Increasing dietary cholesterol induces different regulation of classic and alternative bile acid synthesis

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We investigated the effect of increasing dietary cholesterol on bile acid pool sizes and the regulation of the two bile acid synthetic pathways (classic, via cholesterol 7α-hydroxylase, and alternative, via sterol 27-hydroxylase) in New Zealand white rabbits fed 3 g cholesterol/per day for up to 15 days. Feeding cholesterol for one day increased hepatic cholesterol 75% and cholesterol 7α-hydroxylase activity 1.6 times without significant change of bile acid pool size or sterol 27-hydroxylase activity. After three days of cholesterol feeding, the bile acid pool size increased 83% (P < 0.01), and further feeding produced 10%–20% increments, whereas cholesterol 7α-hydroxylase activity declined progressively to 60% below baseline. In contrast, sterol 27-hydroxylase activity rose 58% after three days of cholesterol feeding and remained elevated with continued intake. Bile drainage depleted the bile acid pool and stimulated down-regulated cholesterol 7α-hydroxylase activity but did not affect sterol 27-hydroxylase activity. Thus, increasing hepatic cholesterol does not directly inhibit cholesterol 7α-hydroxylase and initially favors enzyme induction, whereas increased bile acid pool is the most powerful inhibitor of cholesterol 7α-hydroxylase. Sterol 27-hydroxylase is insensitive to the bile acid flux but is upregulated by increasing hepatic cholesterol.


Introduction

Cholesterol is the obligatory precursor of bile acids in all mammals. Until recently, virtually all bile acids were considered to be produced via a single pathway. The first reaction, the conversion of cholesterol to 7α-hydroxycholesterol, is catalyzed by the microsomal enzyme choles terol 7α-hydroxylase (EC 1.14.13.17) and is considered rate controlling (1, 2). This bile acid synthetic pathway is designated as the classic pathway. The hepatic bile acid flux exerts negative feedback control on cholesterol 7α-hydroxylase (3–5) so that depletion of the bile acid pool upregulates, and feeding bile acid inhibits, cholesterol 7α-hydroxylase and bile acid synthesis via the classic pathway. Recently, it has been shown (6, 7) that bile acids may be synthesized via an alternative pathway (Fig. 1). The initial reaction is the formation of 27-hydroxycholesterol from cholesterol catalyzed by mitochondrial sterol 27-hydroxylase, which may be considered a rate-controlling reaction for this pathway. The alternative pathway begins with the formation of 27-hydroxycholesterol that is then 7α-hydroxylated by microsomal oxysterol 7α-hydroxylase (27-hydroxysterol-7α-hydroxylase), an enzyme that is independent and different from cholesterol 7α-hydroxylase, but both pathways lead to the formation of cholic acid. Information about the regulation of sterol 27-hydroxylase and alternative bile acid synthesis is limited and controversial. Vlahcevic et al. (8) reported that in rats, malabsorption of bile acid produced by cholestyramine stimulated, and bile acid feeding inhibited, hepatic sterol 27-hydroxylase activity. Araya et al. (9) reported that in rabbits, sterol 27-hydroxylase activity did not respond to cholic acid feeding or bile acid malabsorption by cholestyramine treatment that modulated strongly cholesterol 7α-hydroxylation activity and mRNA levels.

