Studies on the link between HMG-CoA reductase and cholesterol 7α-hydroxylase in lymph-fistula rats: evidence for both transcriptional and post-transcriptional mechanisms for down-regulation of the two enzymes by bile acids

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Abstract The mechanism of down-regulation of hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase by cholic acid (CA) and chenodeoxycholic acid (CDCA) was investigated in rats with a lymph fistula. In accordance with the contention that bile acids are able to down-regulate HMG-CoA reductase by a mechanism independent of their effect on cholesterol absorption, inclusion of the above bile acids in diet (1%, wt/wt) reduced the HMG-CoA reductase specific activity by about 80%. The levels of mRNA were reduced by about 50% and the transcription rate (nuclear run-on) by 27% and 35%, respectively. Thus, HMG-CoA reductase appears to be regulated at both a transcriptional and a post-transcriptional level by bile acids. The cholesterol 7α-hydroxylase activity as well as the corresponding mRNA levels were both reduced by about 50% by the same treatments. The transcription rate of the cholesterol 7α-hydroxylase was not significantly affected by the treatment with CDCA, whereas the CA treatment reduced the transcription rate by about 24%. It is evident that bile acids regulate these two enzymes by different mechanisms in this model, in spite of the fact that the levels of mRNA were affected to about the same degree. The most obvious difference was the marked suppression of HMG-CoA reductase activity by cholic acid with relatively modest effects on transcription rate and level of mRNA. The latter finding is interesting in view of recent reports that dietary cholesterol also seems to down-regulate HMG-CoA reductase predominantly by a post-transcriptional mechanism. Our findings are discussed in relation to the well-documented coordination of HMG-CoA reductase and cholesterol 7α-hydroxylase under most experimental conditions.—**Björkhem, I., U. Andersson, E. Sudjama-Sugiaman, G. Eggertsen, and P. Hylemon.** Studies on the link between HMG-CoA reductase and cholesterol 7α-hydroxylase in lymph-fistula rats: evidence for both transcriptional and post-transcriptional mechanisms for down-regulation of the two enzymes by bile acids. *J. Lipid Res.* 1993. **34:** 1497–1503.

Supplementary key words dietary cholesterol • cholesterol synthesis • bile acid synthesis • lymph drainage

Under most experimental conditions, the activity of the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase, changes in parallel with the activity of the rate-limiting enzyme in bile acid biosynthesis, cholesterol 7α-hydroxylase (for reviews, see refs. 1–3). The linkage between the two enzymes is most important for cholesterol homeostasis and an increased synthesis of cholesterol thus always causes a compensatory increase in degradation of cholesterol.

The nature of the link between the two enzymes is still unknown. The fact that bile acids are able to down-regulate both enzymes in a similar way may be important for the coordination of the two enzymes.

It was recently shown that a major part of the down-regulation of cholesterol 7α-hydroxylase by bile acids in bile-fistula rats is due to effects on mRNA levels (4–7) and transcription rate (7). Cholesterol feeding is known to up-regulate cholesterol 7α-hydroxylase and this up-regulation is also mediated by increased levels of mRNA (3, 4, 8). Recent work with lymph-fistula rats suggests, however, that most of the effect of cholesterol feeding on cholesterol 7α-hydroxylase is due to the effect on the flux of bile acids and that bile acids are the primary regulators of the enzyme also under such conditions (8).

As cholesterol is a very effective suppressor of HMG-CoA reductase, it has been suggested that the suppressive effect of bile acids on the enzyme may be related to the

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; HMG, 3-hydroxy-3-methylglutaryl.

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fact that bile acids are needed for absorption of cholesterol (9). According to work with lymph-fistula rats, a major part of the effect of bile acids on this enzyme does not seem to be due to absorption of cholesterol (10, 11). It was recently shown that the down-regulation of HMG-CoA reductase obtained by feeding hydrophobic bile acids to intact rats was associated with decreased mRNA levels (12). The transcriptional activity (nuclear run-on) was decreased by about 50% in rats treated with cholic acid but was not decreased in rats treated with chenodeoxycholic acid. The latter finding would suggest that there may be a post-transcriptional regulation of HMG-CoA mRNA by some hydrophobic bile acids. Due to the fact that the rats had an intact enterohepatic circulation and a normal flux of cholesterol from the intestine to the liver, it cannot be completely excluded that part of the effects observed in the above study may be related to the stimulatory effect of bile acids on cholesterol absorption. According to some recent work, dietary cholesterol seems to down-regulate hepatic HMG-CoA reductase mainly by a post-transcriptional mechanism (13–15).

