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EFFECT OF FEEDING CHOLESTEROL AND SITOSTEROL ON HEPATIC STEROID 12α-HYDROXYLASE ACTIVITY IN FEMALE HAMSTERS

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The effects of dietary cholesterol and sitosterol on the activity of the hepatic steroid 12α-hydroxylase, gallbladder bile acid composition, and serum and liver cholesterol concentrations were studied in female hamsters. The 12α-hydroxylase activity was inhibited by 65% in cholesterol-fed animals and by 30% in sitosterol-fed animals. Cholesterol feeding increased percentages of chenodeoxycholic and lithocholic acids in gallbladder bile with reciprocal decrease of cholic acid, while sitosterol feeding had no significant effect on bile acid composition. Cholesterol feeding increased levels of serum and liver cholesterol, on the contrary, sitosterol decreased both concentrations. A positive correlation between the 12α-hydroxylase activity and the ratio of cholic acid plus its metabolites to chenodeoxycholic acid plus its metabolite was also observed. These results support the proposal that steroid 12α-hydroxylase is a major factor in determining the relative proportion of cholic acid and chenodeoxycholic acid synthesized in the liver and indicate that cholesterol feeding increased percentage of chenodeoxycholic acid by inhibiting the activity of steroid 12α-hydroxylase.

Keywords—steroid 12α-hydroxylase; liver microsome; cholesterol; sitosterol; 7α-hydroxycholesterol-4-en-3-one; 7α, 12α-dihydroxycholesterol-4-en-3-one; chenodeoxycholic acid; cholic acid; bile acid synthesis; hamster

INTRODUCTION

Dietary neutral sterols consist of cholesterol and phytosterols. The latter have alkyl groups at carbon-24 of cholesterol. Cholesterol absorption is limited and about a half of dietary cholesterol is absorbed from the small intestine. Hepatic cholesterol metabolism is sensitive to dietary cholesterol, which inhibits cholesterol synthesis by a negative feedback action at the level of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoAR), and increases bile acid formation by enhancing the activity of cholesterol 7α-hydroxylase presumably due to expansion of the substrate pool. On the other hand, phytosterols, despite their structural similarity to cholesterol, are absorbed in only trace amounts and when they are administered in large doses, they inhibit intestinal absorption of cholesterol, increase fecal excretion of neutral sterols and bile acids, and decrease levels of serum and liver cholesterol concentrations.

Despite these studies, however, not much is known about the effects of dietary cholesterol and phytosterols on the biliary bile acid composition and hepatic microsomal steroid 12α-hydroxylase, which catalyses the conversion of 7α-hydroxycholesterol-4-en-3-one to 7α, 12α-dihydroxycholesterol-4-en-3-one in the pathway of cholic acid biosynthesis and may play an important regulatory role in determining the ratio of the two primary bile acids synthesized in mammalian livers, cholic acid and chenodeoxycholic acid. Although

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Andersén and Hellström found that cholesterol feeding to man influenced the pattern of the two primary bile acids produced,10 there is no study about the effect of dietary sterols on the 12α-hydroxylase activity in human liver. Gustafsson et al. showed that the activity of steroid 12α-hydroxylase in the rat was inhibited by cholesterol feeding and that the relative concentration of chenodeoxycholic acid and its metabolites was significantly increased following cholesterol administration.11 However, the rat might be an inappropriate animal model for further investigation of effect of sterol feeding on biliary bile acid composition, because the rat does not have a gallbladder, and diversion of bile by cannulation of the bile duct may cause major changes in the types and amounts of bile acids synthesized in the liver due to removal of feedback control mechanisms. Furthermore, since bile acid composition of the rat is quite different from that of human,11 the changes of the ratio of cholic acid to chenodeoxycholic acid by sterol feeding could not be easily estimated.

Recently, we proposed that the hamster is a more suitable animal model than the rat for studies on the changes of the 12α-hydroxylase activity and biliary bile acid composition.12 Thus, we have now studied effects of feeding cholesterol and sitosterol on hepatic steroid 12α-hydroxylase and biliary bile acid composition in female hamsters.

