Regulation of classic and alternative bile acid synthesis in hypercholesterolemic rabbits: effects of cholesterol feeding and bile acid depletion

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Abstract The effect of cholesterol feeding (3 g/day) on bile acid synthesis was examined in 10 New Zealand white rabbits (NZW), 8 Watanabe heterozygous and 10 homozygous rabbits with partial and complete deficiencies of LDL receptors. After 10 days of cholesterol feeding, bile fistulas were constructed and bile acid pool sizes were measured. Cholesterol feeding increased plasma and hepatic cholesterol levels in all rabbit groups. Baseline bile acid pool sizes were smaller ($P < 0.01$) in heterozygotes (139 ± 3 mg) and homozygotes (124 ± 30 mg) than NZW rabbits (254 ± 44 mg). After feeding cholesterol, bile acid pool sizes doubled with increased cholic acid synthesis in NZW and, to a lesser extent, in Watanabe heterozygous rabbits but not in homozygotes. Baseline cholesterol 7α-hydroxylase activity in NZW and heterozygotes declined 69% and 53% ($P < 0.001$), respectively, after cholesterol feeding. Sterol 27-hydroxylase activity reflecting alternative bile acid synthesis increased 66% ($P < 0.01$) in NZW and 37% in Watanabe heterozygotes but not in homozygotes after feeding cholesterol. Bile fistula drainage stimulated cholesterol 7α-hydroxylase activity but not sterol 27-hydroxylase activity in all three rabbit groups. These results demonstrated that dietary cholesterol increased hepatic sterol 27-hydroxylase activity and alternative bile acid synthesis to expand the bile acid pool and inhibited cholesterol 7α-hydroxylase in NZW and in Watanabe heterozygous rabbits but not in homozygotes with absent hepatic LDL receptor function. Thus, in rabbits, sterol 27-hydroxylase is up-regulated by the increased hepatic cholesterol that enters the liver via LDL receptors whereas cholesterol 7α-hydroxylase is controlled by the circulating hepatic bile acid flux.—Xu, G., G. Salen, S. Shefer, G. S. Tint, L. B. Nguyen, T. T. Parker, T. S. Chen, J. Roberts, X. Kong, and D. Greenblatt. **Regulation of classic and alternative bile acid synthesis in hypercholesterolemic rabbits: effects of cholesterol feeding and bile acid depletion. *J. Lipid Res.* 1998.** 39: **1608–1615.**

Supplementary key words LDL receptor • bile fistula • cholesterol 7α-hydroxylase • cholic acid • HMG-CoA reductase

Cholesterol is the obligate precursor of bile acids in all mammalian species. According to current views (1, 2), the synthetic pathway is initiated by the formation of 7α-hydroxycholesterol that is catalyzed by the enzyme, cholesterol 7α-hydroxylase (EC 1.14.13.17). This enzyme is located in microsomes and the enzyme activity undergoes diurnal variation, and is sensitive to negative feedback control by the hepatic bile acid flux (3–5). Ring modifications that include 5β-reduction of the double bond, the addition of a 12α-hydroxy group, and epimerzation of the 3β-hydroxy to the 3α-position precede side chain cleavage in the classic pathway of cholic acid formation. Up until recently, this pathway was considered the almost exclusive mechanism for bile acid formation.

New Zealand white (NZW) rabbits are very sensitive to dietary cholesterol and accumulate large amounts in plasma after cholesterol feeding (6). Watanabe heritable hyperlipidemic (WHHL) rabbits with inherited deficiency of LDL receptors also show marked plasma hypercholesterolemia (7, 8). Recently, we described a marked inhibition of cholesterol 7α-hydroxylase in hypercholesterolemic cholesterol-fed NZW rabbits and in untreated WHHL rabbits (9). The effect of cholesterol feeding in the NZW rabbits was opposite to that seen in rats where dietary cholesterol up-regulated cholesterol 7α-hydroxylase (10–13) and prevented the rats from becoming hypercholesterolemic (10). Recently, Rudel et al. (14) reported that African Green monkeys fed cholesterol develop marked plasma hypercholesterolemia associated with elevated hepatic cholesterol levels and inhibited cholesterol 7α-hydroxylase. These investigators believed that increased hepatic cholesterol concentrations inhibited cholesterol 7α-hydroxylase and this mechanism was very sensitive to even small increments in liver cholesterol. In addition, Horton, Cuthbert, and Spady (15) and Pandak et al. (16)

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; WHHL, Watanabe heritable hyperlipidemic; NZW, New Zealand white.

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showed that cholesterol-fed hamsters also manifested plasma hypercholesterolemia with suppressed cholesterol 7α-hydroxylase activity and mRNA levels. The information from different animal species suggested that feeding cholesterol caused suppression of cholesterol 7α-hydroxylase and was associated with plasma hypercholesterolemia. However, a preliminary study in NZW rabbits suggested that the inhibition of cholesterol 7α-hydroxylase by cholesterol feeding was due to expansion of bile acid pool that resulted from increased alternative bile acid synthesis (17).

