Evidence for a lack of regulatory importance of the 12α-hydroxylase in formation of bile acids in man: an in vivo study

Ingemar Björkhem, Mats Eriksson, and Kurt Einarsson

Departments of Clinical Chemistry and Internal Medicine, Karolinska Institutet, Huddinge University Hospital, S-141 86 Huddinge, Sweden

Abstract The possibility that the 12α-hydroxylase involved in formation of bile acids is of regulatory importance for the ratio between cholic acid and chenodeoxycholic acid in bile was studied with an in vivo technique. [4-^{14}C]7α-Hydroxy-4-cholesten-3-one and [6β-^{3}H]7α,12α-dihydroxy-4-cholesten-3-one were synthesized, and a mixture of these two bile acid intermediates was administered intravenously in five healthy subjects and in one patient with severe liver cirrhosis. The patient with liver cirrhosis was included in the study because of a considerable reduction in biosynthesis of cholic acid. Since the [4-^{14}C]-labeled steroid is an intermediate just proximal to and since the [6β-^{3}H]-labeled steroid is an intermediate just distal to the 12α-hydroxylase step, the ^{3}H/^{14}C ratio in the cholic acid formed should reflect the relative 12α-hydroxylase activity. The ^{3}H/^{14}C ratio varied between 1.8 and 3.9 in the cholic acid isolated from the healthy subjects and was 3.6 in the cholic acid isolated from the patient with liver cirrhosis. The ratio between cholic acid and chenodeoxycholic acid varied between 0.6 and 3.9 in the bile from the control subjects and was only 0.4 in the bile from patients with liver cirrhosis. There was no correlation between the ^{3}H/^{14}C ratios and the ratios between cholic acid and chenodeoxycholic acid in bile. The result of the present work, as well as that of a previous in vitro work from our laboratory, is consistent with the contention that the 12α-hydroxylase activity is of little or no importance for the regulation of the ratio between cholic acid and chenodeoxycholic acid in human bile.—**Björkhem, I., M. Eriksson, and K. Einarsson.** Evidence for a lack of regulatory importance of the 12α-hydroxylase in formation of bile acids in man: an in vivo study. *J. Lipid Res.* 1983. **24:** 1451–1456.

Supplementary key words cholic acid • chenodeoxycholic acid

According to available information, the overall rate of conversion of cholesterol into the primary bile acids, cholic acid and chenodeoxycholic acid, is regulated by the activity of the cholesterol 7α-hydroxylase (1). The mechanisms by which the ratio between cholic acid and chenodeoxycholic acid in bile is regulated in man have not been established, however. In view of the fact that the only structural difference between cholic acid and chenodeoxycholic acid is the presence or absence of a 12α-hydroxyl group, the most simple type of regulation should be a regulation of the activity of the 12α-hydroxylase. Such a mechanism seems to be of importance in the rat (2). In previous work from our group it was shown that the ratio between the activity of the microsomal 12α-hydroxylase and that of the microsomal 26-hydroxylase in rat liver varied in parallel with the ratio between cholic acid and chenodeoxycholic acid in bile in response to differences in the thyroid state (2). The mitochondrial 26-hydroxylase activity was relatively constant during the different thyroid states. The reason for the importance of the 26-hydroxylase activity is that introduction of a 26-hydroxyl group in a bile acid precursor seems to have a preventive effect on a subsequent 12α-hydroxylation (2). This effect is, however, less pronounced in man. In view of this, and in view of the fact that the 26-hydroxylase activity is almost exclusively located in the mitochondrial fraction of human liver, 26-hydroxylase activity is probably of little or no regulatory importance in man. In contrast to the rat, thyroid hormone has relatively little effect on the ratio between cholic acid and chenodeoxycholic acid formed in human liver (3).

The possibility that the 12α-hydroxylase activity is of regulatory importance in man was investigated in a previous study from our laboratory (4). We assayed 12α-hydroxylase activity in liver biopsies from patients with different types of hyperlipoproteinemia undergoing elective cholecystectomy. There was no apparent correlation between 12α-hydroxylase activity and the ratio between cholic acid and chenodeoxycholic acid in bile, and we concluded that a reduced 12α-hydroxylase activity could not explain the relatively low rate of synthesis of cholic acid found in some patients with hyperlipoproteinemia type II.

In the present work, we have studied the possibility that the 12α-hydroxylase activity is of regulatory importance in man also by an in vivo technique. The best substrate known for the microsomal 12α-hydroxylase

is 7α-hydroxy-4-cholesten-3-one (1). We have therefore administered [4-^{14}C]7α-hydroxy-4-cholesten-3-one to subjects together with [^{3}H]7α,12α-dihydroxy-4-cholesten-3-one. Since the latter steroid can only be converted into cholic acid, the ratio between ^{3}H and ^{14}C in cholic acids is likely to reflect the degree of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one under the conditions employed. In view of the very rapid conversion of an administered precursor into bile acids, the conversion should be essentially complete after only one passage through the liver. The ratio between ^{3}H and ^{14}C in the cholic acid formed should therefore be constant with time, thus eliminating the need for several determinations. With this method there is no need for determination of specific radioactivity in the isolated cholic acid.

