12α-Hydroxylase activity in human liver and its relation to cholesterol 7α-hydroxylase activity

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Abstract Interruption of the enterohepatic circulation by cholestyramine causes a several-fold increase in bile acid synthesis, reflected in a stimulation of cholesterol 7α-hydroxylase activity; the synthesis of cholic acid being stimulated to a greater extent than chenodeoxycholic acid. It is not known if this preferential increase in cholic acid is due to an increase of the 12α-hydroxylase activity. The present study aimed at investigating the 12α-hydroxylase activity and its relation to cholesterol 7α-hydroxylase activity in liver microsomes of patients with different levels of cholesterol 7α-hydroxylase activity. Liver biopsies were obtained from four gallstone-free patients, and seven untreated and two cholestyramine-treated gallstone patients undergoing cholecystectomy, and four patients with Crohn's disease undergoing intestinal resection. The combined group of cholestyramine-treated and ileum-resected patients had four times higher cholesterol 7α-hydroxylase activity and two times higher 12α-hydroxylase activity than the other patients. A positive correlation was obtained between cholesterol 7α-hydroxylase activity and 12α-hydroxylase activity ($r = +0.69$; $n = 16$). These results indicate that the increased ratio between the synthesis of cholic acid and chenodeoxycholic acid during cholestyramine treatment is due to a compensatory increase of the 12α-hydroxylase activity.—**Einarsson, K., J-E. Åkerlund, E. Reihnér, and I. Björkhem.** 12α-Hydroxylase activity in human liver and its relation to cholesterol 7α-hydroxylase activity. *J. Lipid Res.* 1992. **33:** 1591–1595.

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The initial and rate-limiting step in bile acid biosynthesis is 7α-hydroxylation of cholesterol (1, 2). The cholesterol 7α-hydroxylase catalyzing the 7α-hydroxylation is a cytochrome P450-dependent monooxygenase, which was recently purified and characterized (for a review, see ref. 2). 7α-Hydroxycholesterol is further metabolized to 7α-hydroxy-4-cholesten-3-one, a key intermediate in bile acid synthesis. 7α-Hydroxy-4-cholesten-3-one may be converted to 5β-cholestane-3α, 7α-diol and further metabolized to chenodeoxycholic acid. In cholic acid synthesis, 7α-hydroxy-4-cholesten-3-one is 12α-hydroxylated to yield 7α, 12α-dihydroxy-4-cholesten-3-one. Also the 12α-hydroxylase is a cytochrome P450-dependent microsomal enzyme and seems to play a role in the regulation of the ratio between cholic acid and chenodeoxycholic acid in some species investigated (1, 2). This hydroxylase has not yet been purified to homogeneity.

The possibility that the 12α-hydroxylase activity may be of regulatory importance also in humans has previously been investigated in our laboratory by both in vitro and in vivo techniques (3–5). An in vivo study that used a labeled substrate for the 12α-hydroxylase did not support the contention that the 12α-hydroxylase activity is of major importance for the ratio between cholic acid and chenodeoxycholic acid in bile (5). In another study, 12α-hydroxylase activity was assayed in liver biopsies from patients with different types of hyperlipoproteinemia undergoing cholecystectomy (3). No correlation between 12α-hydroxylase activity and the ratio between cholic acid and chenodeoxycholic in bile was obtained. Apparently factors other than 12α-hydroxylase activity, e.g., different half-lifes and circulation rates of the different bile acids, are more important for composition of bile acids in human bile. On the other hand, treatment of gallstone patients with chenodeoxycholic acid inhibited the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one in liver microsomes by about 50% (4).

Upon interruption of the enterohepatic circulation of bile acids, i.e., by cholestyramine treatment, the synthesis of cholic acid is increased to a greater extent than that of chenodeoxycholic acid (6). It is well known that cholesterol 7α-hydroxylase activity is increased in cholestyramine-treated patients (7), but it is not known if the preferential increase in cholic acid is due to an absolute increase of the 12α-hydroxylase activity. According to some recent work (see ref. 8 for a review) a substantial part of chenodeoxycholic acid synthesis in humans may occur in a pathway that bypasses the regulatory cholesterol 7α-hydroxylase. If so, this may be another ex-

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NADPH, nicotinamide-adenine dinucleotide phosphate.

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planation for the preferential increase in cholic acid synthesis in patients with an up-regulated cholesterol 7α-hydroxylase.

