Hormonal control of cholesterol cholelithiasis in the female hamster

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Abstract Male golden Syrian hamsters from Sasco form cholesterol gallstones when fed a lithogenic diet; in contrast, female hamsters are resistant to stones when fed the identical diet. Upon addition of the synthetic androgen, methyltestosterone, to the diet, the incidence of cholesterol gallstones in female hamsters increased from 0% to 40% after 3 weeks and from 0% to 86% after 6 weeks. Cholesterol cholelithiasis remained high in the males. Biliary cholesterol and phospholipid levels were elevated in the females fed the hormone and approached those of the males. The cholesterol saturation of bile in the females increased from 36% to 75% after 3 weeks and from 54% to 109% after 6 weeks. In addition, an appreciable proportion of the cholesterol in the bile of female hamsters was now present in the form of vesicles. The bile acid *composition* was significantly altered by methyltestosterone even though the total bile acid *concentration* did not change; the bile acid composition of the female hamsters approached that of the males. The glycine to taurine ratio of the bile acids was drastically reduced by methyltestosterone in the females and to a lesser extent in males. In summary, in female hamsters the addition of methyltestosterone to the lithogenic diet induced cholesterol gallstones, elevated total biliary phospholipid and cholesterol, altered the bile acid composition, and changed the distribution of cholesterol from micelles to vesicles. The data obtained so far do not enable us to define the precise mechanism of action of methyltestosterone.—**Ayyad, N., B. I. Cohen, E. H. Mosbach, T. Mikami, Y. Mikami, and A. Ohshima.** Hormonal control of cholesterol cholelithiasis in the female hamster. *J. Lipid Res.* 1995. **36:** 1483–1488.

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A hamster model of cholesterol cholelithiasis has been in use in our laboratory for a number of years (1–5). Male golden Syrian hamsters from Sasco, Inc. gave the highest and most reproducible stone incidence among the various hamster strains studied (3, 5). The hamsters were fed a nutritionally adequate semipurified diet containing 2% corn oil, 4% butterfat, and 0.3% cholesterol. This diet produced cholesterol gallstones in 50–70% of the animals (1, 2). The substitution of 1.2% palmitic acid for butterfat raised the incidence of gallstones to more than 90% (1, 4). Under these conditions no cholesterol stones formed in female hamsters regardless of strain or age (3).

The observed sex difference indicated that in this model, as in humans, hormonal factors play a role in the development of cholesterol gallstones. Consequently, we examined the effect of feeding the synthetic androgen, methyltestosterone (MeT), in the lithogenic diet on the incidence of cholelithiasis. It was found that MeT stimulated the production of cholesterol gallstones in the female hamsters; stone incidence increased from 0% to more than 85% under the conditions used.

MATERIALS AND METHODS

Animals and diets

Four-week-old male (59–67 g) and female (45–62 g) golden Syrian hamsters (*Mesocricetus auratus*) were purchased from Sasco, Inc., Omaha, NE. The animals were maintained with water and rodent chow ad libitum during a 1-week quarantine period prior to the administration of the experimental diets. All hamsters were

Abbreviations: MeT, methyltestosterone; LD, lithogenic diet; CH, cholesterol; LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; ACA, allocholic acid; HDCA, hyodeoxycholic acid; MDCA, murideoxycholic acid; GLCA/TLCA, glycine to taurine ratio of LCA; GDCA/TDCA, glycine to taurine ratio of DCA; GCDCA/TCDCA, glycine to taurine ratio of CDCA; GCA/TCA, glycine to taurine ratio of CA; CA+/CDCA+, ratio of cholic acid metabolites to chenodeoxycholic acid metabolites.

