1) and were repeated during the 76th and 87th wk of the experimental period (Fig. 1), as shown in Table IV. The percentage of cholesterol absorbed was significantly lower (25–30%) during the Monkey Chow period than it was during the experimental diet period. Monkey Chow is a low-fat diet (< 10% of calories) containing a large amount of fiber, and these factors may account for the lower percentage of cholesterol absorption. During the two different studies of the experimental diet period, trends were similar with a lower percent absorption in the HI diet group and a higher percent absorption in the LO diet group, and with the MOD diet group having an intermediate value. These differences show that when more cholesterol is available for absorption, a lower average percentage absorption is found.

Table III. Liver Cholesterol Concentrations

Dietary group	Period	Free cholesterol	Esterified cholesterol
		mg/g protein	
LO	1	12.13±0.31*	1.80±0.46*
	2	20.98±1.91‡	14.35±3.39‡
	3	16.35±0.43§	5.80±0.52§
MOD	1	12.93±0.25*	2.00±0.76*
	2	25.10±2.29‖	20.50±4.85‖
	3	19.85±1.56‡	11.90±1.60§
HI	1	14.40±1.08*	2.18±1.07*
	2	24.48±1.31‖	24.20±4.58‖
	3	21.73±1.70‡‖	19.28±3.18‖

All values are mean±SEM. Different superscript symbols within a column indicate statistically significant differences ($P < 0.05$).

Table IV. Intestinal Cholesterol Absorption

Group	*n*	Cholesterol absorption: Monkey Chow	Period 3: Week 76	Period 3: Week 87
			%	
LO	15	25±4*	56±3	67±2
MOD	15	29±3*	50±3	54±2
HI	15	31±4*	45±2	48±2
One-way ANOVA				
Diet Chol		NS	0.03	0.0001
Fisher's PLSD				
LO-MOD			NS	0.0001
LO-HI			0.008	0.0001
MOD-HI			NS	0.04

All values are mean±SEM. * Significantly different from weeks 76 and 87, paired *t* test. PLSD, protected least significant difference.

The data in Fig. 2 indicate that a statistically significant relationship existed within each diet group between the cholesterol absorption percentage and the total plasma cholesterol (TPC). This occurred in spite of the fact that the average percent absorption was proportionally reduced in the groups receiving the higher dietary cholesterol levels. The data show that a higher percent absorption in animals fed higher dietary cholesterol levels is associated with higher TPC. For example, reading from the regression lines for 60% absorption, the plasma cholesterol concentration would be about 175, 300, and 600 mg/dl in the LO, MOD, and HI groups, respectively. The slope of the line increased significantly with higher dietary cholesterol loads.

Hepatic C7H mRNA abundance and activity were measured since this enzyme is the rate-limiting enzyme in bile acid formation. The data in Fig. 3 *A* show the mean±SEM for C7H mRNA during each diet period for each group. Clearly, the C7H mRNA values for all groups were the highest during period 1 when the diet was Monkey Chow. Values were 62% lower ($P < 0.0001$, paired *t* test) during period 2 when the HI diet was fed to all of the animals. The extent of decrease was similar for all three experimental groups. When experimental diets were fed during period 3, hepatic C7H mRNA abundance in each group was significantly lower ($P < 0.0001$) than during period 1 and was not significantly different from those of period 2. A statistically significant correlation ($r = 0.34$, $P < 0.05$) was seen between hepatic C7H mRNA values in periods 1 and 2. The mRNA abundance in period 3 was compared to that in period 1 by group; highly significant correlations were seen for the MOD and HI groups ($r = 0.89$ and $r = 0.83$, $P < 0.001$, respectively) but no significant correlation was found for the LO group.

