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CHOLESTEROL EXCRETION AND LIVER CHOLESTEROL IN RATS DURING EARLY STAGES OF OROTIC ACID FEEDING

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SUMMARY

The early effects of orotic acid on the concentration of plasma cholesterol, liver cholesterol, and fecal excretion of neutral sterols and bile acids were examined. Feeding rats 1% orotic acid for a week resulted in a decrease in plasma cholesterol and an increase in liver cholesterol. The fecal excretion of bile acids and neutral sterols was decreased significantly ($P < 0.02$). This decreased the total sterol excretion ($P < 0.01$). In the bile there was a decrease in the concentration of chenodeoxycholic acid while an increase was observed in the concentration of β-muricholic acid.

INTRODUCTION

Fatty liver produced in rats by adding 1% orotic acid to the diet [1, 2] is mainly due to the accumulation of triglyceride in that organ. This effect of orotic acid was found to be reversed by incorporating adenine into the orotic acid-containing diet [2]. Windmueller and Levy[3] showed that orotic acid feeding caused total inhibition of the secretion of β-lipoproteins from the liver. Studies by Roheim *et al.*[4] suggested that orotic acid might inhibit steps subsequent to the biosynthesis of protein moiety in assembly of lipid and protein moieties. This finding was confirmed by the studies of Pottenger and Getz[5], who further demonstrated the accumulation of apoproteins of the lipoproteins in the livers of rats fed orotic acid. Feeding of orotic acid causes a decrease in plasma cholesterol concentration and an increase in liver cholesterol [6]. What effect this accumulation of cholesterol has on the excretion of cholesterol and bile acids in the feces is not known. This communication describes the fecal excretion of cholesterol and bile acids in rats fed a diet containing 1% orotic acid.

METHODS

One-month-old Sprague–Dawley rats (female) were used for the study. All rats were first fed a basal diet [1, 2] containing 68% sucrose, 18% casein, 8% vegetable oil, 2% yeast, and 4% salt mixture. After 3 weeks of equilibration on this diet, one group of rats was switched to a diet having the same composition as described except that it contained 1% orotic acid; the remaining rats continued on the high carbohydrate diet. After 1 week on an orotic acid diet, histologic studies and analysis of total lipids in liver confirmed the presence of fatty liver in these rats. Cholesterol excretion studies were started after 1 week on an orotic acid diet by keeping rats in metabolic cages as described previously [7]. At the end of the balance study (7 days), bile was collected from these rats after establishing a bile fistula. For this purpose the rats were anesthetized with pentobarbital and a cannula was placed in the bile duct. Collection of bile was started immediately. The animals were then placed in restraining cages with access to water for 4 h. The animals were conscious soon after the operation and during the collection of bile. The bile flow from control and orotic acid-fed rats was similar (3.7 to 4.0 ml in 4 h). It is reported [8, 9] that the first 4-hour collection of bile after bile duct cannulation would provide mainly the bile acid from the total bile acid pool and very little newly synthesized bile acids.

For cholesterol balance studies the total intake of food during the 7-day study period was determined using preweighed containers. The cholesterol concentration in the diet was 0.39 mg/g as determined by gas–liquid chromatography (g.l.c.). The fecal samples from 1 week were pooled, weighed, and homogenized in a known vol. of water. Aliquots of the fecal homogenates were taken for the analysis of neutral sterols and bile acids. β-Sitosterol, which is normally present in the chow diet (1.15 mg/g) as determined in this laboratory, was used as a nonabsorbable marker to correct for variations in fecal flow and stool [10].

The fecal neutral sterols were extracted and purified as described previously [10, 11] based on the procedure of Miettinen *et al.*[12]. After thin-layer chromatography (t.l.c.) the bands corresponding to cholesterol, coprostanol, and coprostanone were eluted and quantitated as trimethylsilyl ether derivatives by g.l.c. using 5α-cholestane as an internal standard [11]. A Packard 409 model gas chromatograph containing 3.8% W-98 columns (4 ft × 4 mm i.d.) was used for the sterol analysis. Column conditions were: flash heater, 300°C; detector, 270°C; column, 320°C; and carrier gas, helium, 50 ml/min. Losses of sterols during the extraction and thin-layer chromatographic

Table 1. Body weights, dietary intake, and plasma lipids of rat (mean ± S.E.M.)