In addition to the classic pathway, bile acids may be synthesized via an alternative pathway (**Fig. 1**) that is initiated by the formation of 27-hydroxycholesterol from cholesterol catalyzed by mitochondrial sterol 27-hydroxylase (18, 19) which may be considered rate-controlling for this pathway. It is important to emphasize that the alternative pathway begins with the formation of 27-hydroxycholesterol and then is 7α-hydroxylated by microsomal oxysterol 7α-hydroxylase (27-hydroxycholesterol-7α-hydroxylase) which is independent and different from cholesterol 7α-hydroxylase, but also leads to the formation of cholic acid. This alternative pathway is also distinguished from the so-called acidic bile acid synthetic pathway (20, 21) which also begins with 27-hydroxycholesterol but the C-27 hydroxyl group is oxidized rapidly to a carboxylic acid, 3β-hydroxy-5-cholesten-27-oic acid. The latter compound does not form cholic acid (20, 21). The alternative pathway may not be quantitatively important in normal liver but may be essential for bile acid formation when cholesterol 7α-hydroxylase is inhibited. Schwarz et al. (22) recently reported that in mice that survived with disrupted cholesterol 7α-hydroxylase gene (knock out), the alternative bile acid synthetic pathway (via sterol 27-hydroxylase) was active. At this time, little is known about the regulation of sterol 27-hydroxylase in rabbits although Araya, Sjoberg, and Wikvall (23) in a preliminary study showed that sterol 27-hydroxylase activity did not respond to perturbations of the bile acid pool (cholic acid or cholestyramine feedings) that strongly modulated cholesterol 7α-hydroxylase activity and mRNA levels.

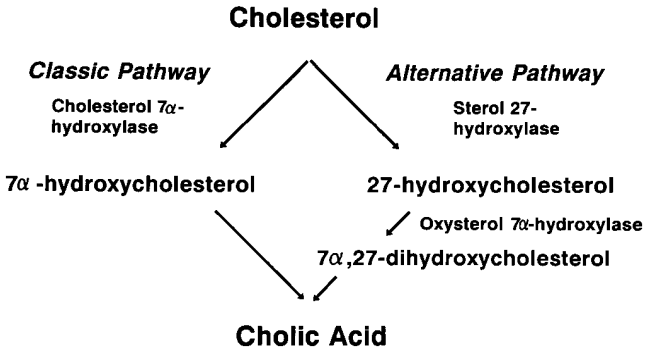

Fig. 1. A flow diagram illustrates two pathways for bile acid synthesis. Cholesterol serves as the sole substrate and is converted to cholic acid via both classic and alternative pathways. Cholesterol 7α-hydroxylase is the initial and rate-controlling enzyme for classic pathway while sterol 27-hydroxylase initiates the alternative pathway and may be rate-limiting. The product 27-hydroxycholesterol is then 7α-hydroxylated by microsomal oxysterol 7α-hydroxylase (27-hydroxycholesterol-7α-hydroxylase) which is different from cholesterol 7α-hydroxylase.

In this paper we have compared the regulation of cholesterol 7α-hydroxylase in the classic pathway and sterol 27-hydroxylase in the alternative pathway by feeding cholesterol and depleting the bile acid pool in normocholesterolemic New Zealand White and hypercholesterolemic WHHL rabbits who are partially (heterozygote) or completely (homozygote) deficient in low density lipoprotein (LDL) receptors. Our results indicated that at baseline, WHHL rabbits had smaller bile acid pool size and lower cholesterol 7α-hydroxylase activity in the liver. Feeding cholesterol induced sterol 27-hydroxylase activity and stimulated the alternative pathway for bile acid synthesis to double the bile acid pool size in both NZW and WHHL heterozygotes, but not homozygotes, with concomitant inhibition of microsomal cholesterol 7α-hydroxylase and classic bile acid synthesis. Depletion of the enlarged bile acid pool by bile drainage in all cholesterol-fed hypercholesterolemic rabbits stimulated cholesterol 7α-hydroxylase and classic bile acid synthesis with no change of sterol 27-hydroxylase activity but with markedly lowered plasma cholesterol levels.

MATERIALS AND METHODS

Materials

Cholesterol (cholest-5-en-3β-ol) and 5α-cholestane were obtained from Sigma Chemical Co., St. Louis, MO and used as standards for the measurements of sterols by capillary gas–liquid chromatography. Regular rabbit chow (Purina Mills Inc., St. Louis, MO) contained less than 0.001% w/w cholesterol. Rabbit chow containing 2% cholesterol was also prepared by Purina Mills Inc., St. Louis, MO. The chow diets all contained 0.57% (w/w) saturated fatty acids, 0.66% (w/w) monounsaturated fatty acids, and 0.97% (w/w) polyunsaturated fatty acids.

Animal experiments

The experiments were carried out in 20 NZW rabbits (Hazleton Labs, Denver, PA) weighing 2.5–3.2 kg, 16 WHHL heterozygotes and 20 homozygotes weighing 2.5–3.5 kg (from the Rogosin Institute, the New York Hospital-Cornell Medical Center, New York). Half of the rabbits (10 NZW rabbits, 8 WHHL heterozygotes, and 10 homozygotes) were fed Purina rabbit chow (Purina Mills Inc., St. Louis, MO). The other half of each group (10 NZW rabbits, 8 WHHL heterozygotes, and 10 homozygotes) was fed 2% cholesterol which was incorporated into the Purina chow diet so that 3 grams of cholesterol per day were fed for 10 days. Blood samples (2–3 ml) were taken from each rabbit immediately before and at the completion of the feeding period for determinations of plasma cholesterol concentrations and liver function tests (plasma alkaline phosphatase, glutamic-pyruvic acid transaminase, and bilirubin levels). After completion of 10 days feeding (chow containing 2% cholesterol or chow, respectively), bile fistulas were constructed in all rabbits under anesthesia (ketamine 35–40 mg/kg body weight combined with xylazine 3–4 mg/kg body weight intramuscularly). Bile was collected for 30 min. The total bile acid outputs (mg/h) measured from the 30-min bile samples represented the baseline hepatic bile acid flux. Half of the cholesterol-fed rabbits (5 NZW, 4 WHHL heterozygotes, and 5 homozygotes) and half of the chow-fed rabbits

(5 NZW, 4 WHHL heterozygotes, and 5 homozygotes) were killed. The livers were removed and portions were immediately frozen for measurements of activities of microsomal cholesterol 7α-hydroxylase and HMG-CoA reductase, and mitochondrial sterol 27-hydroxylase and for determination of hepatic cholesterol concentrations. A separate specimen of the liver was submitted for histologic examination. In the remaining half of the rabbits in each experimental group, bile drainage was continued for 7 days to collect bile without interruption for measurement of bile acid pool size and biliary cholic acid outputs which represented bile acid synthesis. Animals with 7 days bile fistula were given lactated Ringer's solution with 5% dextrose intravenously at 12 ml/h in the first 24 h but this was replaced by 0.9% NaCl after the animals started eating and drinking. This infusion was continued until the experiment was finished to replace the loss of body fluid. The rabbits were then killed and liver and blood were collected for the same biochemical measurements and histologic examinations mentioned above.