In the present work, the 12α-hydroxylase activity has been assayed in liver microsomes of patients with different levels of cholesterol 7α-hydroxylase activity. A positive correlation between cholesterol 7α-hydroxylase activity and 12α-hydroxylase activity in human liver was obtained, indicating a compensatory increase also of the 12α-hydroxylase activity.

MATERIALS AND METHODS

Materials

Deuterium-labeled 7α-hydroxycholesterol and 7α-[6β-^{3}H]hydroxy-4-cholesten-3-one (sp act 11.7 μCi/mg) were synthesized as described previously (9, 10). Ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), and the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Co., St. Louis, MO.

Patients

The study comprised four gallstone-free patients, nine gallstone patients, and five patients with Crohn's disease (**Table 1**). The gallstone-free patients were cholecystectomized because of suspected adenomyoma or polyps of the gallbladder. The gallstone patients underwent elective cholecystectomy. The gallstones were of pigment type in one of the patients and of cholesterol type in the other ones as judged by inspection and/or chemical analysis. Two of the gallstone patients had been treated with cholestyramine (Questran®, Bristol-Myers) in a daily dose of 16 g (8 g b.i.d.) for 3 weeks prior to surgery. One patient with Crohn's disease underwent colectomy. The other four patients were subjected to resection of different lengths of the terminal ileum. Some of these patients had undergone one or more partial ileal or ileocolic resections on previous occasions. The lengths of resected ileum were estimated by adding any previously performed resection to the present one and amounted to 40 to 120 cm. One patient with Crohn's disease was on treatment with prednisolone and had total parenteral nutrition before operation.

TABLE 1. Clinical data of the patients

Patient Number, Sex[a]	Age	Relative Body Weight[b]	Length of Resected Part of Distal Ileum	Extension of Crohn's Disease	Other Clinical Disorders, Medical Treatment
	yr	*%*	*cm*		
Gallstone-free					
1F	20	92			
2F	45	97			
3F	41	104			
4F	50	84			
Pigment gallstone					
5F	51	97			
Cholesterol gallstone					
6F	39	97			
7F	58	100			
8F	41	111			
9F	30	84			
10F	40	90			
11M	58	72			
Cholestyramine-treated					
12F	33	92			
13F	41	86			
Crohn's disease					
14F	39	89		Colon	Cholelithiasis
15F	60	89	40	Distal ileum	
16M	40	92	110	Ileocolic	
17F	33	74	120	Ileocolic	Cholelithiasis, TPN[c], prednisolone
18M	39	89	120	Ileocolic	

[a] F, female; M, male.

[b] Calculated as $\frac{\text{weight (kg)}}{\text{height (cm} - 100)} \times 100\%$.

[c] TPN, total parenteral nutrition.

Informed consent was obtained from each patient before operation. The ethical aspects of the study were approved by the Ethical Committee at Huddinge University Hospital. Data on cholesterol 7α-hydroxylase activity in some of the patients have been included in two previous publications (7, 11).

Experimental procedure

The patients were admitted to the hospital on the day before operation and were given the regular hospital diet containing about 0.5 mmol of cholesterol per day. To prevent possible influence of any diurnal variation in enzyme activity, operation was always performed between 8 and 9 AM after a 12-h fast. Standardized anesthesia was given during the operation (12). A wedge biopsy weighing 2–4 g was taken from the left lobe of the liver immediately after opening the abdomen. The biopsy was placed in ice-cold buffer, and immediately transported to the laboratory.

Preparation of liver microsomes

The liver biopsy was minced and homogenized in nine volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM DTT, and 10 mM EDTA. The homogenate was centrifuged at 20,000 *g* for 15 min. The supernatant fraction was immediately centrifuged at 100,000 *g* for 60 min. The pellet was suspended in homogenizing medium lacking DTT, and recentrifuged at 100,000 *g* for 60 min. The resulting microsomal fraction was suspended in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, to give a final concentration of 10% (w/v). The microsomal content of protein was determined by the method of Lowry et al. (13).

Assay of cholesterol 7α-hydroxylase activity

The activity of cholesterol 7α-hydroxylase was assayed as described recently (14). The assay system consisted of 0.5 ml of the microsomal fraction and 0.5 ml 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM NADPH in a total volume of 1.0 ml. After the incubation was stopped, deuterium-labeled 7α-hydroxycholesterol was added as internal standard. The amount of 7α-hydroxycholesterol formed was determined by combined gas–liquid chromotography–mass spectrometry and was expressed as pmol/min per mg protein.

