The pool of free cholesterol is not of major importance for regulation of the cholesterol 7α-hydroxylase activity in rat liver microsomes

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Abstract The relationship between the cholesterol 7α-hydroxylase activity and the pool of free cholesterol in rat liver microsomes was studied under experimental conditions aimed to stimulate (biliary drainage, cholestyramine treatment, and lymphatic drainage) as well as inhibit (chenodeoxycholic acid treatment) bile acid synthesis. Highly accurate methods based on isotope dilution–mass spectrometry were used both for assay of the cholesterol 7α-hydroxylase activity and the concentration of free cholesterol in the microsomes. In the assay of the cholesterol 7α-hydroxylase, only endogenous cholesterol was used as substrate for the enzyme. Under the experimental conditions employed, the concentration of microsomal free cholesterol remained essentially unchanged in spite of a more than 20-fold variation in enzyme activity. It is concluded that the total pool of free cholesterol in the microsomes is not of major regulatory importance for the cholesterol 7α-hydroxylase in rats.—**Einarsson, K., J-E. Åkerlund, and I. Björkhem.** The pool of free cholesterol is not of major importance for regulation of the cholesterol 7α-hydroxylase activity in rat liver microsomes. *J. Lipid Res.* 1987. **28:** 253–256.

Supplementary key words bile acids • chenodeoxycholic acid • cholestyramine • biliary drainage • lymphatic drainage

The first step in the metabolism of cholesterol to bile acids is the introduction of a hydroxyl group in 7α-position (1, 2). The enzyme catalyzing this reaction is a microsomal P-450 dependent mixed function oxidase. Early studies in the rat gave evidence that this enzyme is also rate-limiting in the biosynthesis of bile acids (3, 4). Further studies have shown that the cholesterol 7α-hydroxylase is subject to regulation by a number of factors including hormones, drugs, dietary components, lymphatic drainage, etc. (1, 2). Based on work with cultured rat hepatocytes, Davis and coworkers (5, 6) suggested that the size of the substrate pool of cholesterol available for the 7α-hydroxylase would regulate the activity of the enzyme. These observations prompted us to examine the relationship between the cholesterol 7α-hydroxylase activity and the pool of free cholesterol in rat liver microsomes under experimental conditions aimed to stimulate and inhibit bile acid biosynthesis in vivo. Highly accurate methods based on isotope dilution–mass spectrometry were used both for assay of the cholesterol 7α-hydroxylase and the concentration of free cholesterol in the microsomes. The results obtained do not support the hypothesis that the concentration of free cholesterol in the microsomes is of major regulatory importance for the cholesterol 7α-hydroxylase activity.

MATERIALS AND METHODS

Materials

Deuterium-labeled 7α-hydroxycholesterol was synthesized as described previously (7). Chenodeoxycholic acid, NADPH, EDTA, and DTT were purchased from Sigma Chemical Co., St. Louis, MO. Cholestyramine was obtained from Mead-Johnson.

Experimental procedure

Male Sprague-Dawley rats weighing 200–250 g were used. They were starved 12 hr prior to being killed at 9 AM. In some experiments the rats were fed a diet containing 5% cholestyramine or 1% chenodeoxycholic acid for 10 days. There was no difference in weight between the treated rats and those eating the control diet.

In some experiments the bile duct or the thoracic lymph duct (8, 9) was cannulated under ether anesthesia. The animals were then kept in restraining cages for 48 hr prior to being killed. They were given the ordinary pellet diet but instead of water they were given 0.9% (w/v) NaCl to drink. The corresponding control animals were sham-operated and then treated in the same way. The animal operations were approved by the local ethical committee for animal experiments.

Preparation of liver microsomes

The livers were excised and homogenized with a loose-fitting Teflon pestle 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 solution was centrifuged at 100,000 *g* for 60 min. The microsomal fraction obtained was suspended in a 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. The microsomal suspension was used for the assay of cholesterol 7α-hydroxylase. The microsomal content of protein was determined by the method of Lowry et al. (10).

Determination of microsomal free cholesterol

Microsomal free cholesterol was determined by isotope dilution–mass spectrometry as described previously (11) with the modifications described in ref. 12.

Assay of cholesterol 7α-hydroxylase activity

The standard assay system consisted of 1.0 ml of the microsomal fraction, 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM NADPH in a total volume of 1.0 ml. The enzyme assay was carried out according to the method of Björkhem and Kallner (7), where the product, 7α-hydroxycholesterol, is measured by isotope dilution–mass spectrometry after addition of deuterium-labeled 7α-hydroxycholesterol as an internal standard.

Statistical analysis

The data are expressed as means ± SEM. The statistical significance of differences was evaluated with Wilcoxon's rank sum test. Correlations were tested with Spearman's method (13).