The animal protocol was approved by Subcommittee on Animal Studies at VA Medical Center, East Orange, NJ and Institutional Animal Care and Use Committee at UMD-New Jersey Medical School, Newark, NJ.

Bile acid pool size

The total bile acid pool sizes were calculated from measurements of the total recovered deoxycholic acid in the bile collected during bile drainage divided by the percentage of deoxycholic acid in the initial bile collected during the first 30 min after the construction of the bile fistula.

Chemical analysis

Assays for sterols. Bile (100 μl) dissolved in 5 ml H_2O with 100 μg glycoursocholic acid added as a recovery standard was deproteinized by passage through a C_{18} Sep-Pak cartridge (1 cc, Waters Chromatography, Milford, MA) which was prewashed with 5 ml methanol and 5 ml H_2O. The bile acid conjugates were then eluted from the Sep-Pak with methanol. The solution was evaporated and the residue was dissolved in 5 ml of 0.02 m acetate buffer, pH 5.6. The bile acids were deconjugated with 30 mg of cholylglycine hydrolase (Sigma Chemical Co., St. Louis, MO) in the presence of 2-mercaptoethanol and EDTA. After overnight incubation at 37°C, the solution was passed through a C_{18} Sep-Pak cartridge prewashed with 5 ml methanol and 5 ml H_2O, then eluted with methanol. The recovered free bile acids were methylated with 3 N methanolic HCl. After the solution was evaporated, trimethylsilyl ether derivatives of the bile acids methylesters were prepared by adding Sil-Prep (a mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane from Alltech, Deerfield, IL). Bile acids were quantitated by capillary gas chromatography (Hewlett-Packard 5890A, Palo Alto, CA) equipped with a (0.25 mm i.d.) 25-m fused silica CP-Sil 5-CB capillary column (24).

Neutral sterols were extracted with hexane from 1 ml plasma or 1 g (wet) pieces of liver after saponification in 1 N ethanolic NaOH. Trimethylsilyl ether derivatives were prepared and quantitated by capillary gas–liquid chromatography as described previously (25). The retention times of the sterol trimethylsilyl ether derivatives relative to the internal standard, 5α-cholestane (retention time 14.62 min) were: cholesterol, 1.40; cholestanol, 1.42; and sitosterol, 1.81.

Hepatic microsomal cholesterol 7α-hydroxylase and HMG-CoA reductase activities. Hepatic microsomes were prepared by differential ultracentrifugation (26), and the protein was determined according to Lowry et al. (27). The assay for HMG-CoA reductase activity was based on the methods of George et al. (28) and Nguyen et al. (29). Briefly, 50–200 μg of microsomal protein was preincubated at 37°C for 5 min in a final volume of 150 μl buffer (50 mm K_2HPO_4, 30 mm EDTA, 10 mm dithiothreitol, 70 mm KCl, pH 7.4) containing an NADPH generating system (34 mm $NADP^+$, 30 mm glucose-6-phosphate, 0.3 U glucose-6-phosphate dehydrogenase) and [^{3}H]mevalonolactone (40,000 dpm) as internal recovery standard. The reaction was started with the addition of 30 nmol [3-^{14}C]HMG-CoA (Amersham Corp., Arlington Heights, IL; sp act, 30 dpm/pmol) and stopped after 15 min at 37°C with the addition of 20 μl 6 N HCl. Zero-time controls were run with each experiment. After lactonization at 37°C for 30 min, the products were separated by thin-layer chromatography, and mevalonolactone was quantitated by liquid scintillation counting (29).

Cholesterol 7α-hydroxylase activity was measured in hepatic microsomes after removal of endogenous lipid by acetone and reconstituting the microsomal protein with cholesterol and optimum amounts of co-factors by the isotope incorporation method of Shefer et al. (26, 30).

Hepatic mitochondrial sterol 27-hydroxylase activity. The method for measurement of sterol 27-hydroxylase activity was the same as previously described by Shefer et al. (31). Hepatic mitochondria were prepared by differential ultracentrifugation (26), and the protein was determined according to Lowry et al. (27). Mitochondrial sterol 27-hydroxylase activity was assayed by an isotope incorporation method. Standard incubation mixtures contained in a volume of 0.5 ml: 100 mm phosphate buffer, pH 7.4; 0.1 mm EDTA; 5 mm DTT (dithiothreitol); 300 μm [4-^{14}C]cholesterol solubilized in 20 μl 2-hydroxypropyl-β-cyclodextrin (0.45% Trappsol, Pharmatec Inc., Alachua, FL); and 50–150 μg mitochondrial protein. The reaction was initiated by the addition of the NADPH generating system containing 2.5 μmol d,l-trisodium isocitrate, 0.1 IU isocitrate dehydrogenase, and 0.6 μmol NADPH, incubated at 37°C for 15 min and stopped with 100 μl aqueous NaOH (50%). The reaction product (27-hydroxycholesterol) was extracted with ethyl acetate and the extract was washed to neutral pH. 27-Hydroxycholesterol was then separated from the substrate (cholesterol) by thin-layer chromatography on silica gel plates (Silica Gel 60, EM Science, Gibbstown, NJ) with hexane–ethyl acetate 1:1 (v/v). The spots corresponding to the individual sterols (27-hydroxycholesterol, R_f 0.56, and cholesterol, R_f 0.81) were removed from the plate and their radioactivity was determined by liquid scintillation spectroscopy using Ecolume (ICN Radiochemicals, Irvine, CA). Proof of the radiopurity of 27-hydroxycholesterol was based on crystallization of the product to constant specific activity with authentic 27-hydroxycholesterol. The mass of 27-hydroxycholesterol was calculated from the recovered radioactivity divided by the initial specific activity of the substrate.