The role of cholesterol in classic bile acid synthesis is less well understood, although studies in rats suggest that feeding cholesterol upregulates cholesterol 7α-hydroxylase and increases bile acid formation (10–13). These results may relate in part to the greater availability of substrate (cholesterol), or to the idea put forward by Björkhem et al. (14) that increased intestinal cholesterol interferes with the reabsorption of bile acids and reduces their return to the liver. However, the effect of cholesterol feeding on bile acid synthesis in New Zealand white (NZW) rabbits was opposite that seen in rats. We reported recently (15) that feeding cholesterol to NZW rabbits unexpectedly inhibited cholesterol 7α-hydroxylase activity and mRNA levels. Similarly, it has been reported that cholesterol fed to African green monkeys (16) or hamsters (17, 18) resulted in inhibited cholesterol 7α-hydroxylase activity and mRNA levels. Rudel et al. (16) suggested that increased hepatic cholesterol concentrations might inhibit cholesterol 7α-hydroxylase, and this mechanism was very sensitive to even small increments in liver cholesterol. However, our preliminary study in NZW rabbits (19) suggested that the suppression of cholesterol 7α-hydroxylase by cholesterol feeding was related to expansion of the bile
Figure 1
Flow diagram illustrating two pathways for bile acid synthesis. Cholesterol serves as the sole substrate and is converted to cholic acid via both classic and alternative pathways. Microsomal cholesterol 7α-hydroxylase is the initial and rate-controlling enzyme for the classic pathway, whereas mitochondrial sterol 27-hydroxylase initiates the alternative pathway and might be rate limiting. The product, 27-hydroxycholesterol, is then 7α-hydroxylated by microsomal oxysterol 7α-hydroxylase (27-hydroxycholesterol-7α-hydroxylase), which is different from microsomal cholesterol 7α-hydroxylase.

In this report, hepatic cholesterol 7α-hydroxylase, sterol 27-hydroxylase, and β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase activities were correlated with the size change in the bile acid pool and hepatic cholesterol levels in NZW rabbits fed increasing amounts of cholesterol. We proposed to test the hypotheses that (a) increasing dietary cholesterol intake does not directly inhibit cholesterol 7α-hydroxylase activity but expands the bile acid pool via increased alternative bile acid synthesis; (b) elevated hepatic cholesterol stimulates sterol 27-hydroxylase activity that is insensitive to feedback regulation by an enlarged or depleted bile acid pool; and (c) cholesterol 7α-hydroxylase is inversely regulated by the hepatic bile acid flux.

Methods
Cholesterol (cholest-5-en-3β-ol) and 5α-cholestane were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA) and used as standards for the measurements of sterols by capillary gas-liquid chromatography. Regular rabbit chow (Purina Mills Inc., St. Louis, Missouri, USA) contained less than 0.001% wt/wt cholesterol. Rabbit Chow containing 2% cholesterol was also prepared by Purina Mills. The chow diets all contained 0.57% wt/wt cholesterol. Rabbit chow containing 2% cholesterol was fed standard Purina rabbit chow (Purina Mills Inc., St. Louis, Missouri, USA) contained less than 0.001% wt/wt cholesterol. We proposed to test the hypotheses that (a) effects of increasing cholesterol feeding on plasma cholesterol concentrations and liver function tests (plasma alkaline phosphatase, glutamic-pyruvic acid transaminase, and bilirubin levels). After completion of various periods of cholesterol feeding, bile fistulas were constructed in all rabbits as described previously (20) under anesthesia (ketamine, 40 mg/kg body weight, combined with xylazine, 4 mg/kg body weight, and acepromazine, 0.5 mg/kg body weight, administered intramuscularly). Bile was collected for 30 min to determine baseline bile acid composition and percentage of deoxycholic acid in the bile. Half of the rabbits from each group (6 controls, 5 fed cholesterol for 1 day, 6 for 3 days, 5 for 5 days, and 6 for 10 days) were sacrificed. 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 determinations 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 to collect bile for 5 days, which ensured total recovery of deoxycholic acid from the bile and evaluation of the effect of bile drainage on activities of the enzymes, because after 3 days of bile drainage, cholic acid synthesis was maximally stimulated and reached a steady-state output (20). During 5 days of bile fistula, all animals were fed regular rabbit chow (without cholesterol) so that we were able to compare enzyme activities and plasma and hepatic cholesterol concentrations between rabbits with and without bile fistula but fed cholesterol for the same number of days (0, 1, 3, 5, and 10 days). Animals with bile fistula were given lactated Ringer’s solution with 5% dextrose intravenously at 12 ml/h in the first 24 h, which was replaced by 0.9% NaCl after the animal started eating and drinking. This infusion was continued until the experiment was finished in order to replace the lost body fluid. The rabbits were then sacrificed, and liver and blood were collected for the same biochemical measurements and histologic examinations already mentioned. To test whether prolonged cholesterol feeding would further increase the bile acid pool size, four rabbits were fed 2% cholesterol for 15 days, and bile fistula was constructed for measurement of bile acid pool size only.