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then fed a pelleted lithogenic diet containing 0.3% cholesterol (LD) either with or without 0.05% methyltestosterone (MeT) (Dyets, Inc., Bethlehem, PA). The composition of the LD was as follows: 43.4% corn starch, 20% casein, 14.6% dyetrose (soluble starch), 10% fiber (cellulose), 5% salt mix (modified U.S.P. XIV salt mix no. 200951), 4% butterfat, 2% corn oil, 0.5% vitamin mix (no. 300000), 0.3% cholesterol, and 0.2% choline chloride. The hamsters were placed into one of the following eight groups: group 1, males fed LD for 3 wk; group 2, males fed LD + MeT for 3 wk; group 3, males fed LD for 6 wk; group 4, males fed LD + MeT for 6 wk; group 5, females fed LD for 3 wk; group 6, females fed LD + MeT for 3 wk; group 7, females fed LD for 6 wk; group 8, females fed LD + MeT for 6 wk. At the end of the feeding period, the hamsters were fasted for 24 h and anesthetized with 20 mg ketamine hydrochloride (Fort Dodge Laboratories, Inc., Fort Dodge, IA). The gallbladder was exposed and bile was aspirated with a syringe and examined for the presence of cholesterol crystals and liquid crystals. Aliquots of the bile were taken immediately for the determination of biliary lipids and biliary vesicle/micelle distribution. The gallbladder was opened and examined for gallstones.

Analytical procedures

The presence of cholesterol gallstones and crystals was confirmed under a polarizing light microscope (Olympus MCHAP microscope, Olympus Corp., Lake Success, NY) and by diffuse reflectance Fourier transform infrared spectroscopy (6).

Biliary bile acids were determined as the methyl ester acetates as previously described (7). The biliary bile acids from the hamsters fed for the 6-wk period were also analyzed by high pressure liquid chromatography (HPLC) to determine the glycine to taurine ratio of the major bile acids. HPLC was performed using a Nova C_{18} 4 μ column (Waters Associates) with 0.01 M potassium phosphate in methanol–water (75:25, pH 5.37, adjusted with 85% phosphoric acid) and a flow rate of 0.9 ml/min as previously reported (8). Biliary phospholipids were determined by an enzymatic-colorimetric procedure (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cholesterol in bile was determined by gas–liquid chromatography as the trimethylsilyl ether derivative as previously reported (9). The distribution of biliary cholesterol and phospholipid in vesicles and micelles was determined by gel filtration chromatography. The cholesterol carriers were separated on Sepharose CL-4B-200 (Sigma Chemical Co., St. Louis, MO) using the method of Pattinson (10) and Pattinson, Willis, and Frampton (11) with minor modifications. The elution buffer contained phosphate-buffered saline (10 mM, pH 7.4) with 0.04% sodium azide and 6 mM sodium taurocholate; this method has been previously described by our laboratory (12). We added 100 μl of bile to the gel filtration column and collected 80 fractions (500 μl each). Ideally, the sample should be eluted from the column with a buffer containing the intermicellar bile salt concentration (IMC). As this was not possible in the present study because of the small amounts of bile obtainable from the hamsters, every sample was eluted with 6 mM taurocholate. This procedure may not yield the absolute ratio of vesicles to micelles in bile (which is very difficult to measure in any case). We believe, however, that the methodology used allows us to compare differences among groups as all samples were separated under identical conditions. The micellar/vesicular profiles obtained in the hamster have been shown to be reproducible under the conditions used (12).

Calculations and statistics

The cholesterol saturation indices were determined using previously published methods (13, 14). All data were reported as mean ± SD. Differences between groups were calculated using ANOVA to determine the F statistic. Student's *t*-test was then used for values for which the F statistic was significant ($P < 0.05$) (15).

RESULTS

All hamsters ingested an average of 10 g of food per day and remained healthy throughout the experimental periods. There were no differences in weight gain between the controls and the hormone-treated groups.

