Influence of Cholesterol Feeding on Bile Acid Metabolism in Young and Aged Germ-Free Rats

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Received December 8, 1995 Accepted March 8, 1996

ABSTRACT—The effects of cholesterol feeding on serum and liver cholesterol levels, fecal and biliary bile acid levels, bile acid pool size and bile acid composition were examined in 2-, 12- and 24-month-old male germ-free rats. The major bile acids in these animals were cholic and β-muricholic acids. Cholesterol feeding increased synthesis of bile acids by 3- to 4-fold, especially that of chenodeoxycholic acid (mainly β-muricholic acid in the rat), decreasing the cholic acid/chenodeoxycholic acid (CA/CDCA) ratio in all rats regardless of age, even though the CA/CDCA ratio increased as a linear function of age in both diet groups. Cholesterol feeding increased the serum cholesterol level markedly in aged rats. This hypercholesterolemia may be produced by the increase in CA/CDCA ratio in aged rats.

Keywords: Dietary cholesterol, Bile acid metabolism, Cholic acid/chenodeoxycholic acid ratio, Germ-free rat, Aging

Bile acids are synthesized from cholesterol in the liver, and the major primary bile acids are cholic acid and chenodeoxycholic acid in many animal species. In rats, chenodeoxycholic acid is further transformed to α- and β-muricholic acids, mainly to β-muricholic acid, in the liver (1). Therefore, cholic and β-muricholic acids, are the two main bile acids formed in the rat.

Bile acids are essential for cholesterol absorption, but their effects on the action of cholic acid and not of bile acids related to chenodeoxycholic acid have been demonstrated in vivo (2-4). When cholesterol absorption was examined in situ by the small intestinal loop method, taurocholic acid was found to enhance cholesterol absorption dose-dependently while tauro-β-muricholic acid showed almost no effect (5). When both of these bile acids were added together to the small intestinal loop, cholesterol absorption was entirely dependent on the concentration of taurocholic acid (5). Therefore, it was presumed that the increase in β-muricholic acid synthesis after cholesterol feeding prevented further increases in cholesterol absorption. This is regarded as a defense mechanism against alimentary hypercholesterolemia in rats (6). When cholesterol was fed to young rats, the liver cholesterol level increased, but that in the serum remained almost unchanged, and the synthesis of bile acids, especially chenodeoxycholic acid, increased markedly (6-8).

On the other hand, bile acid synthesis is altered by aging. Cholic acid synthesis increased and chenodeoxycholic acid (mainly β-muricholic acid in the rat) synthesis decreased with the progress of age, while the total bile acid synthesis remained almost constant in rats (9-11). These changes seem to be a cause for the hypercholesterolemia in aged rats.

Because intestinal bacteria in conventional animals convert the primary bile acids to their secondary bile acids (12), However, fecal bile acid composition is complicated. However, the fecal bile acids are roughly classified into two groups, cholic acid group and chenodeoxycholic acid group, according to their origin. The cholic acid group was comprised of cholic, deoxycholic, 7-oxo-deoxycholic, 12-oxo-deoxycholic and 12-oxo-lithocholic acids, and the chenodeoxycholic acid group was comprised of chenodeoxycholic, α-muricholic, β-muricholic, lithocholic, hyodeoxycholic, ursodeoxycholic, β-hyocholic (ω-muricholic), 7-oxo-lithocholic and 6-oxo-lithocholic acids. The cholic acid/chenodeoxycholic acid (CA/CDCA) ratio in feces was calculated, and we concluded that cholesterol feeding decreased the ratio (6) and aging in-

creased the ratio (9, 10). Although the conversion of bile acids by intestinal bacteria has been extensively examined (12), all the conversions are not fully elucidated and all the secondary bile acids (13) which will be found in feces are not detected quantitatively. In addition, intestinal flora and therefore fecal bile acid compositions are not the same for individual rats even though they are kept under a similar condition.