The animal protocol was approved by the Subcommittee on Animal Studies at Veterans Affairs Medical Center, and by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey Medical School.

Bile acid pool size The total bile acid pool sizes were calculated from measurements of the total recovered deoxycholic acid in the bile continuously 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 bile acids and sterols. Bile (100 μl) dis-
remained significantly higher than the baseline value in the control rabbits. In the cholesterol-fed rabbits, decreased after bile drainage, they still after 5 days of bile drainage. Although hepatic cholesterol concentrations in the cholesterol-fed rabbits decreased after bile drainage, they still remained significantly higher than the baseline value in the control rabbits.

**Figure 3**
Effects of increasing cholesterol feeding and bile fistula on hepatic cholesterol concentrations. Rabbits with bile fistula (+BF) were also fed 2% cholesterol chow for 1, 3, 5, and 10 days, and bile fistulas were constructed after cholesterol was fed. During the fistula period, the rabbits were then fed regular rabbit chow (without cholesterol). The open bars represent hepatic cholesterol concentrations in controls and rabbits after feeding cholesterol, and the crosshatched bars represent hepatic cholesterol levels after 5 days of bile drainage. Although hepatic cholesterol concentrations in the cholesterol-fed rabbits decreased after bile drainage, they still remained significantly higher than the baseline value in the control rabbits.

**Figure 4**
Disappearance of deoxycholic acid (expressed as percent) in the bile after bile fistula in controls and rabbits fed cholesterol for 10 days. Deoxycholic acid represented 83%–88% of the total bile acids in the initial bile and disappeared from the bile after 3 or 4 days of bile fistula in controls and cholesterol-fed rabbits, respectively. There was no significant difference in percent deoxycholic acid between control and cholesterol-fed rabbit groups.
Results
The effects of cholesterol feeding on plasma cholesterol concentrations are illustrated in Fig. 2. Plasma cholesterol concentrations increased 3.6 times after 1 day, 11.7 times after 3 days, 20 times after 5 days, 33 times after 10 days, and 20 times after 15 days of feeding chow that contained 2% cholesterol (3 g per day).

Figure 3 shows the comparison of hepatic cholesterol concentrations in control and cholesterol-fed rabbits and after five days of bile drainage. Hepatic cholesterol levels rose progressively after feeding cholesterol 3 g per day for 1 to 10 days. Cholesterol levels increased 75% (*P* < 0.05) after 1 day, 4 times after 3 days (*P* < 0.0001), 7 times after 5 days (*P* < 0.0001), and 9.6 times after 10 days (*P* < 0.0001) of feeding cholesterol. After 5 days of bile drainage, hepatic cholesterol concentrations decreased in all rabbit groups, but they still remained significantly higher than baseline in rabbits fed cholesterol for 1 day (*P* < 0.05), 3 days (*P* < 0.05), 5 days (*P* < 0.01), and 10 days (*P* < 0.001).

The bile acid pool size was measured by the total recovery of biliary deoxycholic acid (excreted in the bile as the glycine conjugate) after bile fistula divided by the percentage of deoxycholic acid in the initial bile specimens. Figure 4 shows the disappearance of deoxycholic acid in the bile after bile fistula in controls and the rabbits fed 2% cholesterol for 10 days. There was no significant difference in percentage of deoxycholic acid at baseline (87.9% ± 4.6% vs. 83.0% ± 3.7%) between controls and cholesterol-fed rabbits. In both rabbit groups, deoxycholic acid rapidly decreased and eventually disappeared after three to four days of bile drainage (percent deoxycholic acid was 1% ± 0.2% of the biliary bile acids in cholesterol-fed rabbits on the fourth day). In rabbits fed cholesterol for 1, 3, 5, and 15 days, depletion of deoxycholic acid from the bile after bile fistula showed the same pattern. Figure 5 presents bile acid pool sizes in rabbits fed cholesterol from 1 to 15 days. The pool size was expanded when cholesterol was fed so that after 1 day, the pool increased to 265 ± 21 mg (+28%) compared with the baseline 207 ± 58 mg, but the difference was not statistically significant. After 3 days of cholesterol feeding, the bile acid pool rose to 379 ± 85 mg (+83%; *P* < 0.01); after 5 days, to 387 ± 65 mg (+87%; *P* < 0.001); after 10 days, 421 ± 25 mg (+100%; *P* < 0.0001); and after 15 days, 397 ± 27 mg (+92%; *P* < 0.01). However, cholesterol feeding beyond 3 days did not result in significantly larger bile acid pool sizes despite continued accumulation of hepatic cholesterol.