Assay of 12α-hydroxylase activity

The activity of 12α-hydroxylase was assayed essentially as described recently (3). 7α-[6β-^{3}H]hydroxy-4-cholesten-3-one, 100 μg, dissolved in 20 μl of acetone was incubated with 0.75 ml of the microsomal fraction in a total volume of 2 ml of 0.1 M Tris- buffer solution, pH 7.4, containing 1 mM NADPH. After incubation for 10 min at 37°C, 20 vol of chloroform–methanol 2:1 (w/v) was added. Unlabeled 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one were added as internal standards and the incubation products were separated by thin-layer chromatography using toluene–ethyl acetate 1:1 (w/v) as developing solvent. The compounds were located with iodine vapor. The conversion of labeled 7α-hydroxy-4-cholesten-3-one to 7α,12α-dihydroxy-4-cholesten-3-one was measured by scanning the chromatoplate with a radioscanner (Berthold, Wildad, Germany). The enzyme activity was expressed as pmol/min per mg protein.

Statistical analysis

Data are given as means ± SEM. The statistical significance of differences was evaluated with Student's *t*-test. Correlations were calculated by the method of Pearson, and their significances were tested by estimating the correlation coefficient, *r*.

RESULTS

The results are summarized in **Table 2.** In the gallstone-free subjects and the untreated gallstone patients the cholesterol 7α-hydroxylase activity averaged 7.2 ± 1.9 pmol/min per mg protein. The two cholestyramine-treated gallstone patients had a several-fold higher 7α-hydroxylase activity than the untreated patients. The combined group of cholestyramine-treated and ileum-resected patients had four times higher 7α-

TABLE 2. Hepatic microsomal activities of cholesterol 7α-hydroxylase and 12α-hydroxylase (individual values)

Patients Number	Cholesterol 7α-Hydroxylase	12α-Hydroxylase
	pmol/min/mg protein	
Gallstone-free		
1		457
2	9,1	453
3	1,8	559
4		434
Pigment gallstone		
5	19,8	533
Cholesterol gallstone		
6	5,4	430
7	4,1	470
8	6,9	244
9	11,9	718
10	2,2	53
11	3,8	678
Cholestyramine-treated		
12	67,0	959
13	33,3	1122
Crohn's disease		
14	10,4	398
15	12,1	311
16	13,4	515
17	35,1	858
18	31,6	1409

hydroxylase activity than the other patients (32.1 ± 8.1 vs. 7.5 ± 1.7 pmol/min per mg protein, $P=0.002$).

The 12α-hydroxylase activity was about the same in gallstone-free subjects (475 ± 24 pmol/min per mg protein) as in gallstone patients. (447 ± 82 pmol/min per mg protein). The activity was about twice as high in the two cholestyramine- treated patients as compared to the untreated patients. The combined group of cholestyramine-treated and ileum-resected patients had significantly higher 12α-hydroxylase activity than the other patients (862 ± 163 vs 452 ± 51 pmol/min per mg protein, $P=0.007$).

As can be seen in **Fig. 1** there was a positive correlation between the cholesterol 7α-hydroxylase activity and the 12α-hydroxylase activity ($r=0.69$, $P < 0.01$).

DISCUSSION

The present study confirms that cholestyramine treatment and resection of terminal ileum stimulates the cholesterol 7α-hydroxylase activity severalfold (7, 11). A finding of potential importance was that the 12α-hydroxylase activity was also stimulated under the same conditions as the cholesterol 7α-hydroxylase activity. The mechanism behind this stimulation can only be speculated on at the present state of knowledge.

In a previous study in rats we reported that biliary drainage led to an eightfold increase in the 7α-hydroxylation of cholesterol and a twofold increase in the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one (15). In a subsequent study in rats, Johansson (16) compared the effects of biliary drainage and cholestyramine treatment on the two hydroxylations. He confirmed that biliary drainage stimulated the rate of 7α-hydroxylation of cholesterol (sevenfold) as well as the rate of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one (fivefold). Cholestyramine treatment increased the 7α-hydroxylation to about the same extent as biliary drainage. There was some stimulation also of the 12α-hydroxylase but this stimulation was relatively small (about twofold) and did not reach statistical significance. Johansson (16) found that 3 days of starvation stimulated the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one fourfold. The cholesterol 7α-hydroxylase activity was unaffected or reduced by starvation, and other groups have later found a clear inhibiting effect of starvation on this enzyme activity (1). The above early findings made it probable that the different effects on the 12α-hydroxylation system of biliary drainage and cholestyramine were due to low food intake in the rats with a bile fistula. However, the increased 12α-hydroxylase activity in the present cholestyramine-treated and ileum-resected patients cannot be explained by diminished food intake or prolonged starvation. One of the patients with Crohn's disease had total parenteral nutrition prior to operation and all the other patients had fasted not more than 12 h before operation.