Table 1 summarizes the cholesterol gallstone incidence and biliary lipid composition at the time of killing. Female hamsters did not develop gallstones on the lithogenic diet after 3 wk (group 5) or 6 wk (group 7); the cholesterol gallstone incidence in the males increased from 20% after 3 wk (group 1) to 75% after 6 wk (group 3). The addition of MeT did not significantly increase the rate of stone formation in the males. In contrast, MeT stimulated the production of cholesterol stones in the female hamsters: 40% after 3 wk (group 6) and 86% after 6 wk (group 8). Liquid crystals were present in almost all bile samples as seen under the polarizing light microscope. Total biliary lipids were not significantly affected by MeT feeding but were reduced by prolonged cholesterol feeding (16.52 vs. 12.86 g/dl, group 1 vs. group 3 for male controls and 13.69 vs. 9.19 g/dl, group 2 vs. group 4 for males fed MeT; 16.94 vs. 9.51 g/dl, group 5 vs. group 7 for female controls and 17.31 vs. 10.67 g/dl, group 6 vs. group 8 for females fed MeT). The concentrations of biliary phospholipid and cholesterol in the female hamsters were greatly elevated by the addition of MeT to the diet (3.76 vs. 7.91 mole % after 3 wk and 10.77 vs. 18.38 mole % after 6 wk for

TABLE 1. Effect of gender and methyltestosterone feeding on biliary lipids and cholesterol gallstone incidence in the hamster

Group	Feeding Period	MeT	Cholesterol Gallstones	Phospholipids	Cholesterol	Bile Acids	Total Lipids	Cholesterol Saturation
	wk		*(%)*		*mol %*		*g/dL*	*%*
Male								
1	3	–	2/10 (20)	10.81 ± 1.64	4.69 ± 1.05	84.50 ± 2.34	16.52 ± 4.33	91 ± 16
2	3	+	2/10 (20)	11.77 ± 2.56	4.84 ± 1.54	83.39 ± 3.96	13.69 ± 3.50	103 ± 14
3	6	–	15/20 (75)	15.28 ± 2.27[a]	4.67 ± 0.92[a]	79.88 ± 3.15[a]	12.86 ± 2.93[a]	83 ± 15[a]
4	6	+	20/22 (90)	17.22 ± 2.02	6.47 ± 1.09	76.55 ± 2.39	9.19 ± 2.73	110 ± 21
Female								
5	3	–	0/10 (0)[b]	3.76 ± 1.41[c]	1.12 ± 0.48[c]	95.11 ± 1.60[c]	16.94 ± 4.13	36 ± 14[c]
6	3	+	4/10 (40)	7.91 ± 1.82	3.07 ± 0.95	89.01 ± 2.65	17.31 ± 5.80	75 ± 17
7	6	–	0/21 (0)[d]	10.77 ± 2.15[d]	2.28 ± 0.71[d]	86.87 ± 2.65[d]	9.51 ± 2.65	54 ± 13[d]
8	6	+	19/22 (86)	18.38 ± 2.39	7.04 ± 2.48	74.22 ± 4.01	10.67 ± 2.41	109 ± 32

See Materials and Methods for details of the hamster diets. wk, week; MeT, methyltestosterone. Numbers are mean ± SD.
[a]Differs from group 4, $P < 0.01$.
[b]Differs from group 6, $P < 0.05$.
[c]Differs from group 6, $P < 0.01$.
[d]Differs from group 8, $P < 0.01$.

phospholipids; 1.12 vs. 3.07 mole % after 3 wk and 2.28 vs. 7.04 mole % after 6 wk for cholesterol; group 5 vs. group 6 and group 7 vs. group 8, respectively). These concentrations were also significantly increased in the males after 6 wk but not as drastically as in the females. In the female hamsters, there was a corresponding decrease in the mole % of biliary bile acids: 95.11 vs. 89.01 mole % after 3 wk (group 5 vs. group 6) and 86.87 vs. 74.22 mole % after 6 wk (group 7 vs. group 8). The cholesterol saturation of bile was raised in females: 36 vs. 75% after 3 wk (group 5 vs. group 6) and 54 vs. 109% after 6 wk (group 7 vs. group 8). The cholesterol saturation was also elevated by MeT in the male hamsters, but the difference was significant only after the 6-wk feeding period.