Receptor-mediated LDL binding. LDL ($1.019 < d < 1.063$) was isolated from human venous blood of healthy voluntary donors by differential ultracentrifugation (32) and labeled with ^{125}I (New England Nuclear, Boston, MA) by the iodine monohydrochloride method (33). Preliminary experiments showed that rabbit liver membrane bound LDL from a human source as sufficiently as LDL separated from NZW rabbits. Receptor-mediated LDL binding to rabbit liver menbranes was assayed by methods described previously by Nguyen et al. (29) and Kovanen, Brown, and Goldstein (34). Receptor-mediated LDL binding to the liver membranes was determined as the difference between total binding of ^{125}I-labeled LDL (assayed in the absence of unlabeled LDL) and nonspecific binding (assayed in the presence of 40-fold excess unlabeled LDL).

Statistical study

Data are shown as means ± SD and were compared statistically by the Student's *t* test (unpaired). The BMDP Statistical Software (BMDP Statistical Software, Inc, Los Angeles, CA) was used for statistical evaluations.

TABLE 1. Plasma cholesterol levels in NZW and WHHL rabbits

	NZW	WHHL Heterozygote	WHHL Homozygote
		mg/dl	
Control	33 ± 5	80 ± 16	620 ± 157
Ch	871 ± 259[a]	1168 ± 272[a]	1555 ± 573[b]
Ch + BF	530 ± 165[a]	534 ± 205[b]	646 ± 217
BF	25 ± 6	54 ± 17	425 ± 108[c]

Data are shown as mean ± SD. Control: chow fed for 10 days; Ch: 2% cholesterol in chow fed for 10 days. BF: chow-fed rabbits with bile fistula for 7 days. Ch + BF: 2% cholesterol in chow for 10 days followed by 7 days of bile fistula.

[a] $P < 0.001$ as compared to the control.
[b] $P < 0.01$ as compared to the control.
[c] $P < 0.05$ as compared to the control.

RESULTS

Plasma cholesterol levels

In **Table 1** are listed plasma cholesterol concentrations. As expected, plasma cholesterol was 2.4 times higher in WHHL heterozygotes and 18.8 times higher in WHHL homozygotes than in NZW rabbits fed regular rabbit chow. Feeding 2% cholesterol (3 g/day) for 10 days increased plasma levels 26-fold in NZW, 14.6-fold in heterozygotes but only 2.5-fold in homozygotes. After bile drainage for 7 days, plasma cholesterol declined 39% ($P < 0.05$) in the NZW, 54% ($P < 0.01$) in the heterozygotes, and 58% ($P < 0.05$) in the homozygotes. For comparison, depletion of the bile acid pool in chow-fed rabbits reduced plasma cholesterol 24% in NZW, 32% in heterozygotes, and 31% ($P < 0.05$) in homozygotes.

Hepatic cholesterol levels

In **Table 2** are listed hepatic cholesterol concentrations. Despite enormous plasma differences, hepatic cholesterol levels at baseline were similar in all three groups. After 10 days cholesterol feeding, hepatic cholesterol levels rose 12-fold in NZW, 8.4-fold in WHHL heterozygotes but only 3.2-fold in the homozygotes. As a result, hepatic cholesterol was 2.6-fold ($P < 0.001$) higher in NZW and 2-fold ($P < 0.01$) higher in heterozygotes than homozygotes. After 7 days of bile drainage in the cholesterol-fed rabbits, hepatic cholesterol declined 76% ($P < 0.001$) in NZW, 34% ($P < 0.05$) in heterozygotes, and 29% ($P < 0.05$) in homozygotes. In contrast, hepatic cholesterol did not decline significantly after bile acid depletion in chow-fed rabbits.

TABLE 2. Hepatic cholesterol concentrations in NZW and WHHL rabbits

	NZW	WHHL Heterozygote	WHHL Homozygote
		mg/g	
Control	1.8 ± 0.5	2.0 ± 0.1	2.6 ± 0.3
Cholesterol	22 ± 4[a]	16.8 ± 3.2[a]	8.4 ± 1.7[a]
Ch + BF	5.3 ± 3.0[c]	11.0 ± 3.2[b]	6.0 ± 1.5[b]
BF	1.3 ± 0.1	2.3 ± 0.2	2.2 ± 0.4

Data are shown as mean ± SD. Control: chow fed for 10 days; Ch: 2% cholesterol in chow fed for 10 days. BF: chow-fed rabbits with bile fistula for 7 days. Ch + BF: 2% cholesterol in chow for 10 days followed by 7 days of bile fistula.

[a] $P < 0.001$ as compared to the control.
[b] $P < 0.01$ as compared to the control.
[c] $P < 0.05$ as compared to the control.

Bile acid pool sizes

Bile acid pool sizes were measured in chow-fed and chow plus cholesterol-fed rabbits (**Fig 2**). The calculation was based on the observation that more than 80% of rabbit biliary bile acids consists of deoxycholic acid formed by bacterial 7α-dehydroxylation of cholic acid in the intestine. Therefore, the total recovered deoxycholic acid divided by the percentage deoxycholic acid in the initial hepatic bile specimen collected during the first 30 min after construction of the fistula gives the size of total bile acid pool.