Therefore, we examined the influence of cholesterol feeding on bile acid metabolism including bile acid synthesis determined by analyzing fecal bile acids, bile acid pool size and biliary bile acids in 2-, 12- and 24-month-old germ-free rats.

MATERIALS AND METHODS

Germ-free male Wistar rats bred in our laboratory were housed individually under germ-free conditions in plastic isolators (Japan Clea Co., Ltd., Tokyo). Ordinary rat chow diet (Oriental CMF diet; Oriental Kobo Co., Ltd., Tokyo) and a cholesterol diet prepared by adding cholesterol at a concentration of 0.5% to the ordinary diet were sterilized by ^{60}Co irradiation and fed to rats for 8 days ad libitum. Two-day feces were collected during the experiment, and the last 2-day fecal samples were analyzed in the present experiments.

At the end of the experiments, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the bile duct was cannulated with PE-10 polyethylene tubing to collect bile for 30 min, while the rectal temperature was controlled at 37℃ using an electrically heated plate (14). Blood was then withdrawn from the abdominal aorta, and the liver, and small and large intestines with their contents were removed.

Blood was kept for at least 30 min at room temperature, and the blood was centrifuged at 3000 rpm for 15 min to obtain the serum. The serum cholesterol level was determined with an enzymatic colorimetric kit (15). About one gram of liver tissue was homogenized with chloroform-methanol (2:1, v/v, 3×10 ml) and then filtered. The liver cholesterol level was determined by a colorimetric method (15).

Bile was extracted with 40 volumes of ethanol at 90℃ for 10 min. After filtration, a portion of the extract was evaporated to dryness under a stream of nitrogen. After adding 2 ml of 1.25 N NaOH solution to the residue, it was hydrolyzed at 120℃ for 6 hr. Then the bile acids in the hydrolysates were extracted three times with 8 ml of diethylether after acidification with 2 ml of 2 N HCl solution. The bile acids were converted to methylester trifluoroacetate derivatives and quantified by gas-liquid chromatography (GLC) using a Shimadzu Gas Chromatograph Model GC-7A (Shimadzu Co., Ltd., Kyoto) equipped with a hydrogen flame ionization detector. Glass columns (1.6 m×3 mm, i.d.) packed with 1.5% QF-1 and 1.5% AN-600 were used for bile acid analysis (16). The operation temperatures were 235℃ and 210℃ for the 1.5% QF-1 and 1.5% AN-600 columns, respectively, and 290℃ for the detector.

The small and large intestines with their contents were homogenized with three volumes of distilled water and a portion of each homogenate was lyophilized. The lyophilized preparations were extracted with 15 ml of absolute ethanol at 90℃ for 1 hr and then filtered. The extraction procedures were repeated three times. The combined extracts were evaporated to dryness under reduced pressure, and the residue was dissolved in 10 ml of 70% ethanol. Then, neutral fats were extracted with an equal volume of *n*-hexane and discarded. The 70% ethanol layer was concentrated to about 1 ml under reduced pressure and diluted with 2.5 N NaOH solution. The solution was placed in an autoclave at 120℃ for 6 hr to hydrolyze the conjugated bile acids, and then the free bile acids were extracted with diethylether after acidification with 2 N HCl solution. The bile acids were quantified by GLC as described above (16).

The fecal samples were lyophilized and ground with a small mill. A portion (usually 1 g) was extracted with 15 ml of absolute ethanol at 90℃ for 1 hr and filtered. The extraction procedures were repeated three times, and the combined extracts were evaporated to dryness under reduced pressure. The residue was dissolved in 10 ml of 90% ethanol, and a portion of this solution (usually 3 ml) was subjected to PHP-LH-20 column chromatography (17) to separate neutral sterols, free bile acids, glycine-conjugated bile acids and taurine-conjugated bile acids. After hydrolysis, the sterols and bile acids were analyzed by GLC as described above (16, 18).

The pool size of bile acids was calculated by summation of the amounts of bile acids in the bile and the small and large intestines (19). In the steady-state, the amount of fecal bile acids was presumed to correspond to the amount synthesized in the liver.