MATERIALS AND METHODS

Chemicals — 7α-Hydroxycholesterol-4-en-3-one, 5β-cholestan-3α, 7α, 12α-triol, 7α, 12α-dihydroxy-5β-cholestan-3-one, 7α, 12α-dihydroxycholesterol-4-en-3-one, and tritium-labeled 7α-hydroxycholesterol-4-en-3-one (specific radioactivity 49μCi/mg) were synthesized as previously described.13 Cholesterol (Yoneyama Yakuhink Kogyo Co., Ltd. Osaka, Japan) was recrystallized twice from hot ethanol before use. Sitosterol was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and used without further purifications, though gas-liquid chromatographic analysis revealed that it consisted of sitosterol (57%), campesterol (35%), and stigmasterol (4%). All other chemicals were of reagent grade. Chows containing 2% of cholesterol or sitosterol were prepared by mixing 20.0 g of each sterol in 300 ml of ether with 1.0 kg of Standard Powder Chow FM (Oriental Yeast Co., Ltd., Tokyo, Japan, containing 0.6 mg of cholesterol, 0.6 mg of sitosterol, and 0.2 mg of campesterol per gram of food as determined by gas-liquid chromatographic analysis).

Animals — Eighteen female Golden Syrian hamsters with an average weight of 86 g were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The animals were kept in individual cages and were allowed free access to food and water. The temperature of the vivarium was maintained at 25°C and a period of light from 6 AM. to 6 PM. was controlled by an electric timer. After two weeks of the adaptation period, the animals were randomly divided into three groups. Each group was fed one of the standard chow, 2% cholesterol chow, or 2% sitosterol chow for seven weeks. All animals were sacrificed between 9 and 10 AM. for determination of hepatic steroid 12α-hydroxylase activity, biliary bile acid composition, serum cholesterol, and liver cholesterol. Under ether anesthesia, blood was aspirated from the heart, gallbladder was resected, and liver was excised, weighed, and two grams portion of the liver was used for measuring the activity of hepatic steroid 12α-hydroxylase. The remaining liver was lyophilized and used for liver cholesterol determination.

Measurement of Hepatic Steroid 12α-Hydroxylase Activity — The rate of the conversion of 7α-hydroxycholesterol-4-en-3-one to 7α, 12α-dihydroxycholesterol-4-en-3-one was determined as previously described.12 In brief, 50 nmol of tritium-labeled 7α-hydroxycholesterol-4-en-3-one in 10 μl of acetone was added to an incubation mixture containing, in a volume of 2.0 ml: potassium phosphate buffer, pH 7.4, 0.191 mmol; MgCl2, 10 μmol; NADPH, 3 μmol; and 1.0 ml of the microsomal suspension and the mixture was incubated at 37°C in air for 10 min. After extraction with 20 ml of CHCl3-methanol (2/1, v/v), the organic layer was subjected to thin layer
TABLE I. Effect of Cholesterol and Sitosterol Feeding on Food Intake, Weight Gain, and Liver Weight in Female Hamsters (Mean ± S.D.)

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>Cholesterol (n=6)</th>
<th>Sitosterol (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake (g/d)</td>
<td>8.2±1.3</td>
<td>9.8±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.9±0.8</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>85.2±5.6</td>
<td>87.3±6.8</td>
<td>86.2±6.4</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>113.2±7.3</td>
<td>115.5±6.3</td>
<td>116.7±13.3</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>28.0±6.0</td>
<td>28.2±10.2</td>
<td>30.5±12.4</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>4.1±0.8</td>
<td>8.0±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1±0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from control values (p<0.05 by paired t test).

chromatography with solvent system of benzene–ethyl acetate (3/7, v/v) and radioactivity was measured in a liquid scintillation spectrometer.

Analysis of Gallbladder Bile Acids — The determination of the gallbladder bile acid composition was carried out by gas-liquid chromatography on 3% QF-1 column as methyl ester-dimethyl-ethylsilyl ether derivatives.<sup>13</sup>

Determination of Liver and Serum Cholesterol Concentrations — Liver and serum cholesterol concentrations were analyzed by gas-liquid chromatography on 3% OV-17 column as trimethylsilyl ether derivatives as described previously<sup>13</sup> using 5α-cholestan as an internal standard.

Statistical Method — Results are expressed as mean±S.D. Group means were compared by paired Student’s t-test. Bivariate regression analysis was performed by the method of least squares.