A subset of six animals from each of the three groups was

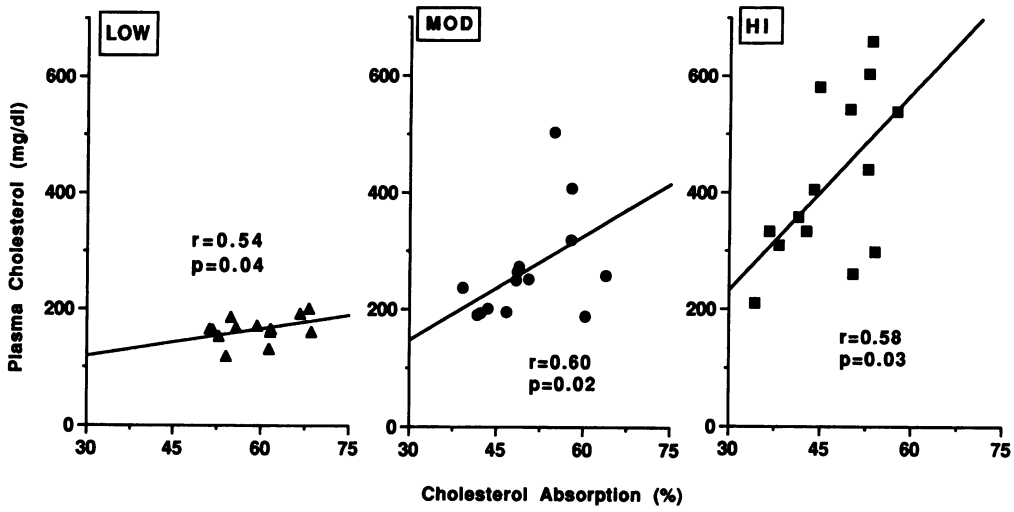

Figure 2. Positive (and statistically significant) correlations were found between TPC and the percentage cholesterol absorption, averaged from two separate measurements taken in each animal during the same time periods (weeks 76 and 87 of period 3).

selected and the activity of C7H was measured in the microsomes prepared from each of the liver biopsies from these animals. The data are shown in Fig. 3 *B*. Patterns of change with diet in hepatic C7H activity were the same as for C7H mRNA abundance. Significantly less ($P < 0.001$) activity was measured in periods 2 and 3 than in period 1, and for each of the groups, the mean of C7H activity was the same in periods 2 and 3. The activity was significantly correlated to the mRNA abundance for each of the three dietary periods ($r = 0.48$ [$P = 0.04$], $r = 0.66$ [$P = 0.003$], and $r = 0.42$ [$P = 0.07$] for periods 1, 2, and 3, respectively).

The abundance for other specific mRNAs potentially involved in regulation of cholesterol metabolism was measured in liver biopsies from each of the experimental periods. The plasma concentrations of LDL and apoB (data not shown) were increased by dietary cholesterol, so hepatic apoB mRNA abundance was evaluated. The data in Fig. 4 *A* show that the apoB mRNA abundance was not different during the three periods for any of the experimental groups. The apoB mRNA abundance in the HI group was consistently higher ($P < 0.05$) than for the other groups except during period 3 when the difference between LO and HI was not statistically significant. Maintenance of these relationships among groups and across diet periods is another indication that individual animal differences were the important variable in determining apoB mRNA abundance, not the type of diet or amount of cholesterol fed. The hepatic lipase mRNA abundance is shown in Fig. 4 *B*. No significant differences among diet periods and/or among groups was found. Thus, the mRNA levels for apoB and hepatic triglyceride lipase were not sensitive to the diet manipulation that resulted in the large plasma LDL and HDL differences among experimental groups in these studies.

Finally, hepatic LDL receptor mRNA abundance was measured in the liver biopsy material. The data are shown in Fig. 5. LDL receptor mRNA abundance was significantly lower ($P < 0.001$, paired t test) during period 2 compared to period 1, although for the LO group alone, no significant difference was apparent. The animals in the LO group had significantly higher ($P = 0.04$, paired t test) LDL receptor mRNA abundance in period 3 than in period 2 while the values for the MOD and HI