Student's *t*-test was used for statistical analyses, and a P-value less than 0.05 was considered significant.

RESULTS

Fecal excretion of cholesterol and bile acids in germ-free rats of various ages fed ordinary and cholesterol diets are shown in Table 1. Although the fecal excretion of cholesterol and bile acids decreased and the CA/CDCA ratio increased in aged rats, cholesterol feeding increased the fecal excretion of cholesterol and bile acids and decreased the CA/CDCA ratios in animals of all ages. These findings suggest that the composition of bile acids

Table 1. Effect of dietary cholesterol on fecal sterol and bile acids in germ-free rats

	2 Months		12 Months		24 Months	
	Ordinary diet	Cholesterol diet	Ordinary diet	Cholesterol diet	Ordinary diet	Cholesterol diet
Fecal cholesterol (mg/day/rat)	9.4±0.31	43.2±2.64[a]	6.5±0.20[b]	39.9±1.95[a]	5.4±0.26[b,c]	21.4±2.40[a,b,c]
Fecal total bile acids (mg/day/rat)	4.1±0.19	12.7±0.68[a]	2.2±0.19[b]	9.9±0.70[a,b]	2.1±0.28[b]	8.2±0.67[a,b]
Cholic acid	1.4±0.06	1.6±0.08	1.0±0.09[b]	2.2±0.27[a]	1.1±0.13	2.4±0.18[a,b]
Chenodeoxycholic acid	0.1±0.01	0.5±0.04[a]	0.1±0.01	0.3±0.04[a]	<0.1	0.2±0.03[a]
α-Muricholic acid	0.1±0.01	0.6±0.05[a]	<0.1	0.4±0.03	<0.1	0.3±0.04[a]
β-Muricholic acid	2.3±0.11	9.6±0.50[a]	1.0±0.09[b]	6.5±0.48[a,b]	0.8±0.13[b]	4.9±0.46[a,b]
Unknown	0.2±0.03	0.5±0.10	0.1±0.01	0.4±0.07[a]	0.1±0.01	0.4±0.09[a]
Fecal CA/CDCA ratio	0.58±0.02	0.17±0.01[a]	0.88±0.02[b]	0.32±0.03[a,b]	1.30±0.08[b,c]	0.46±0.01[a,b,c]

Mean±S.E. of 5 rats. [a]Statistically significant compared to the age-matched ordinary diet group ($P<0.05$), [b]statistically significant compared to 2-month-old rats ($P<0.05$), [c]statistically significant compared to 12-month-old rats ($P<0.05$). CA/CDCA: Cholic acid/chenodeoxycholic acid (mainly chenodeoxycholic acid plus α- and β-muricholic acids).

synthesized in the liver changes with aging, showing an increase in cholic acid production and decrease in chenodeoxycholic acid, and cholesterol feeding increased the synthesis of chenodeoxycholic acid in all age groups at almost the same ratio.

The pool size of bile acids and the bile acid composition are shown in Table 2. The pool size was almost the same for 2- and 12-month-old rats, but that in 24-month-old rats was lower than those in the younger groups. Cholesterol feeding increased the pool size, but the increase in 24-month-old animals was statistically insignificant. Bile acid contents in the large intestine were generally increased after cholesterol feeding.

We next examined the changes in individual bile acids. Cholesterol feeding decreased the level of cholic acid and increased those of chenodexycholic acid-related bile acids, especially β-muricholic acid, resulting in a marked decrease in the CA/CDCA ratio.

Bile flow and biliary bile acid secretion are shown in Table 3. Aging or cholesterol feeding caused no significant changes in either the bile flow or the biliary bile acid secretion when the values were expressed on a per rat basis. Aging, however, increased the CA/CDCA ratio and cholesterol feeding decreased the ratio. These findings coincided well with those regarding the fecal and pool bile acids, but the CA/CDCA values were highest in the bile and lowest in the feces in all the rat groups.