At baseline, the bile acid pool was 83% ($P < 0.01$) larger in NZW (254 ± 44 mg) than WHHL heterozygotes (139 ± 3 mg) and 100% ($P < 0.001$) larger than homozygotes (124 ± 30 mg). After feeding cholesterol for 10 days, the bile acid pool increased 110% in NZW rabbits (533 ± 51 mg, $P < 0.001$) and 123% in WHHL heterozygotes (310 ± 14 mg, $P < 0.001$) but only 35% (P = NS) in homozygotes (168 ± 49 mg). However, the bile acid pool in cholesterol-fed NZW rabbits was still 72% ($P < 0.0001$) larger than WHHL heterozygotes and 3 times ($P < 0.0001$) greater than the homozygotes. After cholesterol feeding, the bile acid pool in WHHL heterozygotes was also significantly ($P < 0.001$) larger than in the homozygotes.

Biliary cholic acid output

After feeding cholesterol, biliary cholic acid outputs increased significantly in NZW and moderately in WHHL heterozygous rabbits but did not change in the homozygotes as compared with the baseline cholic acid outputs in rabbits fed only chow diets (**Fig. 3**). In addition, after cholesterol feeding, biliary cholic acid outputs increased more in NZW rabbits than in WHHL heterozygotes.

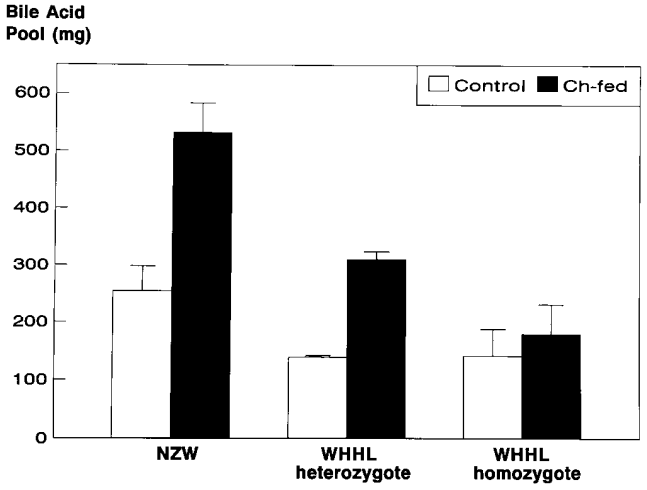

Fig. 2. Effect of cholesterol feeding on bile acid pool size. The open bars represent the bile acid pool in chow-fed rabbits while the black bars represent the bile acid pool in 2% cholesterol-fed rabbits.

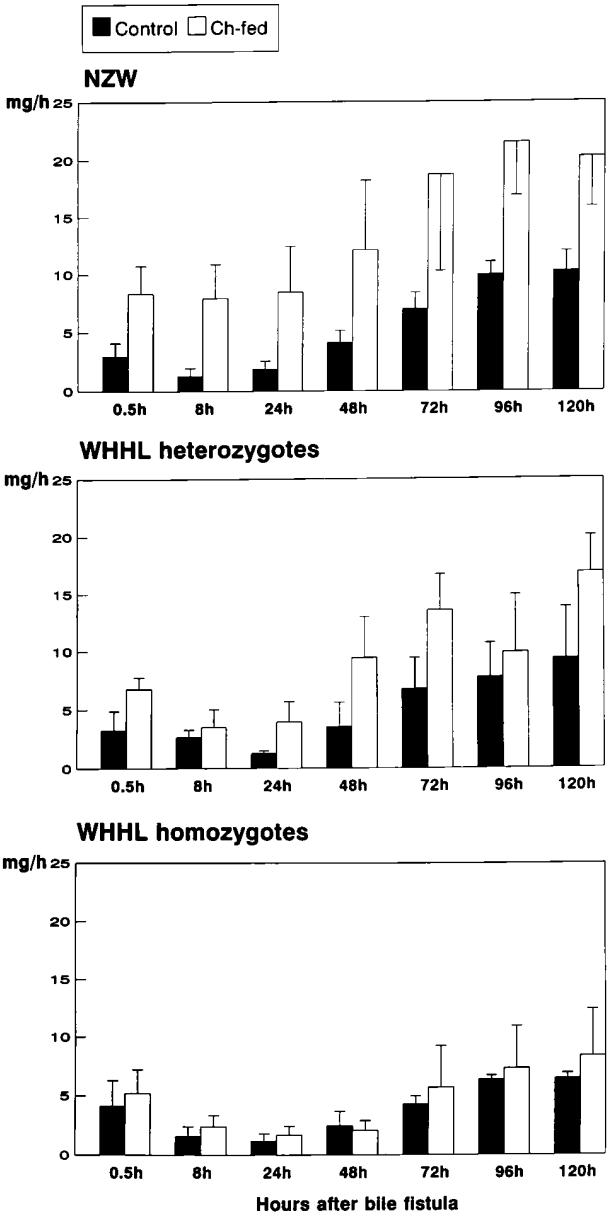

Fig. 3. Effect of 2% cholesterol feeding on biliary cholic acid output (mg/h) in bile fistula NZW and WHHL rabbits. After feeding cholesterol, biliary cholic acid outputs increased significantly in NZW and moderately in WHHL heterozygous rabbits but not in the homozygotes as compared with baseline cholic acid outputs in rabbits fed only chow.