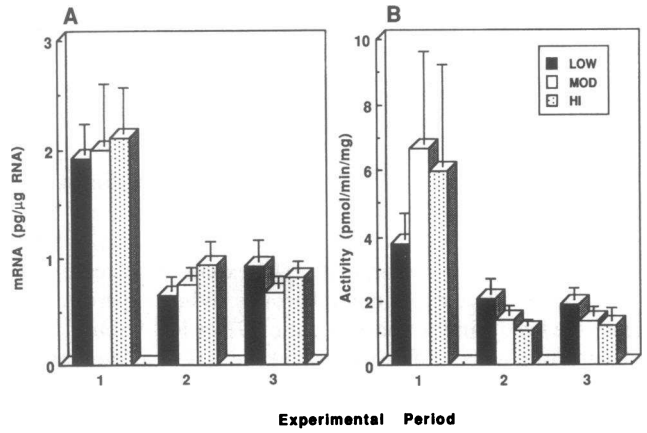

Figure 3. C7H mRNA abundance (*left panel*) measured in sequential liver biopsies taken from each of the animals in each experimental period (see Fig. 1) and activity (*right panel*) measured in the liver biopsies from subgroups of six animals of the LO, MOD, and HI groups. Values during periods 2 and 3 were significantly lower ($P < 0.0001$) than in period 1 for both mRNA abundance and activity. Within a period, values among groups were not significantly different.

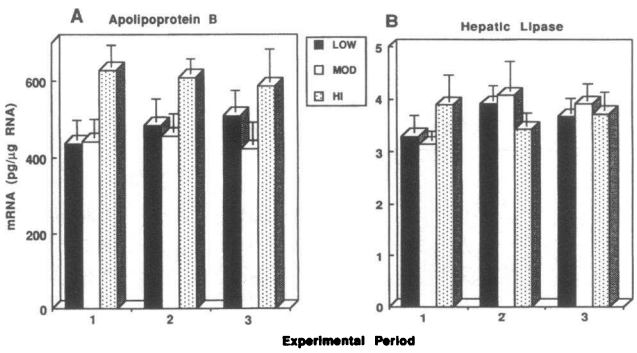

Figure 4. (*A*) Apolipoprotein B mRNA abundance and (*B*) hepatic lipase mRNA abundance expressed as mean±SEM after measurement in the sequential liver biopsies of each of the animals during each of the experimental periods (see Fig. 1). No significant difference among experimental periods was seen for either type of mRNA. ApoB mRNA abundance in the HI group was higher than in the LO or MOD groups in periods 1 and 2 ($P < 0.05$).

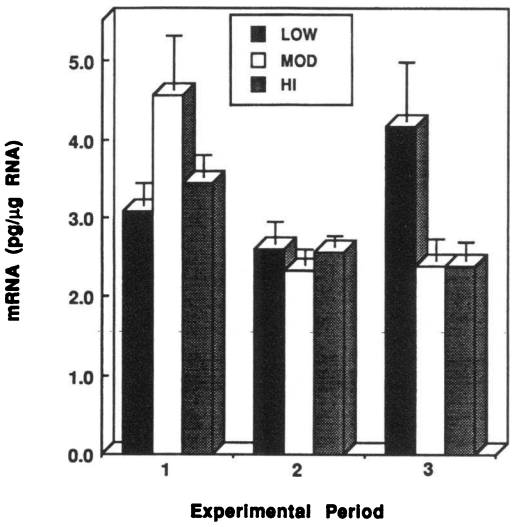

Figure 5. LDL receptor mRNA abundance (mean±SEM) measured in the sequential liver biopsies of each of the animals during each experimental period. Values in period 2 were significantly lower ($P < 0.001$) than in period 1, with the average in period 2 being lower by ~ 30%. The values in the LO group were significantly higher in period 3 ($P = 0.005$) than for the MOD and HI animals.

groups were not significantly different between periods 2 and 3. In addition, during period 3, the LDL receptor mRNA abundance was significantly higher ($P = 0.005$, ANCOVA) for the LO group than for the MOD and HI groups. No statistically significant correlations between LDL receptor mRNA abundance between periods 1 and 2 or between periods 1 and 3 were seen. A statistically significant correlation between LDL receptor mRNA abundance and apoB mRNA abundance was seen for period 2 ($r = 0.43$, $P < 0.05$); no correlation with C7H mRNA abundance was seen for either apoB or LDL receptor mRNA abundance.