Table 4 shows the serum and liver cholesterol levels in these rats. The serum and liver cholesterol levels seemed to increase with aging, and changes in the serum choles-

Table 2. Effect of dietary cholesterol on bile acid pool in germ-free rats

	2 Months		12 Months		24 Months	
	Ordinary diet	Cholesterol diet	Ordinary diet	Cholesterol diet	Ordinary diet	Cholesterol diet
Pool size (mg/rat)	74.4±2.95	108.0±2.86[a]	81.4±4.73	103.9±3.08[a]	52.2±5.53[b,c]	64.9±2.05[b,c]
Bile (mg/30 min/rat)	8.1±0.50	10.0±1.17	9.0±0.83	10.9±1.04	7.3±0.86	9.3±1.25
Small intestine (mg/rat)	62.6±2.47	80.3±1.75[a]	68.5±4.52	73.4±3.76	43.3±5.38[b,c]	45.2±2.15[b,c]
Large intestine (mg/rat)	3.7±0.15	17.7±0.76[a]	3.9±0.91	19.7±1.38[a]	2.1±0.22[b,c]	10.6±0.38[a,b,c]
Bile acid composition (mg/rat)						
Cholic acid	27.4±0.60	11.1±1.03[a]	38.6±3.01[b]	23.8±1.55[a,b]	35.2±3.26	27.5±0.25[b]
Chenodeoxycholic acid	2.1±0.20	5.4±0.14[a]	1.5±0.13	3.2±0.18[a,b]	0.9±0.10	3.0±0.23[b,c]
α-Muricholic acid	1.1±0.11	3.2±0.19	0.9±0.09	2.3±0.17	0.5±0.08	0.8±0.10
β-Muricholic acid	37.0±2.49	79.0±3.47[a]	34.2±1.70	66.6±4.22[a]	12.9±2.78[b,c]	29.5±2.27[a,b,c]
Unknown	0.2±0.03	0.5±0.10	0.1±0.01	0.4±0.07	2.9±0.14	4.0±0.27
Pool CA/CDCA ratio	0.67±0.02	0.18±0.01[a]	1.02±0.07[b]	0.36±0.03[a,b]	2.53±0.13[b,c]	0.84±0.05[a,b,c]

Mean±S.E. of 5 rats. [a]Statistically significant compared to the age-matched ordinary diet group ($P<0.05$), [b]statistically significant compared to 2-month-old rats ($P<0.05$), [c]statistically significant compared to 12-month-old rats ($P<0.05$). CA/CDCA: Cholic acid/chenodeoxycholic acid (mainly chenodeoxycholic acid plus α- and β-muricholic acids).

Table 3. Effect of dietary cholesterol on biliary bile acids in germ-free rats

	2 Months		12 Months		24 Months	
	Ordinary diet	Cholesterol diet	Ordinary diet	Cholesterol diet	Ordinary diet	Cholesterol diet
Bile flow	1.24±0.04	1.34±0.02	1.28±0.02	1.42±0.06	1.38±0.08	1.62±0.09
Biliary bile acids (mg/hr/rat)	16.2±1.01	17.7±0.68	18.0±1.66	21.8±2.08	14.1±1.72	18.1±2.51
Bile acid composition (%)						
Cholic acid	49±2.0	19±0.3[a]	56±1.6[b]	32±2.0[a]	68±4.0[b,c]	38±3.7[a]
Chenodeoxycholic acid	4±0.3	9±0.6[a]	3±0.1	5±0.3[a]	3±0.3	9±0,8[a]
α-Muricholic acid	<1	1±0.2	<1	1±0.1	<1	1±0.1
β-Muricholic acid	44±2.6	69±0.6[a]	39±1.6	60±3.4[a]	17±9.3[b]	36±8.9[a,b]
Unknown	2±0.2	2±0.1	2±0.2	1±0.1	12±2.4[b,c]	16±1.4[a,b]
Biliary CA/CDCA ratio	1.02±0.09	0.24±0.04[a]	1.34±0.09[b]	0.49±0.04[a,b]	3.60±0.48[b]	0.84±0.12[a,b,c]

Mean±S.E. of 5 rats. [a]Statistically significant compared to the age-matched ordinary diet group ($P<0.05$), [b]statistically significant compared to 2-month-old rats ($P<0.05$), [c]statistically significant compared to 12-month-old rats ($P<0.05$). CA/CDCA: Cholic acid/chenodeoxycholic acid (mainly chenodeoxycholic acid plus α- and β-muricholic acids).