Group	Body weight (g)	Dietary intake	Plasma lipids (mg/dl) Cholesterol*	Triglycerides*
Control rats (5)	224.7 ± 10.6	10.8 ± 2.1	70.0 ± 5.3	112.0 ± 27.5
Orotic acid-fed rats (5)	217.7 ± 12.2	11.4 ± 1.2	37.0 ± 7.1	32.0 ± 7.5

* $P < 0.02$ for difference between groups.

procedures were corrected on the basis of recovery of [^{14}C]-cholesterol added to the fecal homogenate before saponification.

The fecal bile acids were analyzed by a procedure described previously [13, 14] using hyocholic acid (added to the homogenate and followed through the entire analysis) as the internal standard. The bile acids were chromatographed as methyl ester trifluoroacetates on 3% QF-1 columns (4 ft × 4 mm i.d.). A gas chromatograph (F & M model 402) was used for this analysis. Column conditions were: column, 220°C; flash heater, 240°C; detector, 250°C; and carrier gas, helium, 50 ml/min.

The bile samples were saponified with 2.5 M NaOH for 4 h at 110°C [13]. The mixture was diluted with water and extracted with petroleum ether to obtain the neutral sterols. After acidification of the mixture, the bile acids were extracted with diethyl ether [15]. The bile acid fraction was methylated with diazomethane, converted into trifluoroacetate derivatives, and analyzed on a 3% QF-1 column as described previously [14]. Cholic and chenodeoxycholic acids were separated by t.l.c. on silica gel G using the solvent system, iso-octane-isopropyl ether-acetic acid 50:25:40 by vol. [15]. Blood samples (2 to 3 ml) were withdrawn from the heart of the rats at the end of the experimental period to measure the plasma cholesterol and triglyceride levels. Plasma cholesterol and triglycerides were determined by the method of Levine and Zak[16] and Ellefson and Caraway[17], respectively. Liver cholesterol content was determined by lipid extraction [18] followed by saponification and g.l.c. as described for fecal samples.

Statistical analysis of the data was made using Student's t-test and a value of $P < 0.05$ was set as the level of significance.

RESULTS

Table 1 shows the body weights and plasma lipids of control rats and those fed orotic acid. As can be seen, the body weights and dietary intakes of the two groups of animals were not significantly different. The plasma cholesterol and triglyceride concentrations of rats fed orotic acid were significantly lower than those of the control rats.

Table 2 shows the content of liver cholesterol and the excretion of neutral sterols and bile acids by control and orotic acid-fed rats. The concentration of cholesterol in the livers of the orotic acid-fed group was almost four times higher than that of the control rats. However, the excretion of both neutral sterols and bile acids was significantly decreased in the liver of orotic acid-fed rats. This resulted in a significantly lower excretion of total fecal steroids by the orotic acid-fed rats.

Table 3 shows the bile acid composition of the bile and feces of control and orotic acid-fed rats. There was a significant decrease in the percentage composition of chenodeoxycholic acid and a significant increase in the percent composition of β-muricholic acid in the bile of orotic acid-fed rats. No difference in the composition of fecal bile acids was noted between the two groups.

DISCUSSION

This study has confirmed the observation that in rats the plasma lipids (including cholesterol) decrease during feeding of orotic acid. Also, feeding orotic acid increases cholesterol in the liver and decreases the excretion of bile acids in the rat. This is surprising because usually an increase in cholesterol in the liver stimulates [19] cholesterol catabolism to bile acids in the rat. The excretion of neutral sterols was also reduced after feeding orotic acid, a finding that suggests that orotic acid, either directly or due to its ability to inhibit lipoprotein synthesis, causes impairment in the excretion of neutral sterols and bile acids. The dietary intake of cholesterol in the control and orotic acid-fed rats was similar and represented 4.7

Table 2. Liver cholesterol and fecal excretion of neutral sterols and bile acids in rats (mean ± S.E.M.)