Bile acid production was quantitated in a separate group of animals (four on the LO diet, one on the HI diet) in which the common bile duct was cannulated and bile acid production was measured (Fig. 6). Distinct diet effects in this small group of animals were not seen so that data from all animals were averaged together. The rate of bile acid appearance varied between 70 and 300 μmol/h (Fig. 6, *left panel*). Bile acid secretion tended to be highest in the morning after the morning meal (at 0 and 24 h) and to be lowest during the middle of the night. Subsequently, the enterohepatic circulation was completely interrupted and the rate of bile acid secretion was measured for 72 h (Fig. 6, *right panel*). Interruption of the enterohepatic recirculation resulted in a prompt decline in bile acid appearance rate to an apparent minimum near 20 μmol/h, a rate that did not change significantly during the remainder of this period. A suggestion of a 24-h cycle may be present in the data. The lack of a subsequent increase in bile acid appearance rate several hours after complete interruption of bile acid return to the liver suggests that production of new bile acids was already operating at a maximal rate (~ 20 μmol/h) and that bile acid derepression of C7H activity did not occur.

Discussion

The major new finding in this study was that atherogenic diets appear to downregulate bile acid production in the African green monkey, an important primate model for experimental atherosclerosis (36, 37) and cholesterol gallstone disease (12). The downregulation of bile acid availability appeared to occur at the level of production, with C7H mRNA abundance and enzyme activity both being equivalently affected. C7H catalyzes the reaction at the first dedicated step in the conversion of cholesterol to bile acids (10). Downregulation of this enzyme in the primate model was surprising since the bulk of existing evidence, principally from rats, is that dietary cholesterol upregulates C7H both at the level of enzyme activity and mRNA abundance (13, 38). However, the finding of cholesterol-induced downregulation of C7H in primates was convincing since the result was obtained in over 40 animals comparing period 1 (Monkey Chow diet) to period 2 (fat and cholesterol-enriched diet), and remained present when comparing period

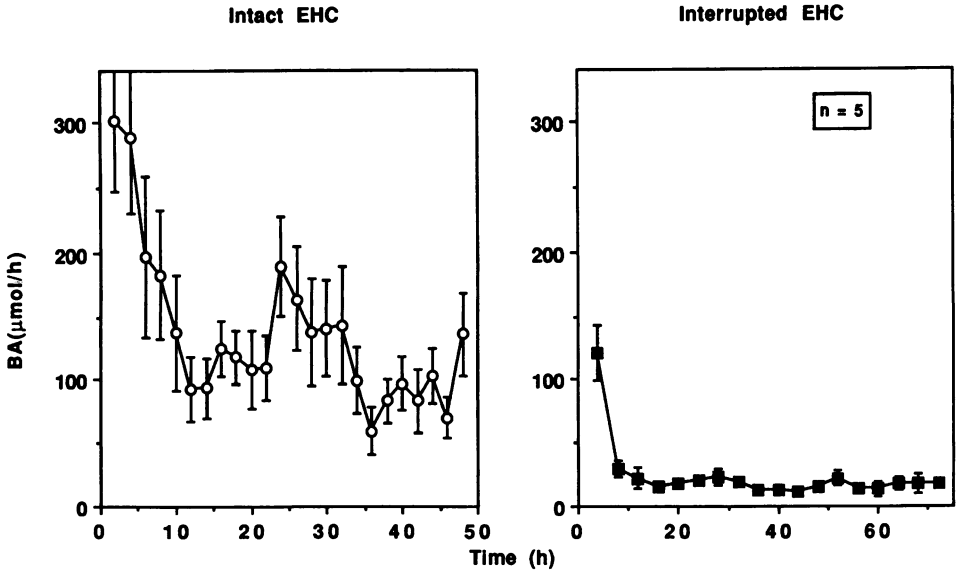

Figure 6. Bile acid (BA) secretion rate as measured in animals with surgically implanted bile fistulae. Each point is the mean±SEM for five observations. For intact enterohepatic circulation (EHC), 5% of the bile was sampled periodically (*left panel*); in contrast, bile was quantitatively removed the animal and collected during the 72-h period of interrupted EHC (*right panel*). The animals were fed in the morning at 8 a.m. (zero time) and in the afternoon at 2 p.m. and lights were on from 7 a.m. until 7 p.m. Interrupted EHC measurements were made immediately after the intact EHC measurements in each of the animals.