In view of the importance of bile acids for the absorption of cholesterol, and in view of the possibility that feeding of bile acids to rats may affect HMG-CoA reductase and cholesterol 7α-hydroxylase by different mechanisms, we have now studied the effect of bile acids on the two enzymes in lymph-fistula rats. In this animal model there is no transport of absorbed cholesterol to the liver, and thus the stimulatory effect of bile acids on cholesterol absorption is minimized. The results show that bile acids can suppress both enzymes also under these conditions and that different mechanisms are involved in the down-regulation.

MATERIALS AND METHODS

Materials

[4-^{14}C]cholesterol and [3-^{14}C]HMG-CoA with specific radioactivities of 55–60 mCi/mmol were obtained from NEN Research Products, Dreieich, Germany. The labeled cholesterol was purified by aluminium oxide chromatography immediately prior to use (14). 7α-[$^2H_2$]hydroxycholesterol was prepared as described previously (14). The cholic and chenodeoxycholic acids used in the feeding experiments were obtained from Sigma Chemical (St. Louis, MO) and had a purity greater than 98%. All cofactors were also obtained from Sigma Chemical. All solvents were of highest analytical grade.

Animal and animal treatments

Male Sprague-Dawley rats (200–250 g) were used. The control animals were given free access to a commercial powdered diet containing no addition, 1% chenodeoxycholic acid, or 1% cholic acid. The powdered diet (R3, Astra Ewos, Södertälje, Sweden) contained 5% fat, 4% fiber, 22% protein, 52% carbohydrate, and 0.2% sterols) The rats were fed the three different diets for 4 days and were then operated on under Hyponorm anesthesia. The thoracic lymph duct was cannulated just proximal to the cisterna magnum through an abdominal approach (11). The proximal part of the lymph duct was ligated. The cannula was drawn out at the back of the animal. The lymph was allowed to flow freely from the animals to the bottom of the metabolic cages and was not routinely collected. Under these conditions the rats could move freely in the cages during the lymphatic drainage. They were given free access to 0.6% (w/v) NaCl instead of water to compensate for loss of electrolytes. The rats were kept on the above diets for an additional 3 days after the operation. During these 3 days the rats consumed 20–25 g daily of the powdered diet. After the first 4 days on the three diets (control diet, cholic acid, and chenodeoxycholic acid), the weights of the animals were 81 ± 5%, 82 ± 3%, and 85 ± 4%, respectively, of the original weight. In a few cases the rats refused to eat the diet. These rats were excluded from the study. All rats were killed at 9 AM. The animal operations were approved by the local ethical committee for animal experiments.

The loss of protein, cholesterol, and triglycerides through the lymph drainage was similar in all three groups of animals. The loss of protein was 312 ± 132, 304 ± 112, and 297 ± 38 mg/24 h, respectively. The loss of cholesterol was 33 ± 14, 44 ± 14, and 29 ± 2 μmol/24 h, respectively. The loss of triglycerides was 0.29 ± 0.13, 0.35 ± 0.12, and 0.25 ± 0.05 mmol/24 h, respectively.

It was observed that the normal diurnal rhythm of the cholesterol 7α-hydroxylase with a peak at midnight was retained in the lymph-fistula rats. There was no significant diurnal rhythm of the HMG-CoA reductase in the lymph-fistula rats.

Preparation of subcellular fractions

Homogenates of rat liver were prepared in 50 mM Tris-Cl buffer, pH 7.4, containing 0.3 M sucrose, 50 mM NaCl, 1 mM EDTA, and 10 mM DTT (10% homogenate, w/w). In some experiments 50 mM NaCl was replaced with 50 mM NaF. A microsomal fraction was prepared by centrifugation at 20,000 *g* for 15 min and recentrifugation of the supernatant at 100,000 *g* for 1 h. Half of the microsomal fraction was resuspended in the homogenizing medium and recentrifuged at 100,000 *g* for 1 h. This fraction was used for assay of HMG-CoA reductase. Half of the original microsomal fraction was recentrifuged at 100,000 *g* in an homogenizing medium lacking DTT. The resulting fraction was used for assay of cholesterol 7α-hydroxylase activity.