RESULTS

During the present experimental period, the hamsters receiving each diet ingested almost the same weight of food per day and also gained almost the same body weight. At laparotomy, the livers of cholesterol-fed hamsters were grayish white in color, large in size, and significantly heavy in weight (i.e. fatty liver) (Table I).

Steroid 12α-Hydroxylase

The effect of dietary cholesterol and sitosterol on the activity of the hepatic steroid 12α-hydroxylase is shown in Fig. 1. The activity was

![Graph showing the effect of cholesterol and sitosterol feeding on the activity of the hepatic microsomal steroid 12α-hydroxylase in female hamsters.](attachment:image)

<sup>a</sup> Significantly different from control values (p<0.05).
63.8±14.9 pmol/mg protein/min for the control group. Feeding of cholesterol significantly decreased the activity to 23.7±5.0 pmol/mg protein/min (p<0.0005). Administration of sitosterol also decreased the activity (44.6±3.6 pmol/mg protein/min, p<0.025). The extent of the inhibition by sitosterol feeding was much less than that by cholesterol feeding (p<0.0005).

**Bile Acid Composition in Gallbladder Bile**

The bile acid composition in each group is shown in Table II. It can be seen that the control and the cholesterol groups differed significantly in the percentages of cholic, chenodeoxycholic, and lithocholic acids. There were no significant differences between the two groups in the percentages of deoxycholic and 7-ketodeoxycholic acids (3α, 12α-dihydroxy-7-oxo-5β-cholan-24-oic acid). Sitosterol feeding did not change the bile acid composition compared with the control group. The ratio of cholic acid (plus its metabolites, *i.e.* deoxycholic acid and 7-ketodeoxycholic acid) to chenodeoxycholic acid (plus its metabolite, *i.e.* lithocholic acid) was also significantly different between control and cholesterol groups, whereas it was not statistically different between control and sitosterol groups. Percentages of the secondary bile acids (*i.e.* deoxycholic acid plus 7-ketodeoxycholic acid plus lithocholic acid) were almost the same label in the three groups.

As shown in Fig. 2, the ratio between cholic acid plus its metabolites and chenodeoxycholic acid plus its metabolite was correlated very well (*r* = 0.80, p<0.0005, *n* = 18) with the activity of the steroid 12α-hydroxylase.

**Serum and Liver Cholesterol Concentrations**

Effects of sterol feeding on serum and liver cholesterol concentrations are shown in Table III. The serum cholesterol level was markedly elevated by 180% by cholesterol feeding (p<0.0005), and was pulled down by 44% by sitosterol feeding (p<0.025). Liver cholesterol concentration (mg/g dry weight) surprisingly increased to 26 times of the control values in cholesterol-fed hamsters (p<0.0005) and decreased by 32% in sitosterol-fed hamsters (p<0.05).

**DISCUSSION**

The result of the present study indicates that the steroid 12α-hydroxylase activity in female hamsters was inhibited markedly by cholesterol feeding and moderately by sitosterol feeding (Fig. 1). The inhibitory effect of cholesterol on the enzyme is consistent with the result of Gustafsson *et al.*, who investigated effects of cholesterol feeding on liver microsomal enzymes in rats, and found increased 7α-hydroxylation of cholesterol.

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**TABLE II. Effect of Cholesterol and Sitosterol Feeding on Gallbladder Bile Acid Composition in Female Hamsters (Mean ± S.D.)**

<table>
<thead>
<tr>
<th>Bile acid</th>
<th>Control (n = 6)</th>
<th>Cholesterol (n = 6)</th>
<th>Sitosterol (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>70.3±5.0</td>
<td>58.7±2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.0±1.1</td>
</tr>
<tr>
<td>Chenodeoxycholic acid</td>
<td>19.0±49</td>
<td>30.3±48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.3±3.4</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>6.8±5.4</td>
<td>5.8±2.9</td>
<td>3.8±1.8</td>
</tr>
<tr>
<td>Lithocholic acid</td>
<td>1.1±0.3</td>
<td>2.4±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>7-Ketodeoxycholic acid</td>
<td>2.9±0.7</td>
<td>2.8±0.2</td>
<td>2.8±0.9</td>
</tr>
<tr>
<td>CA/CDC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.29±1.32</td>
<td>2.11±0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.03±0.85</td>
</tr>
<tr>
<td>Secondary bile acids (%)</td>
<td>10.8±5.1</td>
<td>11.3±2.8</td>
<td>7.7±2.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from control values (p<0.05 by paired t test).