1 to period 3, when liver free and esterified cholesterol content was elevated in all three experimental groups. Downregulation of C7H by dietary cholesterol should limit bile acid availability that would, in turn, result in decreased cholesterol absorption efficiency by the intestine (10). As a result, the liver would process less cholesterol returning in chylomicron remnants.

The likelihood that the dietary effect on C7H was due to the cholesterol content of the diet is good although it was not definitively established in the present experimental design. The C7H activity and mRNA abundance in the LO group in period 3 did not return to baseline values even though the animals were fed Monkey Chow for at least 10 wk and the LO diet for another 10–12 wk before the third liver biopsy was taken. While plasma cholesterol levels (Table II) and hepatic LDL receptor mRNA abundance (Fig. 5) had returned to baseline levels in the LO group during period 3, liver free and esterified cholesterol were both significantly higher than during the baseline period. Liver cholesterol may remain elevated due to incomplete washout from the challenge diet and/or because the 0.06 mg/kcal of cholesterol in the LO diet may be enough to raise liver cholesterol levels without affecting plasma cholesterol or liver LDL receptor mRNA. The correlation for C7H mRNA abundance in period 1 to the level in period 3 in the MOD and HI groups was very high ($r > 0.8$) while it was not significant for LO animals. This suggests that the mRNA was not at a stable response level in many of the LO animals in period 3, i.e., the moderately elevated free and/or esterified cholesterol levels may have been insufficient to completely downregulate the enzyme. While no significant difference among diet groups in C7H mRNA or activity was found in period 3 (Fig. 3), it appears that the mean values tend to be higher in the LO diet group of period 3 compared to periods 1 and 2. The data for C7H mRNA and activity in the LO group in period 3 were surprising in that plasma cholesterol and hepatic LDL receptor mRNA had normalized. However, since the liver cholesterol concentration was significantly elevated, the likelihood is that C7H is very sensitive to even small increases in liver cholesterol.

The possibility that bile acid availability was limiting in monkeys fed dietary cholesterol was supported by the cholesterol absorption data. Bile acids are known to be necessary for cholesterol absorption to occur from the intestine (10), presumably because they result in the formation of cholesterol-containing micelles, from which cholesterol diffusion and absorption is facilitated (39). The amounts of dietary cholesterol fed during a 24-h period were ~ 30, 200, and 400 mg in the LO, MOD, and HI groups, respectively. If we assume that the amount of biliary cholesterol secreted into the intestinal lumen was ~ 50 mg/d, as estimated from our in vivo measurements of bile secretion in the bile fistula monkeys, then the approximate amount of intralumenal cholesterol in the three diet groups was 80, 250, and 450 mg/d (207, 650, and 1160 μmol/d), respectively, for the three dietary groups. We assume that the biliary and dietary cholesterol are essentially completely mixed during intestinal transit (40). For equivalent micellization and absorption of these five- to six-fold different amounts of lumenal cholesterol, bile acid secretion would need to vary proportionally. The molar ratio of bile acid to cholesterol (BA/CHOL) measured in bile of African green monkeys has been found to vary from ~ 15 to 60, or fourfold (M.W. Scobey and L.L. Rudel, unpublished results). Our data indicate that the maximum secretion of bile acid per day in an animal would be ~ 7,200 μmoles (estimated based on data in Fig. 6, 300 μmol/h × 24). Under these conditions, the molar ratio of bile acid to cholesterol in the intestine of the HI group would be ~ 6, considerably below that in bile or in the intestine of a LO diet animal (BA/CHOL = 37). If bile acid availability is further limited when bile acid binding resins are fed, cholesterol absorption would be expected to be even further decreased and this was found (L.L. Rudel, unpublished observations). These data suggest that with increasing dietary cholesterol, bile acid may become a limiting factor for cholesterol micellization. The proportional decrease in the percentage absorption with higher dietary cholesterol content is consistent with this possibility and further experimentation seems warranted.