Activity of microsomal cholesterol 7α-hydroxylase, the rate-limiting enzyme for the classic bile acid synthetic pathway, unexpectedly rose 1.6 times (from 27.7 ± 6.8 to 45.1 ± 9.0 pmol/mg/min; *P* < 0.01) after 1 day of cholesterol feeding (Fig. 6), at a time when hepatic cholesterol concentrations had increased 75% (*P* < 0.05) but the bile acid pool size had not enlarged significantly. However, as the daily dietary intake of cholesterol (3 g per day) continued, cholesterol 7α-hydroxylase activities progressively decreased so that after 3 days of cholesterol feeding, enzyme activity declined to 28.7 ± 11.7 pmol/mg/min (*P* < 0.05 compared with upregulated activity, 45.1 ± 9.0 pmol/mg/min, on day 1 of cholesterol feeding); after 5 days, the activity fell to 19.5 ± 2.4 pmol/mg/min (*P* < 0.05, vs. baseline); and after 10 days, to 10.6 ± 6.1 pmol/mg/min (*P* < 0.01, vs. baseline).

Figure 6 also shows that 5 days of bile drainage strongly stimulated cholesterol 7α-hydroxylase activity in every group except for the rabbits fed cholesterol for 1 day when cholesterol 7α-hydroxylase had already been induced. In control rabbits, cholesterol 7α-hydroxylase activity rose from baseline 27.7 ± 6.8 to 52.7 ± 8.3 pmol/mg/min (*P* < 0.001) after 5 days bile drainage. However, the highest increases of cholesterol 7α-hydroxylase activity were observed after bile drainage in the rabbits fed cholesterol for 5 days (72.1 ± 12.7 pmol/mg/min; *P* < 0.0001) and 10 days (115.4 ± 33.8 pmol/mg/min; *P* < 0.0001), when hepatic cholesterol levels were the highest.

Activity of hepatic mitochondrial sterol 27-hydroxylase (Fig. 7), the rate-limiting enzyme for alternative bile acid synthetic pathway, did not change after 1 day of cholesterol feeding (21.2 ± 4.7 pmol/mg/min compared with the baseline 25.1 ± 8.5 pmol/mg/min) but rose signifi-
Effects of increasing cholesterol feeding and bile fistula on cholesterol 7α-hydroxylase activity. The open bars represent cholesterol 7α-hydroxylase activity in controls and after different days of cholesterol feeding; the crosshatched bars represent the activity after 5 days of bile drainage. Cholesterol 7α-hydroxylase activity unexpectedly increased significantly after 1 day of 2% cholesterol feeding and then progressively decreased in rabbits fed 2% cholesterol for more than 3 days. The activities after bile drainage were all significantly higher than the baseline value (the open bar in controls). The highest levels were seen after bile drainage in the rabbits fed 2% cholesterol for 5 and 10 days.

Liver function tests showed normal values for plasma alkaline phosphatase, glutamic-pyruvic acid transaminase, and bilirubin in all cholesterol-fed groups. Liver histology showed only increased deposition of fat in the cholesterol-fed rabbits, with no hepatocyte damage, inflammation, or disruption of the architecture.