In order to obtain subjects with a relatively wide range of ratios between cholic acid and chenodeoxycholic acid, we have included one patient with severe liver cirrhosis in our study. It is well established that such patients often have markedly reduced synthesis of cholic acid (5–13), and the possibility has been discussed that this is due to a reduced activity of the 12α-hydroxylase in the liver (5, 10, 11).

MATERIALS AND METHODS

Preparation of [4-^{14}C]7α-hydroxy-4-cholesten-3-one and [6β-^{3}H]7α,12α-dihydroxy-4-cholesten-3-one

[4-^{14}C]7α-Hydroxy-4-cholesten-3-one was prepared from [4-^{14}C]cholesterol (obtained from Radiochemical Centre, Amersham, England) according to a previously described procedure (14). The material was purified by preparative thin-layer chromatography, using toluene–ethyl acetate 1:1 (v/v) as solvent. The material obtained was pure as judged by thin-layer chromatography and by gas–liquid chromatography on 1.5% SE-30 (as trimethylsilyl ether). The specific radioactivity was 3.0 × 10^{6} cpm per mg.

[6β-^{3}H]7α-Hydroxy-4-cholesten-3-one was prepared by the same route as described above for synthesis of [4-^{14}C]7α-hydroxy-4-cholesten-3-one, with the exception that unlabeled and ^{3}H-labeled lithium aluminium hydride (obtained from New England Nuclear Corp., Boston, MA) were used (cf. ref. 15). The material obtained had a specific radioactivity of 2.0 × 10^{6} cpm per mg, and was pure according to the same criteria as above. Part of this material was enzymatically converted into [6β-^{3}H]7α,12α-dihydroxy-4-cholesten-3-one by incubation with the microsomal fraction of a rat liver homogenate and NADPH, under the conditions previously described (16). The product was purified twice by thin-layer chromatography, using ethyl acetate as solvent. The purified material was pure as judged by thin-layer chromatography and gas–liquid chromatography (as trimethylsilyl ether). The specific radioactivity was 2.0 × 10^{6} cpm per mg.

The [4-^{14}C]- and [6β-^{3}H]-labeled steroids were mixed together in a solution of aqueous (30%, v/v) ethanol. The ethanol solution was passed through a Millex 0.22-μm filter (Millipore Co., Bedford, MA) and stored in 2-ml aliquots at 4°C in sealed glass ampoules prior to use. Each ampoule contained 0.28 × 10^{6} cpm ^{3}H and 0.41 × 10^{6} cpm ^{14}C when assayed for radioactivity as described below.

Subjects

Five apparently healthy subjects (four males and one female) served as controls (**Table 1**). One subject (#5) had increased levels of alanine aminotransferase and as-

TABLE 1. Basal data of the healthy subjects and the patient with liver cirrhosis

Subject	Sex[a]	Age	Relative Body Weight[b]	Previous History	Bilirubin μmol/l[c]	ASAT μkat/l[c]	ALAT μkat/l[c]	AP μkat/l[c]	PTB %[c]	Albumin g/l[c]	IgG g/l[c]
No cirrhosis											
1	M	29	82		8	0.65	0.67	3.0		51	10
2	M	30	89		11	0.36	0.10	1.9	90	51	14
3	F	51	104		8	0.33	0.21	4.7	100	49	5
4	M	34	85		8	0.56	0.56	2.2	90	44	12
5	M	30	96		7	0.84	1.56	2.1		45	12
Cirrhosis											
6	F	69	152	Bleeding from esophageal varices	63	1.47	0.70	6.4	36	31	12

[a] M, male; F, female.
[b] Relative body weight, calculated as weight (kg)/height (cm) − 100) × 100%.
[c] Normal range: bilirubin, <26; ASAT (aspartate aminotransferase), <0.70; ALAT (alanine aminotransferase), <0.70; AP (alkaline phosphatase), <4.2; PTB (prothrombin complex) 70–130%; albumin, 38–55 g/l; IgG (immunoglobulin G), 7–17 g/l.

partate aminotransferase but no signs of liver cirrhosis. All the other subjects had normal laboratory values. One patient with portal liver cirrhosis participated in the study (**Table 1**). The cirrhosis was most probably due to an excessive intake of ethanol. This patient had had an episode with bleeding from esophageal varices. At the time of the study the body weight and the liver function test had reached a constant level, suggesting that the patient was in a steady state.