From the data on average percent cholesterol absorption (Table IV), the amount of cholesterol transported into the body each day can be calculated. An average of 0.62 × 80 or 50 mg, 0.52 × 250 or 130 mg, and 0.465 × 450 or 210 mg of cholesterol are the estimates in the LO, MOD, and HI diet groups, respectively. Therefore, the mass of cholesterol that the liver must accommodate is approximately four-fold different among diet groups even after attempts to regulate cholesterol absorption.

The degree to which individual animal differences in cholesterol absorption reflect the degree of hypercholesterolemia was estimated. Statistically significant correlations between the percentage cholesterol absorption and total plasma cholesterol were found (Fig. 2). These relationships establish cholesterol absorption as an important variable that is significantly related to the extent of the hyperlipoproteinemic response to dietary cholesterol, a response in green monkeys (as shown here in Table II) that is similar to that reported in humans at high risk for premature coronary heart disease (41, 42). In this context, the extent of bile acid availability and its role in determining cholesterol absorption needs to be quantified.

Our findings suggest that regulation of C7H by dietary cholesterol could be a major component of hepatic bile acid production. It is widely assumed that downregulation by bile acid is the primary form of C7H regulation (10). Our data open the possibility that in some individuals, downregulation of C7H by bile acid occurs only after the very potent effect of dietary cholesterol to downregulate this enzyme. This is of potentially great interest in human beings since the Western diet includes such high levels of fat and cholesterol. If dietary cholesterol is as effective in regulating C7H in human beings as was apparent in the LO monkeys of period 3 of this study, then further regulation by bile acid may be small. Fig. 3 shows that C7H activity and mRNA abundance in the LO animals of period 3 were practically the same as when the presumed maximal downregulation of period 2 was present. This level of enzyme production was apparently adequate to maintain the low but constant 20 μmol/h secretion rate of bile acids in the bile fistula animals (Fig. 6), a value comparable to that found early after a bile fistula was established in an earlier primate study (19). There was no increase in bile acid appearance in the subsequent 72 h in our studies, suggesting that even after release of feedback inhibition of bile acid production by bile fistula, an increase in bile acid production did not occur. In the data of Dowling et al. (19), in female rhesus monkeys, a presumed upregulation of bile acid synthesis occurred several hours after interruption of bile return to the liver, but the animals in their studies were fed

Monkey Chow. When we fed this diet, we saw maximal C7H activity and mRNA abundance in the liver of our animals. Therefore, the presumed difference between these studies is diet.

The present studies in African green monkeys show that hepatic C7H regulation may be of major importance in the establishment of the type of hyperlipoproteinemia (increased LDL with lowered HDL) that is most highly correlated with premature CHD in this primate species (36, 37). Together with the present data, we have now examined several potential regulatory sites in the liver that could be involved in promoting the increased LDL concentrations induced by dietary cholesterol, including apoB production, apoE production, hepatic lipase activity, and LDL receptor activity (6, 8, 25, 43). We have made a combination of mRNA and activity measurements and have measured hepatic secretion rates of individual apolipoproteins; i.e., we have monitored several steps in the regulation of the expression of several genes by atherogenic diets. To date, the regulation of C7H gene, as demonstrated here, is larger than any other effect we have detected.

Perhaps the downregulation of C7H by dietary cholesterol is also related to the propensity of the African green monkey to develop cholesterol gallstones, but further work is needed to be certain. Clearly, this primate is unique among Old World monkeys in its proclivity to develop cholesterol gallstones and comparisons to other species that do not develop gallstones may help indicate the relative importance of C7H regulation by dietary cholesterol in the development of this disease. The relationships of dietary cholesterol to cholesterol gallstone disease identified in this monkey model appear similar to those seen by Everson and colleagues in studies in human beings (44).

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