Discussion

This study has expanded our understanding and better defined the role of dietary cholesterol intake and the hepatic bile acid pool on regulation of classic (cholesterol 7α-hydroxylase) and alternative (sterol 27-hydroxylase) bile acid synthesis and hepatic cholesterol formation (HMG-CoA reductase). The study confirmed that an enlarged bile acid pool, but not increased hepatic cholesterol concentrations, inhibited cholesterol 7α-hydroxylase activity (classic bile acid synthesis). Feeding cholesterol initially increased cholesterol 7α-hydroxylase activity, but the expansion of the bile acid pool was a much more powerful downregulator, and cholesterol 7α-hydroxylase became inhibited. Mitochondrial sterol 27-hydroxylase was also stimulated by increasing hepatic cholesterol but did not respond to expansion or depletion of the bile acid pool. This study also suggested that although cholesterol was a potent inhibitor of hepatic HMG-CoA reductase activity, interruption of the enterohepatic circulation was a more powerful stimulus for upregulation of this enzyme activity even when hepatic cholesterol levels remained high.

This study showed that after one day of cholesterol feeding, hepatic cholesterol rose significantly and cholesterol 7α-hydroxylase activity was initially stimulated because the bile acid pool had not yet enlarged significantly. However, HMG-CoA reductase responded immediately to the increased hepatic cholesterol pool and was significantly inhibited. After 3, 5, and 10 days of cholesterol feeding, the bile acid pool expanded and cholesterol 7α-hydroxylase activity progressively declined. This observation suggested that increased hepatic cholesterol stimulated cholesterol 7α-hydroxylase activity, perhaps by providing extra substrate, but that the circulating bile acid pool was a much more powerful regulator of cholesterol 7α-hydroxylase; so, when the bile acid pool size significantly expanded, enzyme activity decreased. The bile
acid pool size had almost reached its maximum plateau after 3 days of cholesterol feeding; further intake (5, 10, and 15 days) did not raise the pool size significantly. The fact that enlargement of the bile acid pool size occurred first and was followed by inhibition of cholesterol 7α-hydroxylase confirmed the hypothesis that expanded bile acid pool was responsible for downregulation of cholesterol 7α-hydroxylase activity in the cholesterol-fed NZW rabbits. After 5 days of bile fistula, when the bile acid pool was depleted entirely, inhibited cholesterol 7α-hydroxylase activity rebounded in all rabbit groups. After bile fistula, the hepatic cholesterol levels in the cholesterol-fed rabbits were still higher than the baseline value, but the more the cholesterol deposited, the greater the upregulation of cholesterol 7α-hydroxylase activity. Again, these results further demonstrated that (a) the circulating bile acid pool was the most powerful regulator of cholesterol 7α-hydroxylase and that it was the enlarged bile acid pool size that inhibited cholesterol 7α-hydroxylase in cholesterol-fed rabbits; and (b) increased hepatic cholesterol was not an inhibitor but a stimulator that initially induced cholesterol 7α-hydroxylase activity and resulted in greater upregulation of cholesterol 7α-hydroxylase after removal of the bile acid pool.

In distinction, mitochondrial sterol 27-hydroxylase responded differently to cholesterol feeding. Sterol 27-hydroxylase activity was upregulated by the increased hepatic cholesterol pool but was insensitive to the feedback regulatory effect of the enlarged circulating bile acid pool. Sterol 27-hydroxylase activity that increased in response to cholesterol feeding did not change significantly when the bile acid pool was removed by drainage. We believe that activated sterol 27-hydroxylase was responsible for the expansion of the bile acid pool size, because sterol 27-hydroxylase continued actively making bile acids via alternative bile acid synthesis regardless of the size of the expanded bile acid pool. After bile drainage, when cholesterol 7α-hydroxylase activity was induced with significant increase of newly synthesized cholic acid (19), sterol 27-hydroxylase activity remained at upregulated levels, because hepatic cholesterol concentrations were still high. Therefore, activation of sterol 27-hydroxylase was not simply compensation for the inhibition of classic bile acid synthesis and cholesterol 7α-hydroxylase but was a result of continued stimulation by increased hepatic cholesterol levels. We recently reported (29) that in Watanabe hyperlipidemic homozygous and heterozygous rabbits with total and partial hepatic low-density lipoprotein (LDL) receptor deficiency, cholesterol feeding did not stimulate (in homozygotes), or insignificantly increased (in heterozygotes), sterol 27-hydroxylase activity. In the cholesterol-fed Watanabe rabbits, hepatic cholesterol levels also significantly rose, whereas either total (in homozygotes) or most (in heterozygotes) hepatic cholesterol entered the liver via non-LDL receptor pathways. These results not only supported our contention that increased hepatic cholesterol stimulated sterol 27-hydroxylase activity but also suggested that only increased hepatic cholesterol that was delivered via LDL receptors could upregulate sterol 27-hydroxylase activity.