Table 4. Effect of dietary cholesterol on serum and liver cholesterol levels in germ-free rats

	2 Months		12 Months		24 Months	
	Ordinary diet	Cholesterol diet	Ordinary diet	Cholesterol diet	Ordinary diet	Cholesterol diet
Body wt (g)	307±9.7	305±4.7	437±7.1[b]	439±5.1[b]	429±7.0[b]	441±5.2[b]
Serum cholesterol (mg/100 ml)	75±3.7	84±2.7	105±6.3[b]	122±6.3[b]	95±5.4[b]	164±8.1[a,b,c]
Liver wt (g)	9.8±0.38	11.0±0.31	11.6±0.45[b]	11.8±0.30	10.8±0.38	11.6±0.42
Liver cholesterol (mg/g)	2.8±0.12	10.9±0.52[a]	3.5±0.24	12.9±0.29[a]	3.5±0.08[b]	13.3±0.85[a]

Mean±S.E. of 5 rats. [a]Statistically significant compared to the age-matched ordinary diet group ($P<0.05$), [b]statistically significant compared to 2-month-old rats ($P<0.05$), [c]statistically significant compared to 12-month-old rats ($P<0.05$).

terol level after cholesterol feeding were markedly different in animals at different ages. Young rats showed only a slight increase (12% on average), while the old rats showed a marked increase (72%) in serum cholesterol level after cholesterol feeding. However, the increases in liver cholesterol level after cholesterol feeding were similar in all groups.

DISCUSSION

Cholesterol feeding increased fecal excretion of bile acids (6–8), mainly those derived from chenodeoxycholic acid in young (2- to 3-month-old) conventional rats (6). On the other hand, fecal bile acid composition was reported to change with age in conventional rats, where the bile acids derived from cholic acid increased and those derived from chenodeoxycholic acid decreased with age (9, 10). Since intestinal bacteria transform the primary bile acids, the fecal bile acid compositions become much more complex, and since the fecal flora are different in individual animals, even if they are kept under similar conditions, their fecal bile acid compositions are different among individuals. In addition, it will be difficult to determine all the secondary bile acids including minor components in the feces by GLC. To avoid these complications, we employed germ-free rats to examine age-related changes in bile acid metabolism and the effect of cholesterol feeding on it.

The present study demonstrated that cholesterol feeding increased the synthesis of bile acids by 3- to 4-fold in rats compared with animals maintained on an ordinary diet. Cholic acid and chenodeoxycholic acid are primarily synthesized from cholesterol in the liver, and chenodeoxycholic acid is further transformed to muricholic acids, mainly β-muricholic acid, in the rat (1). Therefore, the major bile acids in the rat are cholic acid and β-muricholic acid. In the present study with germ-free rats, cholic acid and β-muricholic acid accounted for 34% and 56% of the total bile acids in the feces, 37% and 50% in the pool, and 49% and 44% in the bile of young (2 months old) rats. Although cholesterol feeding increased bile acid synthesis (6–8), it mainly increased the synthesis of chenodeoxycholic acid (mainly β-muricholic acid in rats), resulting in a marked decrease in the CA/CDCA