Assay of cholesterol 7α-hydroxylase activity

After preparation of a microsomal fraction as above, incubations with 10 μg of [4-^{14}C]cholesterol dissolved in 1 mg of Tween 80 were performed, as described previously, in a total volume of 3 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA (16). 7α-[$^{2}H_2$]hydroxycholesterol was added to the incubation mixture after the incubation, before the extraction steps. The conversion of exogenous [4-^{14}C]cholesterol into 7α-hydroxycholesterol was determined by radioscanning after thin-layer chromatography; the corresponding conversion of endogenous cholesterol was determined by combined gas–liquid chromatography–mass spectrometry as described previously (16). In all experiments, the total conversion of both the endogenous microsomal cholesterol and the exogenous [4-^{14}C]cholesterol was calculated.

Assay of HMG-CoA reductase activity

After preparation of the microsomal fraction as outlined above, incubations with [3-^{14}C]HMG-CoA and subsequent analysis of incubation mixtures were performed essentially as described by Brown, Goldstein, and Dietschy (17). In this assay, the microsomal fraction, 40 μl, is preincubated for 15 min at 30°C in a total volume of 200 μl containing 0.1 M phosphate buffer, pH 7.4, 10 mM imidazole buffer, pH 7.4, 5 mM dithiothreitol, 10 mM EDTA, 3 mM NADP, 12 mM glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase. The reaction is then initiated with the addition of 90 nmol (0.5 μCi) of [3-^{14}C]HMG-CoA, dissolved in 25 μl of distilled water, giving a total substrate concentration of 400 μM. The incubation is continued for 15 min and is stopped by the addition of 25 μl of 6 M HCl. Tritium-labeled mevalonic acid (0.01 μCi) together with 3 mg of unlabeled mevalonic acid was added to the incubation mixture, which was then further lactonized, subjected to thin-layer chromatography, and analyzed for radioactivity.

Assay of microsomal protein

The protein concentration was determined according to the method of Lowry et al. (18).

Assay of cholesterol in liver microsomes

The amount of cholesterol in liver microsomes was measured with isotope dilution mass spectrometry (19). The total cholesterol content in liver microsomes from the untreated control rats was 33 ± 4 μg/mg protein (mean ± SEM, n = 8). The content in liver microsomes from rats treated with cholic acid and chenodeoxycholic acid was 36 ± 7 and 36 ± 5 μg/mg protein, respectively (n = 7). The corresponding levels of free cholesterol were 28 ± 3, 29 ± 5, and 26 ± 4 μg/mg protein, respectively.

RNA isolation and Northern blot analysis

Total cellular RNA was isolated from rat liver slices by the LiCl–urea method (20). Poly A RNA was prepared from total RNA using Dynabeads Oligo (dT) (Dynal AS, Oslo, Norway) according to the manufacturer's instructions. Electrophoresis of total RNA and poly A RNA in agarose gels containing formaldehyde and blotting of the separated RNA onto nylon membranes (Hybond N, Amersham, U.K.) was carried out by standard procedures (21). For the hybridization, cDNA probes for rat cholesterol 7α-hydroxylase (kindly supplied by Dr. Li et al. (5)), for hamster HMG-CoA reductase (22), and for human glyceraldehyde 3-phosphate dehydrogenase (GAP) (23) were labeled with ^{32}P using the Pharmacia Oligolabeling kit (Pharmacia, Uppsala, Sweden). Hybridization of the blots with the labeled probes was done according to Gehring et al. (24), and the blots were thereafter exposed to Fuji New RX X-ray films at −70°C. mRNA for HMG-CoA reductase was only quantitated from poly A RNA, as hybridization of total RNA yielded nonspecific interactions between the probe and 18S ribosomal RNA. Semiquantitive analysis of the amount of increase in mRNA was estimated by densitometry.