Gel filtration column chromatography showed that vesicles were present in bile of male hamsters on both diets. Vesicles appeared to form in the bile of the females only when MeT was added to the diet.

Table 2 summarizes the effect of MeT feeding for 6 wk on the biliary bile acid composition of male (group 3 vs. group 4) and female (group 7 vs. group 8) hamsters. Deoxycholic acid (DCA) was significantly elevated in the males (9.37 vs. 14.77%) and reduced in the females (17.08 vs. 11.14%). Chenodeoxycholic acid (CDCA) was reduced in the males (26.21 vs. 21.99%) and elevated in the females (16.26 vs. 23.84%). Cholic acid (CA) was unchanged in the males but was significantly reduced in the females (51.07 vs. 41.79%). Hyodeoxycholic acid (HDCA) and murideoxycholic acid (MDCA) were both significantly raised in the females (4.24 vs. 8.13% and 1.78 vs. 2.41%, respectively) but unchanged in the males. It is noted that in all cases, the bile acid composition of the female hamsters fed MeT approached that of the male hamsters fed the LD. The glycine to taurine ratios (G/T) of lithocholic acid (LCA), DCA, CDCA, and CA were all significantly reduced in the males given MeT. These conjugate ratios were even further reduced in the females except for the CA conjugates which remained relatively unchanged. The ratio of CA plus metabolites (CA + ACA + DCA = CA+) to CDCA plus metabolites (LCA + CDCA + HDCA + MDCA = CDCA+) [CA+/CDCA+] was elevated in the males and drastically reduced in the females (3.05 vs. 1.48). Again, the bile acid composition of the females approached that of the males in all respects when the diet was supplemented with 0.05% MeT.

DISCUSSION

It has already been shown that diet, age, sex, and strain affect experimental cholesterol cholelithiasis in the hamster model (1, 2). All attempts to induce cholesterol cholelithiasis in female hamsters with our lithogenic diets failed (3). Male hamsters gave variable results as a function of specific dietary components (4, 5) and source of hamster (3, 5). The aim of this study was *a)* to determine whether the gender dependence of cholelithiasis is hormonal in origin and *b)* to determine whether hormones affect biliary lipids and cholesterol carriers in hamster bile. Because the Sasco strain was the most susceptible to the formation of gallstones, we used it for this study (3, 5).

The addition of MeT, a synthetic androgen, to the lithogenic diet of female hamsters induced cholesterol gallstones. The gallbladders of most of the animals were completely filled with cholesterol stones and crystals. As early as 3 weeks after MeT administration, 40% of the females had developed gallstones. The hormone also produced a small increase in gallstone incidence in male hamsters. Evidently, in this strain of hamster there

TABLE 2. The effect of methyltestosterone feeding for 6 weeks on biliary bile acid composition in the hamster