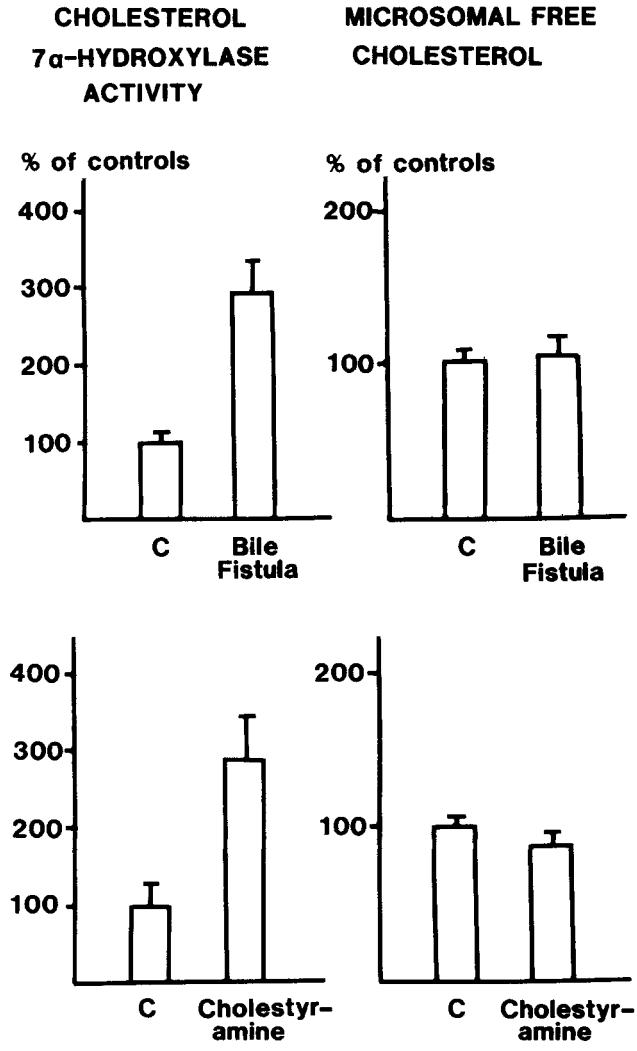

Fig. 1. Influence of biliary drainage (upper panel) and cholestyramine treatment (lower panel) on the cholesterol 7α-hydroxylase activity and the pool of free cholesterol in rat liver microsomes. Each bar represents mean ± SEM of six animals. The values are expressed as % of the controls (C).

RESULTS

In the control animals the 7α-hydroxylase activity averaged 33.7 ± 4.2 pmol • min $^{-1}$ • mg protein $^{-1}$ and the amount of free cholesterol in the microsomal fraction was 62.0 ± 3.0 nmol • mg protein^{-1}.

The corresponding values in the treated animals expressed as % of the controls are shown in Figs. 1 and 2.

Bile-fistula rats had a threefold higher 7α-hydroxylase activity than the controls (**Fig. 1**). Similarly, cholestyramine treatment caused a threefold rise of the 7α-hydroxylase activity (Fig. 1). Lymph-fistula rats had on an average 50% higher enzyme activity than the corresponding controls (**Fig. 2**), which is in agreement with previous work (14). Treatment with chenodeoxycholic acid markedly reduced the 7α-hydroxylase activity to about 10–15% of the control values (Fig. 2).

As demonstrated in Figs. 1 and 2, the amount of microsomal free cholesterol was about the same in bile-fistula and lymph-fistula rats as in the corresponding control animals. Also, treatment with cholestyramine and chenodeoxycholic acid did not significantly affect the amount of microsomal free cholesterol.

DISCUSSION

The hypothesis that the level of free cholesterol in the liver microsomes is of regulatory importance for the cholesterol 7α-hydroxylase would afford a simple and at-

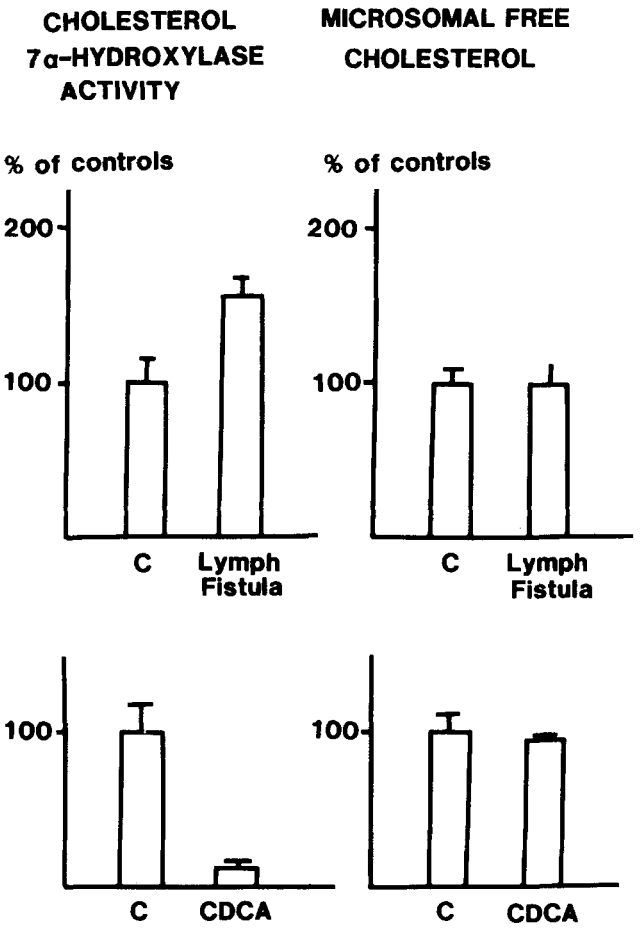

Fig. 2. Influence of lymphatic drainage and chenodeoxycholic acid (CDCA) treatment on the cholesterol 7α-hydroxylase activity and the pool of free cholesterol in rat liver microsomes. Each bar represents mean ± SEM of six animals. The values are expressed as % of the controls (C).