The finding that alternative bile acid synthesis is not controlled by expanded or depleted bile acid pool has important implications with respect to bile acid synthesis, especially in cholestatic liver diseases. In these conditions, there is interruption of the hepatic excretion of bile acids that are accumulated in the liver. Classic bile acid synthesis and cholesterol 7α-hydroxylase are inhibited. However, as observed in the cholesterol-fed NZW rabbits, sterol 27-hydroxylase remains active in the presence of an enlarged intrahepatic bile acid pool, and alternative bile acid synthesis continues to add more detergent bile acids to the liver. Thus, alternative bile acid synthesis that is present to supplement the classic pathway can actually contribute detergent bile acids that may further damage the liver in cholestatic liver diseases.

With respect to cholesterol biosynthesis, HMG-CoA reductase, the rate-determining enzyme, was inhibited as expected after one day of cholesterol feeding and remained suppressed throughout the cholesterol feeding period. However, removal of the bile acid pool by drainage upregulated HMG-CoA reductase in control and cholesterol-fed rabbits when hepatic cholesterol levels still remained significantly higher than baseline. Thus, hepatic cholesterol biosynthesis that is very sensitive to negative-feedback control also responded to interruption of enterohepatic circulation. In this case, depletion of the bile acid pool obviously overrode the effects of elevated hepatic cholesterol levels. Figure 8 shows that, opposite to cholesterol 7α-hydroxylase (Fig. 6), after bile drainage, the more hepatic cholesterol accumulated, the less increase of HMG-CoA reductase activity. This suggests that retaining hepatic cholesterol still exerts an inhibitory effect on hepatic HMG-CoA reductase. There is a difference, however, between the rats and rabbits. Cholesterol fed to rats increased classic bile acid synthesis and

Figure 8
Effects of increasing cholesterol feeding and bile fistula on HMG-CoA reductase activity. The open bars represent HMG-CoA reductase activity in controls and after different days of cholesterol feeding; the cross-hatched bars represent the activity after 5 days of bile drainage. Enzyme activities were inhibited immediately after 1 day of 2% cholesterol feeding and remained suppressed as the feeding continued. After 5 days of bile drainage, the activities significantly rebounded in each group. However, the more hepatic cholesterol, the less the increase of HMG-CoA reductase activity. HMG-CoA, β-hydroxy-β-methylglutaryl coenzyme A.
cholesterol 7α-hydroxylase without stimulating alternative bile acid synthesis and sterol 27-hydroxylase activity. The hepatic cholesterol pool rises, but plasma cholesterol levels do not. Thus, the rat is protected against dietary cholesterol–induced hypercholesterolemia by increasing the conversion of cholesterol to bile acids via the classic pathway and excreting the excess cholesterol as bile acids in the feces. As already mentioned, in rabbits, cholesterol 7α-hydroxylase activity is initially induced by increasing hepatic cholesterol but is inhibited after cholesterol feeding as expanded bile acid pool, which is a more powerful regulator. The question is why cholesterol 7α-hydroxylase activity is not inhibited in cholesterol-fed rats. Is it because hepatic cholesterol is a more powerful inducer or because cholesterol feeding does not enlarge the bile acid pool size in rats? Perhaps the difference resides in ileal bile acid transport.

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