Bile Acid Composition	Male Group 3 LD	Male Group 4 LD + MeT	P	Female Group 7 LD	Female Group 8 LD + MeT	P
	%			%		
LCA	1.84 ± 0.34	2.07 ± 0.33	< 0.035	2.00 ± 0.95	2.43 ± 0.55	NS
DCA	9.37 ± 2.28	14.77 ± 5.17	< 0.01	17.08 ± 4.25	11.14 ± 3.79	< 0.01
CDCA	26.21 ± 3.67	21.99 ± 3.31	< 0.01	16.26 ± 4.10	23.84 ± 3.65	< 0.01
CA	44.03 ± 4.94	41.58 ± 4.75	NS	51.07 ± 5.14	41.79 ± 1.97	< 0.01
ACA	3.33 ± 1.08	2.69 ± 0.77	< 0.035	0.31 ± 0.32	3.03 ± 0.88	< 0.01
HDCA	7.56 ± 3.01	6.21 ± 2.83	NS	4.24 ± 2.20	8.13 ± 2.49	< 0.01
MDCA	1.86 ± 0.56	1.88 ± 1.08	NS	1.78 ± 0.98	2.41 ± 0.82	< 0.03
GLCA/TLCA	0.28 ± 0.24	0.14 ± 0.09	< 0.025	0.55 ± 0.41	0.18 ± 0.14	< 0.01
GDCA/TDCA	0.77 ± 0.36	0.44 ± 0.20	< 0.01	1.30 ± 0.79	0.52 ± 0.15	<0.01
GCDCA/TCDCA	1.09 ± 0.41	0.54 ± 0.16	< 0.01	2.72 ± 0.92	0.62 ± 0.17	< 0.01
GCA/TCA	1.08 ± 0.23	0.75 ± 0.13	< 0.01	0.94 ± 0.40	1.12 ± 0.28	NS
CA+/CDCA+	1.46 ± 0.23	1.92 ± 0.49	< 0.01	3.05 ± 0.68	1.48 ± 0.22	< 0.01

LD, lithogenic diet; MeT, methyltestosterone; *P*, statistical *P* value; NS, statistically not significant; LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; ACA, allocholic acid; HDCA, hyodeoxycholic acid; MDCA, murideoxycholic acid; GLCA/TLCA, glycine/taurine ratio of LCA; GDCA/TDCA, glycine/taurine ratio of DCA; GCDCA/TCDCA, glycine/taurine ratio of CDCA; GCA/TCA, glycine/taurine ratio of CA; CA+/CDCA+, ratio of CA metabolites (CA, ACA, DCA) to CDCA metabolites (CDCA, LCA, HDCA, MDCA).

seems to be a powerful relationship between "maleness" and cholesterol gallstone formation. These results differ from those found in humans in which women are almost twice as likely to develop stones as men (16) and the administration of female steroid hormones as well as pregnancy promote cholelithiasis (17–19). This is the first time that the reverse of the human situation, i.e., gallstone induction by male hormones, has been studied in the hamster. These factors lead to the conclusion that, in humans, hormonal imbalance may be a risk factor for cholelithiasis.

As can be seen in Table I, all hamsters that developed cholesterol gallstones did not have a cholesterol saturation index above unity. In all cases, however, the CSI of the animals fed the gallstone-inducing diet was higher than the CSI of the hamsters fed the control diet. We and others have previously reported that animals developed cholesterol gallstones even though measurement of the CSI at time of killing gave values below unity (3, 20, 21). Presumably, from equilibrium dynamics, the CSI must have been above unity at an earlier time point for cholesterol to precipitate from solution and form crystals and stones. The measurement of the CSI was calculated using a phase diagram that was based on an ideal model system that is at thermodynamic equilibrium (14, 22). It is possible that the residence time of hamster bile in the gallbladder may not have allowed for this equilibrium to be reached, resulting in a value for the CSI that was below unity.

Gilloteaux et al. (23–27) studied the effect of female sex steroids on epithelial surface changes in the gallbladder and the formation of mucus and crystalline "gallstone-like deposits" in male and female hamsters fed identical diets. Female sex steroid receptors were detected in the epithelium (28). Cell proliferation has been found to be a response of the gallbladder to many injuries such as those produced by lithogenic diets or steroid drugs (29). Changes in the gallbladder epithelium can cause an irritation leading to increased mucus secretion followed by rapid nucleation and stimulation of cholelithogenesis. The effect of MeT on biliary nucleating proteins such as mucin must also be considered as the latter has already been shown to have pronucleating properties (30–32). These factors may play a role in our hamster model, but further studies are needed.