The cirrhotic patient was older than the control subjects. This fact should not influence the results however, since previous investigations have shown that the pool size and synthesis of cholic acid are about the same in young adults and elderly people (17, 18).

Ethical aspects

The ethical aspects of this study were approved by the Ethical Committee at Huddinge Hospital. Informed consent was obtained from each subject prior to the experiment.

Administration of labeled steroids and collection of bile

Immediately prior to infusion, the ethanolic solution with the labeled steroids was mixed with 18 ml of a sterile solution of sodium chloride in water (0.9%, w/v), and the mixture was slowly infused intravenously. All infusions were performed in the morning, 8 AM. No complications were observed during or after the infusion. The next morning, 8–9 AM, cholecystokinin was administered intravenously, and concentrated duodenal bile was collected through a thin polyvinyl tube. In one subject (cf. Results), duodenal bile was also collected 48 and 96 hr after the infusion of labeled steroids.

Bile analysis

The bile samples were hydrolyzed with 1 M KOH at 110°C for 12 hr. After acidification, the deconjugated bile acids were extracted with diethyl ether, and methylated with diazomethane. The methyl ester of cholic acid was isolated from the remaining part of the material by thin-layer chromatography, using system S11 (19). The purified methyl cholate was mixed with unlabeled methyl cholate (about 30 mg) and crystallized three times from acetone–water and methanol–water mixtures. The radioactivity in the recrystallized material was measured with an Intertechnique SL 30 liquid scintillation spectrometer, using Luma gel (LUMAC, BV, The Netherlands) as scintillation fluid. Under the conditions employed, the counting efficiency was 71% for ^{14}C and 49% for ^{3}H. Under the conditions used, no corrections for quenching were found to be necessary.

A small part of the methylated material was converted into trimethylsilyl ether and the relative composition of the bile acids was determined by gas–liquid chromatography using 1% HiEff 8 BP column. The responses of the individual bile acids were checked repeatedly.

RESULTS

The pattern of bile acids in the duodenal bile from the individual subjects is given in **Table 2.** Patient #6 had low relative concentrations of cholic acid and deoxycholic acid and a concomitant increase in chenodeoxycholic acid, a bile acid pattern considered to be typical for advanced cirrhosis (8). The ratio between cholic acid and chenodeoxycholic acid varied between 0.6 and 3.9 in the healthy subjects and was 0.4 in the patient with cirrhosis (**Table 3**).

The results of the ^{3}H- and ^{14}C-determinations are given in Table 3. The ratio between ^{3}H and ^{14}C varied between 1.8 and 3.9 in cholic acid isolated from the healthy subjects and was 3.6 in the cholic acid isolated from the patient with liver cirrhosis. No correlation was found between the $^{3}H/^{14}C$ ratio and the relative percentage of cholic acid and chenodeoxycholic acid in bile or the ratio between these two bile acids.

In one healthy subject (#4), bile samples were taken

TABLE 2. Biliary bile acid composition (%) in the healthy subjects and the patient with liver cirrhosis

Subject	Cholic Acid	Chenodeoxycholic Acid	Deoxycholic Acid	Ursodeoxycholic Acid	Lithocholic Acid
No cirrhosis					
1	76.9	19.7	3.4	trace	trace
2	24.1	38.3	33.0	4.6	trace
3	37.7	31.9	28.4	2.0	trace
4	42.1	37.9	20.0	trace	trace
5	41.1	52.0	6.9	trace	trace
Mean ± SEM	44.3 ± 8.7	36.0 ± 11.7	18.3 ± 5.8	1.3 ± 0.9	
Cirrhosis					
6	27.9	68.9	3.2	trace	trace

TABLE 3. Ratio between cholic acid and chenodeoxycholic acid and the ratio between ^3H and ^{14}C in cholic acid in bile of the healthy subjects and the patient with liver cirrhosis

Patient		Cholic Acid/ Chenodeoxycholic Acid	$^3H/^{14}C$
No cirrhosis			
1		3.90	2.2
2		0.63	1.8
3		1.18	2.9
5		0.79	2.4
4	24 hr	1.11	3.9
	48 hr		4.5
	96 hr		4.7
Cirrhosis			
6		0.40	3.6

Cholic acid was isolated from hydrolyzed bile by extraction and thin-layer chromatography as methyl ester. The material was crystallized three times together with unlabeled methyl cholate prior to assay for radioactivity (cf. Methods).

also 48 and 96 hr after the injection. The ratio $^3H/^{14}C$ was slightly increased in the latter two samples as compared to the first bile sample.