Hepatic microsomal cholesterol 7α-hydroxylase activity

As noted previously, microsomal cholesterol 7α-hydroxylase activity was 3.8-fold higher ($P < 0.001$) in NZW than WHHL homozygotes at baseline; heterozygotes showed intermediate levels of enzyme activity (**Table 3**). After cholesterol feeding, cholesterol 7α-hydroxylase activity declined 69% ($P < 0.001$) in NZW and 53% ($P < 0.001$) in WHHL heterozygotes but did not significantly decrease in the homozygotes. In the cholesterol-fed NZW rabbits, 7 days of bile drainage completely depleted the bile acid pool which stimulated cholesterol 7α-hydroxylase activity

TABLE 3. Hepatic cholesterol 7α-hydroxylase activity in rabbits

	NZW	WHHL Heterozygote	WHHL Homozygote
	pmol/mg protein/min		
Control	50.2 ± 12.3	33.8 ± 6.8	13.0 ± 5.0
Cholesterol	15.7 ± 5.0[a]	15.8 ± 3.9[a]	9.5 ± 0.6
Ch + BF	179.4 ± 68.5[b]	35.2 ± 6.8	38.0 ± 9.1[a]
BF	131.6 ± 25.1[a]	93.4 ± 7.6[a]	84.9 ± 13.9[a]

Data are shown as mean ± SD. Control: chow fed for 10 days; Ch: 2% cholesterol in chow fed for 10 days. BF: chow-fed rabbits with bile fistula for 7 days. Ch + BF: 2% cholesterol in chow for 10 days followed by 7 days of bile fistula.

[a] $P < 0.001$ as compared to the control.

[b] $P < 0.01$ as compared to the control.

11.4-fold, that was 3.5-fold higher than baseline value in the chow-fed rabbits. Similarly, cholesterol 7α-hydroxylase activity was increased 2-fold ($P < 0.001$) in WHHL heterozygotes and 4-fold ($P < 0.001$) in homozygotes after bile drainage eliminated the bile acid pool in the cholesterol-fed rabbits. In comparison, in the chow-fed rabbits, depleting the bile acid pool increased cholesterol 7α-hydroxylase activity 2.6-fold ($P < 0.001$) in NZW, 2.8-fold ($P < 0.0001$) in WHHL heterozygotes, and 6.5-fold ($P < 0.0001$) in homozygotes.

Hepatic mitochondrial sterol 27-hydroxylase activity

Mitochondrial sterol 27-hydroxylase catalyzes the formation of 27-hydroxycholesterol, the first committed precursor in the alternative bile acid synthetic pathway (Fig. 1). At baseline in NZW rabbits, sterol 27-hydroxylase activity was 42% less than cholesterol 7α-hydroxylase activity, while sterol 27-hydroxylase activity in WHHL heterozygotes was 47% less than in NZW rabbits with similar levels in homozygotes (**Table 4**). After cholesterol feeding, sterol 27-hydroxylase activity increased 66% ($P < 0.05$) in NZW, 37% in WHHL heterozygotes (P = NS), and 29% in homozygotes (P = NS). In contrast to cholesterol 7α-hydroxylase, elimination of the bile acid pool in these cholesterol-fed rabbits did not significantly change hepatic sterol 27-hydroxylase activity in either NZW or WHHL heterozygotes and homozygotes. Similarly, in chow-fed rabbits, bile drainage produced no significant change in sterol 27-hydroxylase activity.

TABLE 4. Hepatic sterol 27-hydroxylase activity in rabbits

	NZW	WHHL Heterozygote	WHHL Homozygote
	pmol/mg protein/min		
Control	29.0 ± 5.5	17.9 ± 5.7	23.0 ± 4.5
Cholesterol	48.1 ± 14.0[a]	24.5 ± 5.8	29.7 ± 4.4
Ch + BF	38.6 ± 10.4	24.4 ± 3.7	40.7 ± 13.2[a]
BF	24.0 ± 9.9	18.0 ± 2.9	33.8 ± 9.8

Data are shown as mean ± SD. Control: chow fed for 10 days; Ch: 2% cholesterol in chow fed for 10 days. BF: chow-fed rabbits with bile fistula for 7 days. Ch + BF: 2% cholesterol in chow for 10 days followed by 7 days of bile fistula.

[a] $P < 0.05$ as compared to the control.

TABLE 5. Hepatic HMG-CoA reductase activity in rabbits

	NZW	WHHL Heterozygote	WHHL Homozygote
	pmol / mg protein / min		
Control	34.0 ± 11.5	47.4 ± 5.8	47.2 ± 9.4
Cholesterol	9.4 ± 3.9[b]	7.3 ± 1.7[a]	9.2 ± 3.8[a]
Ch + BF	87.8 ± 29.4[b]	18.8 ± 6.8[a]	118.7 ± 16.8[a]
BF	205.6 ± 55.8[a]	337.8 ± 41.4[a]	366.7 ± 62.6[a]

Data are shown as mean ± SD. Control: chow fed for 10 days; Ch: 2% cholesterol in chow fed for 10 days. BF: chow-fed rabbits with bile fistula for 7 days. Ch + BF: 2% cholesterol in chow for 10 days followed by 7 days of bile fistula.

[a] $P < 0.001$ as compared to the control.

[b] $P < 0.01$ as compared to the control.

HMG-CoA reductase activity

Microsomal HMG-CoA reductase activity was similar at baseline in the three rabbit groups despite the marked differences in plasma cholesterol concentrations. As expected, HMG-CoA reductase activity declined substantially in all three rabbit groups after 10 days of cholesterol feeding. Removal of the bile acid pool by 7 days of bile drainage, strongly up-regulated HMG-CoA reductase activity in both the chow and chow plus cholesterol-fed rabbits although plasma and liver cholesterol concentrations were still significantly higher in the later group than the baseline values (**Table 5**).