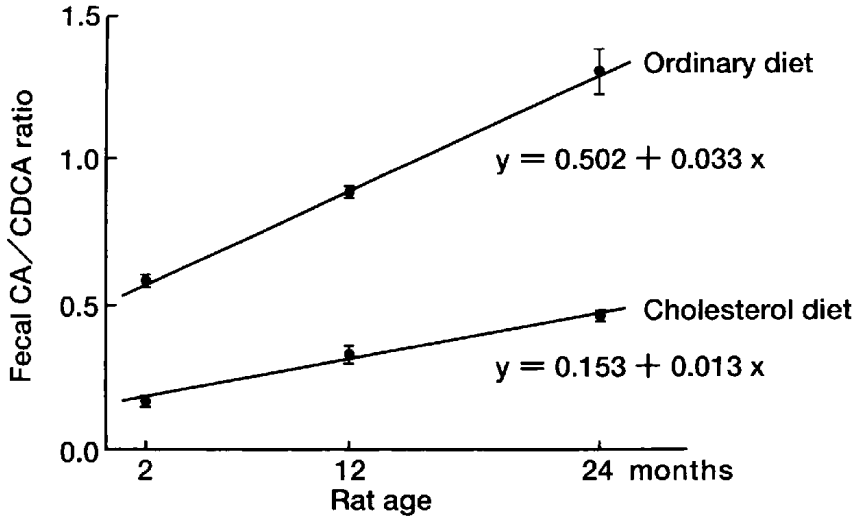

Fig. 1. Age-related increases of cholic acid/chenodeoxycholic acid (CA/CDCA) ratio in germ-free rats fed ordinary and cholesterol supplemented diets.

ratio.

On the other hand, it is known that bile acid metabolism is affected by aging. The present experiments with germ-free rats showed that bile acid synthesis decreased with aging, biliary bile acid secretion remained almost constant, and the pool size of bile acids also remained constant by the age of 12 months, but was decreased in 24-month-old rats. The pool size of cholic acid, however, increased in the aged rats and the value at 24 months of age was comparable with that at 12 months of age. The bile acid composition showed more marked changes than the amounts; cholic acid increased and chenodeoxycholic acid (β-muricholic acid in the rat) decreased, resulting in an age-dependent increase in the CA/CDCA ratio. These changes are shown in Fig. 1, which clearly indicates that cholesterol feeding decreased the CA/CDCA ratio, and the ratios increased almost linearly as a function of age in both diet groups.

These findings obtained from the present experiment were principally the same as those obtained in conventional rats, but the data were more clear-cut in germ-free rats, probably due to the absence of intestinal bacteria. Some differences, however, were found between germ-free rats and conventional animals. The amount of fecal bile acids excreted in a day, which corresponds to the amount of bile acids synthesized in the liver, was decreased in aged germ-free rats but not in conventional rats (10, 11). The pool size of bile acids was decreased in 24-month-old germ-free rats but not in conventional rats (11). The reasons for these differences are not yet known.

In addition, the serum cholesterol level in germ-free rats increased after cholesterol feeding and the increase was remarkable in aged rats, but no hypercholesterolemia was produced in conventional rats (11), although no experiment was performed in aged conventional rats. The liver cholesterol level increased after feeding of a cholesterol diet in both germ-free and conventional rats, but the increase in germ-free rats was more remarkable than that in conventional rats (11). This difference between germ-free and conventional rats will be due a larger pool size of taurocholic acid in germ-free rats.

Our previous experiments (3–5) demonstrated that bile acids enhanced cholesterol absorption resulting in increases in serum and liver cholesterol levels, but the effects of bile acids were found only in the action of cholic acid and not of bile acids related to chenodeoxycholic acid including β-muricholic acid. Taking these observations into account, it is conceivable that the observed increase in β-muricholic acid synthesis is a defense mechanism in rats against hypercholesterolemia induced by cholesterol feeding, preventing increases in cholesterol absorption, and that aging, in contrast, results in a defect in this defense mechanism, leading to a decrease in synthesis of β-muricholic acid.

The mechanisms underlying these changes are not yet known, but diabetic rats lose the ability to synthesize chenodeoxycholic acid (mainly β-muricholic acid) and increase the synthesis of cholic acid (20–22). Since serum insulin levels decrease with aging in rats, we presume that the changes in bile acid synthesis with aging may be related to a decrease in insulin secretion with aging.

Acknowledgment

We thank Miss Michiko Katayama of Shionogi Research Laboratories for preparing the manuscript.

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