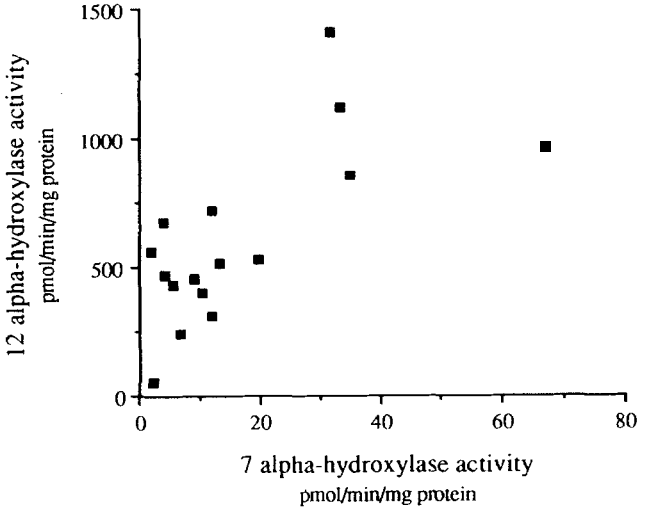

Fig. 1. Relationship of the microsomal cholesterol 7α-hydroxylase activity and the microsomal 12α-hydroxylase activity in human liver tissue in 16 patients, see Table 1 ($r=0.69$, $P < 0.01$).

The positive correlation between the 12α-hydroxylase and the cholesterol 7α-hydroxylase activities may indicate that the two hydroxylases have a regulatory factor in common. Previously Danielsson et al. (17) reported that the activity of reconstituted 12α-hydroxylase could be stimulated by a protein fraction isolated from rabbit liver microsomes. This fraction also stimulated reconstituted cholesterol 7α-hydroxylase activity. Later, however, Lidström-Olsson (18) could separate the factor stimulating 12α-hydroxylase from that stimulating the cholesterol 7α-hydroxylase, indicating that the activity of these two enzymes might be separately regulated by different specific proteins in the microsomal membranes.

We recently reported that cholesterol 7α-hydroxylase activity in human liver is regulated by the portal inflow of bile acids (7). Cholestyramine stimulated the enzyme activity severalfold whereas treatment with chenodeoxycholic acid inhibited the 7α-hydroxylase activity considerably. The composition of individual bile acids seemed to be more important than the total concentration of bile acids in the portal vein for the regulation of the cholesterol 7α-hydroxylase. In a previous study we found that treatment of gallstone patients with chenodeoxycholic acid inhibited the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one by about 50% without affecting several other cytochrome P450-dependent steroid hydroxylases (4). The most plausible explanation for the increased 12α-hydroxylase activity in the present patients is, therefore, that the 12α-hydroxylase to some extent is also influenced by the portal inflow of bile acids to the liver.

Patients with the rare disease cerebotendinous xanthomatosis (CTX) have a markedly up-regulated cholesterol 7α-hydroxylase due to a reduced formation of bile acids, in particular chenodeoxycholic acid (for a review see ref. 19). Salen et al. (20) have reported that liver microsomes from such patients have three times higher capacity than normal to catalyze 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one. Also, this finding is in accord with the contention that there is a correlation between cholesterol 7α-hydroxylase and 12α-hydroxylase in human liver.

As outlined in the introduction it has been discussed whether or not the 12α-hydroxylase is of regulatory importance in man. In the two previous studies referred to above we could not demonstrate that the 12α-hydroxylase activity is of major importance for the regulation of the ratio between cholic acid and chenodeoxycholic acid in human bile (3, 5). During treatment with cholestyramine the synthesis of cholic acid is increased to a greater extent than that of chenodeoxycholic acid (6). The possibilities have been discussed that cholic acid is formed to a greater extent than chenodeoxycholic acid from newly synthesized cholesterol (21) and that part of the chenodeoxycholic acid may be formed in a pathway bypassing the regulatory cholesterol 7α-hydroxylase (8). Another explanation for the increased ratio between the synthesis of cholic acid and chenodeoxycholic acid during cholestyramine treatment could be a compensatory increase in the 12α-hydroxylase activity. The latter explanation is in accord with the present findings.

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