<sup>b</sup> (cholic acid + deoxycholic acid + 7-ketodeoxycholic acid)/(chenodeoxycholic acid + lithocholic acid).
and 6β-hydroxylation of lithocholic acid and decreased 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one.\textsuperscript{11} This raises interesting questions concerning the physiological role of cholesterol as a regulator of microsomal metabolism of bile acid. Hydroxylation of these steroids is catalyzed by membrane-bound enzymes embedded in a lipid environment. It may be speculated that cholesterol forms a major part of this environment and that the microsomal concentration of cholesterol may influence the catalytic activity of the hydroxylating enzymes. Another possibility is that cholesterol affects the synthesis of specific cytochrome P-450 species participating in these hydroxylations. Physiological meaning of decreased 12α-hydroxylation by cholesterol feeding is obscure. Low 12α-hydroxylase activity would result in decreased conversions of cholest-5-en-3β, 7α-diol, 7α-hydroxycholest-4-en-3-one, and 5β-cholestan-3α, 7α-diol to their 12α-hydroxylated compounds, and consequently decrease the ratio of cholic acid to chenodeoxycholic acid synthesized in the liver. Chenodeoxycholic acid has a lesser ability on cholesterol secretion from the liver than cholic acid does\textsuperscript{13} and may increase deposit of cholesterol in the liver. On the other hand, since chenodeoxycholic acid suppresses intestinal absorption of cholesterol,\textsuperscript{13,14} increased proportion of chenodeoxycholic acid in bile might be reasonable for animals to make accumulation of cholesterol minimum. Furthermore, the greater turnover of chenodeoxycholic acid compared to cholic acid\textsuperscript{15} may also result in an increased elimination of cholesterol via the biosynthesis of bile acids.

Effect of sitosterol feeding on the activity of steroid 12α-hydroxylase has not yet been studied.

![Graph](image)

FIG. 2. Correlation Between the Activity of the Hepatic Microsomal Steroid 12α-Hydroxylase and the Ratio of Cholic Acid Plus Its Metabolites to Chenodeoxycholic Acid Plus Its Metabolite in Gallbladder Bile in Control, Cholesterol-fed, and Sitosterol-fed Female Hamsters

The best-fit linear function was determined by the method of least squares. a) (Cholic acid + deoxycholic acid + 7-ketodeoxycholic acid)/(chenodeoxycholic acid + lithocholic acid). The symbols indicate the different treatment groups (control (■), cholesterol-fed (▲), and sitosterol-fed (●)).

<table>
<thead>
<tr>
<th>TABLE III. Effect of Cholesterol and Sitosterol Feeding on Serum and Liver Cholesterol Concentrations in Female Hamsters (Mean±S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dl)</td>
</tr>
<tr>
<td>Liver cholesterol (mg/g liver)</td>
</tr>
</tbody>
</table>

\textsuperscript{a)} Significantly different from control values (p < 0.05 by paired t test).
Since sitosterol is not well absorbed,\textsuperscript{6} the effect on the liver enzyme may be mediated through its inhibition of cholesterol absorption in the small intestine.\textsuperscript{2} The decreased cholesterol absorption causes hypocholesterolemia, reduces cholesterol content in the liver,\textsuperscript{8} and increases HMGCoAR activity,\textsuperscript{3} \textit{i.e.} opposite effects as observed in cholesterol feeding. Thus, we hypothesized that sitosterol feeding would have the reverse effect on steroid 12$\alpha$-hydroxylase compared with the case of cholesterol feeding. However, the activity of steroid 12$\alpha$-hydroxylase was inhibited by 30\% in sitosterol-fed animals. Indeed, further studies are required to elucidate mechanisms by which sitosterol suppresses the enzyme, while there are at least two possible mechanisms explaining this result. First, decreased cholesterol concentration returning to the liver stimulates cholesterol synthesis in the liver microsome,\textsuperscript{3} and increased level of this newly synthesized cholesterol may affect enzyme system of bile acid synthesis, such as cholesterol 7$\alpha$-hydroxylase and steroid 12$\alpha$-hydroxylase, in the same subcellular compartment. This speculation may be supported by the result of Shefer \textit{et al.}, who showed the increased 7$\alpha$-hydroxylase activity in sitosterol-fed rats.\textsuperscript{3} Second, sitosterol might directly suppress the activity of steroid 12$\alpha$-hydroxylase. Salen \textit{et al.} reported that less than 5\% of dietary sitosterol was absorbed from the intestine, 20\% of the absorbed sitosterol was converted to bile acids, and that the remainder was excreted in bile as free sterol.\textsuperscript{6} Moreover, Tabata \textit{et al.} reported that intravenously injected phytosterol decreased levels of serum and liver cholesterol and increased bile acid excretion.\textsuperscript{16} These facts suggest that small amounts of absorbed sitosterol may directly affect hepatic enzymes concerning cholesterol and bile acid metabolism, including 12$\alpha$-hydroxylase.