DISCUSSION

It should be pointed out that there are always some theoretical pitfalls in studies in vivo with labeled precursors. After intravenous administration of a bile acid precursor, it may be taken up by the Kuppfer cells in the liver and modified prior to entrance into the hepatocytes. In addition, due to compartmentation, the metabolic fate of the precursor reaching the hepatocyte might be different from that of the same compound when it has been formed within the cell. Normally, the different precursors are present within the cells in trace amounts only (21), and a sudden expansion of the pool of a specific precursor may lead to an abnormal metabolism. In spite of all these theoretical objections, detailed conclusions have been drawn from a great number of in vivo experiments in man with different labeled bile acid precursors (13, 22–30), and in general, the results obtained have been in good consonance with results obtained in vitro.

In the present work, the aim was to study whether or not the 12α-hydroxylase activity is of regulatory importance for the ratio between cholic and chenodeoxycholic acid in bile. The general technique used here, to inject a mixture of the intermediate just proximal to and just distal to the metabolic step studied (with different label in each intermediate), has previously been used successfully in a study on patients with cerebrotendinous xanthomatosis, who lack the mitochondrial 26-hydroxylase in their liver (31). A great advantage with this technique is that the errors and inconvenience related to estimation of absolute rates of synthesis can be avoided (cf. 31).

In use of the present technique, it must be assumed that the two precursors reach the hepatic metabolic site simultaneously. In view of the simultaneous injection and the relative small difference in structure, this assumption seems reasonable. In view of the previously demonstrated very rapid conversion of bile acid precursors into bile acids also in patients with severe liver disease (13, 22), the assumption should be valid also in the patient with liver cirrhosis. As a consequence of the rapid conversion of the precursor into cholic acid, the ratio between ^3H and ^{14}C should be constant once the cholic acid has been formed. In an attempt to confirm this, we found a small increase in $^3H/^{14}C$ when we isolated cholic acid different days after the injection. The explanation for this small increase is most probably that cholic acid is continuously converted into deoxycholic acid. This conversion involves a *trans* diaxial loss of the 7α-hydroxyl group together with the 6β-hydrogen (32). Since the tritium label was located in the 6β-position, there may be an isotope effect which may lead to a slight increase in the ratio $^3H/^{14}C$ in cholic acid with time. This small increase will not, however, influence the general results of our study.

The $^3H/^{14}C$ ratio in cholic acid formed varied much less between the different subjects (about twofold) than did the ratio between cholic acid and chenodeoxycholic acid in bile (about tenfold). There was no significant difference between the patient with and those without liver cirrhosis with respect to the ratio between $^3H/^{14}C$ in the cholic acid formed. From the lack of correlation between $^3H/^{14}C$ and the ratio between cholic acid and chenodeoxycholic acid in bile, it may be concluded that the activity of the 12α-hydroxylase is of little or no regulatory importance. Our previous in vitro work is also in consonance with this contention (4).

The patient with liver cirrhosis was included in our study because of the low ratio between cholic acid and chenodeoxycholic acid in bile. It should be mentioned, however, that this is not an obligatory finding in patients with liver cirrhosis. The present patient was selected from a group of five patients with severe liver cirrhosis. The other four patients all had a ratio between cholic acid and chenodeoxycholic acid within the normal range. The mixture of [4-^{14}C]7α-hydroxy-4-cholesten-3-one and [6β-^3H]7α,12α-dihydroxy-4-cholesten-3-one was in fact administered also to these patients, and in these cases the ratios between ^3H and ^{14}C in the isolated cholic acid were within the normal range (results not shown).

Patteson et al. (13) and Goldman et al. (22) have

shown that patients with liver cirrhosis are able to efficiently convert 7α-hydroxycholesterol, as well as several metabolites distal to 7α-hydroxycholesterol, into cholic acid. The formation of cholic acid from exogenous 7α-hydroxycholesterol was reduced only to a small degree as compared to control subjects. These investigators concluded that other factor(s) than a reduced 12α-hydroxylase activity are important for the reduced synthesis of cholic acid found in some patients with liver cirrhosis. Our results are in consonance with this conclusion and, in addition, seem to demonstrate that under normal conditions the activity of the 12α-hydroxylase is of little or no importance. It is possible that different pools of cholesterol are utilized in the biosynthesis of cholic acid and chenodeoxycholic acid, and that the relative sizes of these two pools may be a determinant for the ratio between cholic acid and chenodeoxycholic acid formed (33). Studies on this possibility are in progress.

It should be pointed out that the ratio between cholic acid and chenodeoxycholic acid in bile is not only influenced by the relative rate of synthesis of the two bile acids in the liver, but also by the differential rates of enterohepatic cycling, and the differential rates of intestinal absorption and degradation. Since we have not been able to measure the rate of synthesis of cholic and chenodeoxycholic acid in our patients, we cannot exclude the possibility that there may be some degree of correlation between the relative rate of synthesis of the two bile acids and the activity of the 12α-hydroxylase. From a physiological point of view, the final ratio between cholic acid and chenodeoxycholic acid in bile should be most important, and our results seem to conclude that the 12α-hydroxylase activity is of little or no importance for this ratio.

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