Hepatic receptor-mediated LDL binding

At baseline, receptor-mediated LDL binding in WHHL heterozygotes and homozygotes was only 23.6% ($P < 0.001$) and 11% ($P < 0.001$) respectively, as high as the value in NZW rabbits (**Table 6**). After feeding cholesterol, LDL receptor-mediated binding was reduced 58% ($P < 0.01$) in NZW rabbits but insignificantly in WHHL heterozygotes and homozygotes. In the cholesterol-fed rabbits, 7 days of bile drainage increased the LDL receptor-mediated binding 4-fold ($P < 0.01$) in NZW rabbits, which was even 66% ($P < 0.05$) higher than the baseline value. In WHHL heterozygotes and homozygotes, LDL receptor-mediated binding returned to the baseline value. In chow-fed NZW rabbits, 7 days of bile drainage raised hepatic LDL receptor-mediated binding 85% ($P < 0.01$) as compared with the baseline value but did not change hepatic LDL receptor-mediated binding in either WHHL heterozygotes or homozygotes.

TABLE 6. Receptor-mediated LDL binding to rabbit liver membranes

	NZW	WHHL Heterozygote	WHHL Homozygote
	ng / mg protein		
Control	160 ± 14	38 ± 35	18 ± 31
Cholesterol	67 ± 39[a]	17 ± 22	10 ± 24
Ch + BF	266 ± 86[b]	37 ± 13	16 ± 31
BF	296 ± 87[a]	44 ± 26	11 ± 34

Data are shown as mean ± SD. Control: chow fed for 10 days; Ch: 2% cholesterol in chow fed for 10 days. BF: chow-fed rabbits with bile fistula for 7 days. Ch + BF: 2% cholesterol in chow for 10 days followed by 7 days of bile fistula.

[a] $P < 0.01$ as compared to the control.

[b] $P < 0.05$ as compared to the control.

Liver function tests and histology examination

Plasma liver function tests showed that plasma alkaline phosphatase, glutamic-pyruvic acid transaminase, and bilirubin were all within the normal range in both the cholesterol-fed and control rabbits and after 7 days of bile drainage. Liver histology showed only increased deposition of fat in the cholesterol-fed rabbits without hepatocyte damage, inflammation, or disruption of the architecture.

DISCUSSION

The results of this investigation extend our understanding of the effect of cholesterol feeding on bile acid metabolism in normocholesterolemic NZW rabbits and hypercholesterolemic receptor-deficient WHHL homozygotes and heterozygotes. Not only were baseline bile acid pool sizes much lower in the WHHL homozygotes and heterozygotes as compared to NZW rabbits, but the reduced bile acid pool sizes in the hypercholesterolemic WHHL rabbits could be related, in part, to down-regulated cholesterol 7α-hydroxylase activity and reduced bile acid synthesis via the classic pathway. Moreover, after cholesterol feeding, bile acid pool sizes doubled in the NZW (with normal hepatic LDL receptor function) and WHHL heterozygotes (with partial hepatic LDL receptor function) which resulted in further inhibition of cholesterol 7α-hydroxylase activity to the depressed levels found at baseline in the WHHL homozygotes. Interestingly, neither the bile acid pool size nor already reduced cholesterol 7α-hydroxylase activity changed further after cholesterol feeding in the WHHL homozygotes where hepatic LDL receptors were almost nil.

In this study, we demonstrated that the accumulation of hepatic cholesterol, via LDL receptors after cholesterol feeding, stimulated sterol 27-hydroxylase and alternative bile acid synthesis that increased the bile acid pool in NZW rabbits with normal LDL receptors and also moderately in WHHL heterozygotes with partially deficient LDL receptor function. In contrast, WHHL homozygotes that showed almost absent LDL receptor function did not respond to cholesterol feeding by an increase in either sterol 27-hydroxylase activity or bile acid pool size. Therefore, we believe that the effect of dietary cholesterol on bile acid synthesis mentioned above is mediated by hepatic cholesterol uptake via LDL receptors. However, we are not certain whether the accumulation of dietary cholesterol in the liver would simply stimulate sterol 27-hydroxylase and alternative bile acid synthesis. After cholesterol feeding, hepatic cholesterol concentrations in WHHL homozygotes were much lower than those in NZW rabbits and WHHL heterozygotes, but still increased 3-fold ($P < 0.01$) while sterol 27-hydroxylase activity did not rise nor did synthesis of cholic acid and bile acid pool size increase. The biliary cholic acid outputs that reflected bile acid synthesis in the cholesterol-fed rabbits after bile drainage increased significantly only in NZW rabbits

and moderately in WHHL heterozygotes but not in homozygotes. In addition, the increase of biliary cholic acid outputs and expansion of bile acid pool size were much greater in NZW rabbits than in WHHL heterozygotes. We hypothesize that cholesterol that enters the liver via non-LDL receptor pathways may not be available for alternative bile acid synthesis, whereas only cholesterol taken up via LDL receptors could be delivered to the compartment where alternative bile acid synthesis is initiated. One must notice that after cholesterol feeding, the increased hepatic cholesterol in the homozygotes was accumulated via non-LDL receptor pathway and therefore, had no effect on alternative bile acid synthesis and sterol 27-hydroxylase. Although hepatic cholesterol increased significantly more in the heterozygotes, because of partial deficiency of LDL receptor function, the entry of cholesterol through LDL receptors was limited. As a result, although bile acid synthesis and sterol 27-hydroxylase activity increased in the cholesterol-fed heterozygotes, the rise was still not significant.