The administration of MeT to female hamsters increased the cholesterol and phospholipid concentrations in bile. The phospholipid molar ratio was elevated almost 2-fold while the cholesterol molar ratio increased 3-fold. The cholesterol saturation was also raised 2-fold and was above 100% after 6 wks, indicating that the bile was saturated with cholesterol. Bile usually becomes saturated when the biliary secretion of cholesterol is increased and/or the biliary secretion of bile acids is decreased. Both the cholesterol and phospholipid levels were elevated in bile by MeT feeding; this suggests an increase in vesicle formation (33). Cholesterol-phospholipid vesicles are the predominant forms of biliary cholesterol secretion (34–37). Male hamsters that formed stones on our lithogenic diet had bile that contained cholesterol in the form of vesicles with a high cholesterol/phospholipid ratio as observed by gel filtration column chromatography. These vesicles were unstable and therefore cholesterol "precipitated" from bile. Female hamsters fed the lithogenic diet had a lower cholesterol concentration in bile relative to males and

their bile contained only micelles with a low cholesterol/phospholipid ratio. These carriers were stable and therefore no stones formed. The addition of MeT to the diet of females resulted in increased amounts of vesicles in gallbladder bile.

Other studies have shown that hormones are capable of shifting the proportion of cholesterol carriers in bile. Thyroid hormone administration in rats increased the secretion of cholesterol-rich vesicles in bile in a manner similar to the administration of MeT (38). This supported the hypothesis that secretion of biliary cholesterol and phospholipids involved a vesicle-specific pathway. Gilloteaux (23) showed that after a 10-day estradiol treatment in male Syrian hamsters, large vesicles developed and "stone-like deposits" were evident in the gallbladder. Ethinylestradiol (EE) also increased cholesterol saturation in bile due to increased biliary cholesterol output (39). Hormones, in general, seem to increase the cholesterol-phospholipid output and tend to shift cholesterol into vesicles.

The biliary bile acid composition of the female hamsters was significantly altered by MeT feeding and approached that of the male hamsters. The males fed the lithogenic diet had higher CDCA and lower CA concentrations than the females. MeT raised the CDCA and lowered the CA concentrations in the female to levels found in males: the CA+/CDCA+ ratio was reduced in females. This was due in part to the reduction in CA and DCA and the increase in CDCA, MDCA and HDCA. The chemical structure of bile acids has been shown to influence the magnitude of lipid secretion in the bile of hamsters. It has been reported that the conjugates of CDCA induced a greater secretion of biliary phospholipid and cholesterol than those of CA when these bile acids were infused into the proximal small intestine of a bile fistula hamster (40). As observed in our hamster model, biliary CDCA was elevated in the females fed MeT; cholesterol and phospholipid levels in bile were also increased implying a bile acid link to lipid secretion.

The 12α-hydroxylase and 6-hydroxylase enzymatic pathways, which regulate the composition of primary and secondary bile acids in bile, seemed to be affected by hormonal factors in our model. Kuroki et al. (41) demonstrated a sex-related difference in the ratio of CA to CDCA in the hamster. He found that the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one was 2 times greater in the female hamsters than the males. Thus, the higher 12α-hydroxylase activity in the females would result in an increase in CA relative to CDCA. The addition of MeT to the diet presumably reduced the activity of this enzyme as the % of CDCA was increased at the expense of CA. HDCA and MDCA were also elevated in the females fed MeT. These bile acids are formed from LCA by a 6α-hydroxylase and a 6β-hydroxylase, respectively (42). The LCA 6β-hydroxylase has been characterized as a P450 family 3 protein, CYP3A10, (42) and the level has been found to be higher in male than female hamsters. MeT could elevate 6β-hydroxylation as this P450 has been shown to be hormone-dependent (43). Estrogen has also been found to alter bile acid composition in the hamster (44, 45). Thus, in hamsters, the bile acid composition seems to be hormone-dependent.

We conclude that MeT stimulates the secretion of cholesterol and phospholipid into bile and induces cholesterol gallstones in the female golden Syrian hamster. The hormone alters the biliary cholesterol distribution among its carriers. The hormone-induced changes in biliary bile acid composition may play a role in the increased cholelithiasis but further studies in this area are needed.

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