Group	Liver cholesterol (mg/g)*	Fecal excretion (mg/kg/day) Neutral sterols*	Bile acids†	Total steroids*
Control rats (5)	2.6 ± 0.1	35.2 ± 1.6	9.1 ± 0.7	44.3 ± 2.5
Orotic acid-fed rats (4)	9.1 ± 0.3	22.0 ± 2.8	5.6 ± 0.8	27.6 ± 1.3

* $P < 0.01$ for difference between groups.
† $P < 0.02$ for difference between groups.

Table 3. Bile acid composition of bile and feces of rats

Bile acid	Composition (% ± S.E.M.)			
	Control rats (6)		Orotic acid-fed rats (6)	
	Bile	Feces	Bile	Feces
3α-Hydroxy-5β-cholanoic acid	—	6.8 ± 0.7	—	4.7 ± 0.6
3β,12α-Dihydroxy-5β-cholanoic acid	0.3 ± 0.1	9.5 ± 4.2	0.4 ± 0.1	7.8 ± 5.0
3α,12α-Dihydroxy-5β-cholanoic acid	1.2 ± 0.1	19.3 ± 7.1	0.5 ± 0.1	15.4 ± 2.1
3α,12β-Dihydroxy-5β-cholanoic acid	—	13.7 ± 4.9	0.2 ± 0.1	5.8 ± 3.7
3α,7α-Dihydroxy-5β-cholanoic acid	13.3 ± 1.0*	—	6.8 ± 1.1	—
3α,6α-Dihydroxy-5β-cholanoic acid	10.0 ± 0.5	18.8 ± 13.1	6.2 ± 0.6	34.3 ± 19.7
3α,7α,12α-Trihydroxy-5β-cholanoic acid	57.6 ± 1.7	15.9 ± 2.6	56.0 ± 2.5	10.3 ± 4.8
7-Keto,3α-hydroxy-5β-cholanoic acid	6.3 ± 0.5	9.6 ± 6.9	5.1 ± 0.4	14.7 ± 5.3
3-Keto,7α-hydroxy-5β-cholanoic acid	1.9 ± 0.8	6.8 ± 2.9	4.8 ± 0.8	7.0 ± 1.5
3α,6β,7β-Trihydroxy-5β-cholanoic acid	9.6 ± 1.0†	—	19.2 ± 2.9†	—

* $P < 0.01$ for difference between two groups.
† $P < 0.05$ for difference between two groups.

to 6.0 mg/day. Hence, the difference in bile acid excretion represents a difference in endogenous synthesis of bile acids.

Recently Carrella *et al.*[20] noted that orotic acid feeding had no effect on cholesterol 7α-hydroxylase, the rate-limiting enzyme of cholesterol biosynthesis. Such discrepancy in the rate of excretion of bile acid and the activity of cholesterol 7α-hydroxylase has been noted previously [7, 21], wherein cholesterol feeding for 1 week stimulated bile acid excretion but showed no change in the activity of cholesterol 7α-hydroxylase. Also, Carrella *et al.* [20] used male rats in their study, whereas this study was done on female rats. This difference is important because the response in terms of bile acid metabolism in male and hepatotoxic female rats to other compounds was found to be different [22]. Carrella *et al.*[20] also found that 12α-hydroxylation of 7α-hydroxy-4-cholestane-3-one (a key step in cholic acid biosynthesis) was decreased while 6β-hydroxylation of lithocholic acid was increased. The biliary bile acid composition noted in this study partly supports this observation, as an increase in β-muricholic acid was found. In a recent study, Kern *et al.*[9] found that in rat bile, in addition to β-muricholic acid, there were two other trihydroxy bile acids with 6β,7β-dihydroxy structure but possessing double bonds in the side chain and in the ring, respectively. In our study the β-muricholic acid peak was not further characterized and might represent a sum of these bile acids. In this study no difference in the percentage of cholic acid was noted between the two groups. Further work on the detailed sequential monitoring of bile acid composition and the key enzymes of bile acid biogenesis after feeding orotic acid needs to be examined.

In conclusion, this study has shown that during early stages of orotic feeding in the rat, cholesterol accumulates in the liver and the fecal excretion of neutral sterols and bile acids is impaired.

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