In the cholesterol-fed NZW and WHHL heterozygous rabbits, the expansion of the bile acid pool occurred because of increased cholic acid synthesis via the alternative bile acid synthetic pathway as evidenced by both increased sterol 27-hydroxylase activity and biliary cholic acid outputs with inhibited cholesterol 7α-hydroxylase activity. The first reaction in the alternative pathway involves the 27-hydroxylation of cholesterol that is catalyzed by the mitochondrial enzyme, sterol 27-hydroxylase. The next step is 7α-hydroxylation carried out by a microsomal enzyme, oxysterol 7α-hydroxylase, which is different from cholesterol 7α-hydroxylase to yield 7α, 27-dihydroxycholesterol. The remaining steps and relevant enzymes have not been completely characterized. However, it is clear that in these rabbits, 12α-hydroxylation occurred as cholic acid was still the major product of the increased bile acid output (synthesis) even though cholesterol 7α-hydroxylase and classic bile acid synthesis were inhibited after cholesterol feeding. Thus, unlike the acidic synthetic pathway, where a carboxylic acid on the side chain prevents 12α-hydroxylation and further formation of cholic acid, the alternative synthetic pathway which begins with formation of 27-hydroxycholesterol does not limit 12α-hydroxylation and cholic acid formation. What is important is that both the classic and alternative pathways co-exist but are under different feedback regulation as seen in the cholesterol-fed rabbits.

In response to cholesterol feeding, sterol 27-hydroxylase activity rose 66% in NZW rabbits and 33% (P = NS) in WHHL heterozygotes with increased formation of cholic acid to expand the bile acid pool 2-fold in both groups. Thus, sterol 27-hydroxylase activity and alternative bile acid synthesis were up-regulated by cholesterol feeding. It has been suggested (35) that availability of cholesterol in the inner mitochondrial membrane where sterol 27-hydroxylase is located may limit synthesis of 27-hydroxycholesterol. Increasing cholesterol feeding may activate certain intracellular pathways that correlate with LDL receptors and deliver cholesterol to the mitochondria in hepatocytes. Importantly, in the cholesterol-fed rabbits, removal of the bile acid pool by biliary drainage through a fistula stimulated suppressed cholesterol 7α-hydroxylase. As a result, total bile acid synthesis was greater than that at baseline in normocholesterolemic chow-fed NZW rabbits because of increased cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities as well as the greater supply of cholesterol substrate in the liver. Clearly, the enlarged bile acid pool with cholesterol feeding suppressed cholesterol 7α-hydroxylase activity while removal of the pool up-regulated cholesterol 7α-hydroxylase activity in all three rabbit groups whether fed cholesterol or not. Thus, cholesterol 7α-hydroxylase is exquisitely sensitive to the circulating bile acid pool and hepatic bile acid flux. In contrast, sterol 27-hydroxylase was stimulated by cholesterol feeding but was not affected when the bile acid pool was either expanded or removed. Similar results were reported by Araya et al. (23) who showed that in rabbits, cholesterol 7α-hydroxylase was sensitive to bile acid feeding while sterol 27-hydroxylase activity was not regulated by the bile acid flux.

It is noteworthy that rats and rabbits respond differently to cholesterol. Rats fed cholesterol show increased cholesterol 7α-hydroxylase activity and classic bile acid synthesis. Sterol 27-hydroxylase activity does not play a role and the bile acid pool does not expand. It had been suggested that excess cholesterol in the intestine may interfere with the reabsorption of intestinal bile acids (36). Clearly, this is not the case in rabbits as cholesterol 7α-hydroxylase was inhibited after cholesterol feeding (9). Thus, the ileal apical Na^+-dependent bile acid transporter may remain active and the bile acid pool is conserved and even expanded when bile acid syntheis via the alternative pathway is stimulated in the cholesterol-fed rabbits. Not only does this limit the elimination of cholesterol by inhibiting classic bile acid synthesis catalyzed by cholesterol 7α-hydroxylase but the expansion of the bile acid pool may be responsible for the liver damage often observed in long term cholesterol-fed rabbits (37, 38). Retention of the bile acids in the liver exposes hepatocyte lipid membranes to the strong lipid solubilizing (detergent) effects of bile acids with ensuing damage to membranes. However, in the cholesterol-fed rabbits of this study, no toxic or histological changes were noted in the liver and liver function tests were not abnormal during the 10-day treatment periods.

An important consideration concerns the effect of dietary cholesterol in humans. Some individuals respond to dietary cholesterol similar to rats to up-regulate classic bile acid synthesis and cholesterol 7α-hydroxylase (39) so that excess cholesterol is eliminated and plasma levels and the risk of atherosclerosis are not increased. Alternatively, others respond to dietary cholesterol similar to rabbits where absorbed cholesterol reaches the liver and may stimulate sterol 27-hydroxylase and alternative bile acid synthesis so that the bile acid pool enlarges to inhibit cholesterol 7α-hydroxylase and classic bile acid synthesis. One consequence of the larger bile acid pool is to facilitate further absorption of cholesterol, which combined with reduced classic bile acid synthesis increases plasma and tissue cholesterol levels to raise the risk for atherosclerosis.

In summary, cholesterol feeding stimulated sterol 27-hydroxylase activity and alternative bile acid synthesis that expanded the bile acid pool and down-regulated cholesterol 7α-hydroxylase in NZW and WHHL heterozygotes.

WHHL homozygotes showed inhibited baseline cholesterol 7α-hydroxylase and small bile acid pool sizes and did not respond to cholesterol feeding with increased sterol 27-hydroxylase activity perhaps because of deficient LDL receptor function that diminishes hepatic cholesterol uptake via LDL receptors. We postulate that only cholesterol taken up through hepatic LDL receptors is used as substrate for alternative bile acid synthesis.

This study was supported by VA Research Service and US Public Health Service Grants HL 17818, HL 18094, DK 26756. We also thank Bibiana Pcolinsky, Susan Hauser, and Eva Paroulek for their excellent technical assistance and Barbara Rouse for preparing the manuscript.

Manuscript received 9 February 1998 and in revised form 9 April 1998.

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