tractive explanation for the coupling between the rate-limiting enzyme in the cholesterol synthesis, the HMG-CoA reductase, and the rate-limiting enzyme in bile acid synthesis, the cholesterol 7α-hydroxylase (cf. ref. 2). According to this hypothesis, the changes in the activity of the cholesterol 7α-hydroxylase would be secondary to the changes in the activity of the HMG-CoA reductase. Increased activity of the HMG-CoA reductase, as seen, for example, during biliary drainage, lymphatic drainage, and cholestyramine feeding, would increase the pool of free cholesterol available for the cholesterol 7α-hydroxylase, which would lead to increased activity of the latter enzyme. Decreased activity of the HMG-CoA reductase, as seen, for example, during treatment with chenodeoxycholic acid (15), would decrease the pool of free cholesterol available for the cholesterol 7α-hydroxylase. Since the flux of cholesterol is of major importance for the regulation of the HMG-CoA reductase (16), it would also be of major importance for the cholesterol 7α-hydroxylase. The increased activity of the cholesterol 7α-hydroxylase obtained after biliary drainage and cholestyramine feeding may then be secondary to malabsorption of cholesterol rather than due to a released negative feed-back inhibition by bile acids returning to the liver in the portal blood.

The results of the present investigation seem to rule out the above simple model as being of major importance for the regulation of the cholesterol 7α-hydroxylase in rats. Thus the concentration of free microsomal cholesterol was found to be about the same in spite of a more than 20-fold variation in the activity of the cholesterol 7α-hydroxylase. It should be pointed out that the method used for assay of cholesterol is highly accurate, and has been suggested as a definitive method for determination of cholesterol (11, 12, 17). Our results are partly at variance with those reported by Davis and coworkers (6) who found that in bile-fistula rats not only was the cholesterol 7α-hydroxylase activity stimulated, but the content of microsomal free cholesterol was increased also. The reasons for this discrepancy are not quite apparent. It could be argued that the methods used to isolate microsomes are different. Thus Davis et al. (6) used a method which includes NaF in the buffers. In addition, there were differences in centrifugation and washing. However, in repeated experiments, we have used a microsomal isolation procedure similar to that used by Davis et al. including NaF or NaCl in the buffers without finding any differences in the microsomal content of free cholesterol (Einarsson, K., J-E. Åkerlund, and I. Björkhem, unpublished experiments). The present study does not exclude the possibility that the differences are due to biological variation.

It may be argued that the pool of cholesterol available for the cholesterol 7α-hydroxylase is only a fraction of the total pool of cholesterol in the microsomes, and that important changes in the size of a small pool near the enzyme may occur without significant changes in the total concentration of free cholesterol in the microsomes. Such a mechanism cannot be excluded, but it seems unlikely that it is of major regulatory importance. Based on Arrhenius plots of HMG-CoA reductase activity in rat liver microsomes, Mitropoulos et al. (18) suggested that the intracellular pool of cholesterol that is in the enviroment of the HMG-CoA reductase and the cholesterol 7α-hydroxylase was increased after treatment of the rats with mevalonate. The total concentration of cholesterol in the microsomes was unchanged. The authors suggested that the twofold increase in the activity of the cholesterol 7α-hydroxylase activity that occurred after the mevalonate treatment could be due to an increased concentration of cholesterol near the cholesterol 7α-hydroxylase.

A regulatory mechanism based on increased binding of cholesterol to the active site of the cholesterol 7α-hydroxylase would require a highly unsaturated enzyme. On the contrary, however, preliminary experiments in our

laboratory seem to suggest that the degree of saturation of the enzyme is high and relatively constant under different experimental conditions (Björkhem, I., unpublished experiments). Treatment of rats with dietary cholesterol leads to a significant increase in the concentration of free cholesterol in the microsomes and a 50–100% increase in cholesterol 7α-hydroxylase activity (19, 20). According to results of experiments in our laboratory, however, only part of the stimulatory effect of the cholesterol feeding on cholesterol 7α-hydroxylase activity can be due to the increased availability of substrate (20).

Even if the level of free cholesterol in the rat liver microsomes was found to be of little or no importance for the regulation of the cholesterol 7α-hydroxylase under the condition used in the present study, the situation may be different in other species. Whether this may be the case in human liver is presently being studied in our laboratory.

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