As shown in Table II, the gallbladder bile acid composition was altered by cholesterol feeding to become rich in chenodeoxycholic acid with reciprocal reduction in the percentage of cholic acid. This change can be explained in view of the above-mentioned fact that the activity of steroid 12$\alpha$-hydroxylase is inhibited by diet rich in cholesterol. Same effect of cholesterol feeding on the bile acid composition has been also reported in rats\textsuperscript{11,17} and in human.\textsuperscript{10} These results suggest that there is a close relation between the bile acid composition and 12$\alpha$-hydroxylase activity and that steroid 12$\alpha$-hydroxylase activity in human liver microsome might be also suppressed by cholesterol feeding.

Sitosterol feeding did not change the bile acid composition compared with the control. Although the activity of steroid 12$\alpha$-hydroxylase did decrease in sitosterol-fed animals, the extent of the inhibition is less than that in cholesterol feeding, and may be too small to change the bile acid composition. Because, there was a positive correlation between 12$\alpha$-hydroxylase activity and the ratio of cholic acid (plus its metabolites) to chenodeoxycholic acid (plus its metabolite) in the control, cholesterol-fed, and sitosterol-fed hamsters (Fig. 2). This correlation again emphasizes the central role of 12$\alpha$-hydroxylase in regulation of the ratio of the two primary bile acids,\textsuperscript{12} and suggests minimal and invisible change in the bile acid composition in sitosterol-fed animals.

Cholesterol feeding increased serum cholesterol concentration to 2.8 times of the control values. The liver became fatty and cholesterol content of the liver strikingly increased (Table III). Not all species react in the same manner to excess dietary cholesterol. Feeding a diet rich in cholesterol produced extreme hypercholesterolemia in rabbits, ground squirrels, and prairie dogs, moderate hypercholesterolemia in chickens and hamsters, and slight elevation of serum cholesterol in rats and dogs.\textsuperscript{18} All animals developed fatty liver due to cholesterol. These species differences may be due to the efficiency of intestinal absorption of cholesterol, the extents of inhibition of cholesterol synthesis and increment of bile acid synthesis, and/or difference in lipoprotein metabolism.\textsuperscript{18,22}

Sitosterol significantly decreased levels of serum and liver cholesterol as previously reported in rats and chickens.\textsuperscript{8,19} As described above, its hypocholesterolemic action is thought to be due to inhibition of cholesterol absorption. The
mechanisms for this inhibitory effect are not well understood. There are at least three possible sites where sitosterol might act to reduce cholesterol absorption. First, within the intestinal lumen, the presence of excess sitosterol may reduce solubility of cholesterol by excluding it from mixed micelles. Second, sitosterol might compete with cholesterol for uptake by the intestinal mucosal cells. And finally, it may interfere with incorporation of cholesterol into chylomicrons, possibly by interfering with its esterification. It should also be kept in mind that there is a possibility of direct hypocholesterolemic ability of small amounts of absorbed sitosterol.6,18)

In conclusion, steroid 12α-hydroxylase activity is markedly inhibited by cholesterol feeding and moderately by sitosterol feeding in female hamsters.

REFERENCES
1) This paper is part XIII of a series entitled “Metabolism of Bile Acids”. Part XII: S.Kuroki and T.Hoshita: Effect of bile acid feeding on hepatic steroid 12α-hydroxylase activity in hamsters, Lipids, submitted.