In accordance with previous work (5), the cholesterol 7α-hydroxylase showed three transcripts for the hybridi-

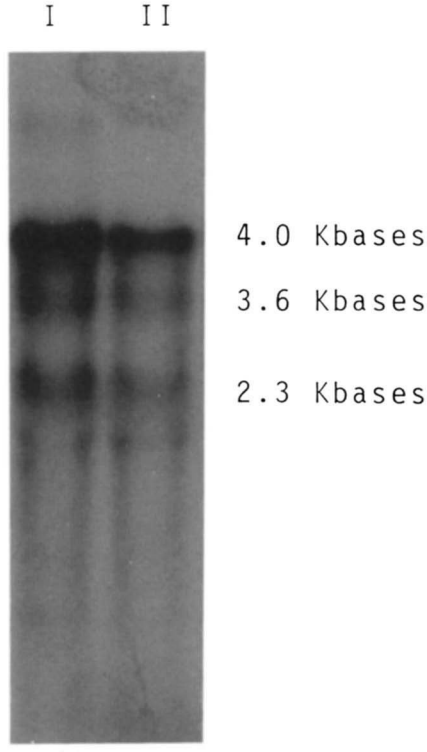

Fig. 1. Northern blot of cholesterol 7α-hydroxylase mRNA in the liver of untreated (I) and treated (II) lymph fistula rats. For experimental details, see Materials and Methods.

zation of the 7α-hydroxylase probe, corresponding to 2.3, 3.6, and 4.0 kbases. A typical Northern blot for cholesterol 7α-hydroxylase of a control rat and a lymph-fistula rat is shown in **Fig. 1.** The major band at 4.0 kbases corresponded to about 80% of the total message and the quantitations were based on the ratio between this band and that of GAP. In separate experiments, however, it was shown that cholic acid and chenodeoxycholic acid affected the three bands similarly (which is also seen in Fig. 1).

Nuclear run-on studies

Nuclei were isolated from one gram of rat liver as previously described (12). RNA labeling, isolation, and hybridization from hepatic nuclei were performed as previously described (12). Nitrocellulose strips containing spots of dilutions of p-Red 227 (Hamster cDNA of HMG-CoA reductase gene), p BSK-7α 6 (rat liver cholesterol 7α-hydroxylase), and pRSA 57 (rat albumin cDNA) were made and allowed to hybridize with ^{32}P-labeled nuclear RNA.

RESULTS

A large advantage with the present animal model was that the rats were allowed to move freely during the experiments. In previous work the rats had to be fixed in restraining cages in order to allow a complete collection of the lymph. The lymphatic drainage was tolerated much better with the model used in this study and with few exceptions there was a normal consumption of the diet. In the previous study, we gave extensive details with respect to losses of lymph, cholesterol, triglycerides, and albumin in lymph-fistula rats during the first 3 days after the operation (11). There were no significant differences among the three different groups of animals with respect to loss of albumin, cholesterol, and triglycerides through the lymph fistula.

The data in **Fig. 2** summarize the effects of the bile acid feedings on the HMG-CoA reductase activity, mRNA level, and transcriptional activity. The results obtained from each individual rat fed cholic acid or chenodeoxycholic acid were compared to the result obtained from a corresponding control rat operated on at the same occasion and fed the control diet without bile acid. It may be mentioned that in accordance with our previous work (11) the absolute level of HMG-CoA reductase activity was about threefold higher in the lymph fistula rats fed the control diet as compared to sham-operated rats fed the same diet (results not shown). The HMG-CoA reductase mRNA was about 100% higher in lymph-fistula rats as compared to sham-operated controls (results not shown).

The enzymatic activity was reduced by 83% and 77% by cholic acid and chenodeoxycholic acid, respectively. The mRNA levels were reduced by 49% and 51%, respectively. The transcription activity was reduced by 27% and 35%, respectively.

The HMG-CoA reductase activity was also measured in homogenates prepared in the presence of NaF. This activity was 12 ± 0%, 14 ± 3%, and 15 ± 4% (mean ±SD) of that obtained with homogenates prepared in NaCl in the three groups of rats (control rats and rats treated with cholic acid and chenodeoxycholic acid, respectively).

The results in **Fig. 3** summarize the effects of bile acid feeding on cholesterol 7α-hydroxylase activity, mRNA level, and transcriptional activity. The absolute level of cholesterol 7α-hydroxylase activity was about 70% higher in the lymph-fistula rats fed the control diet compared to intact rats fed the same diet (cf. ref. 11).

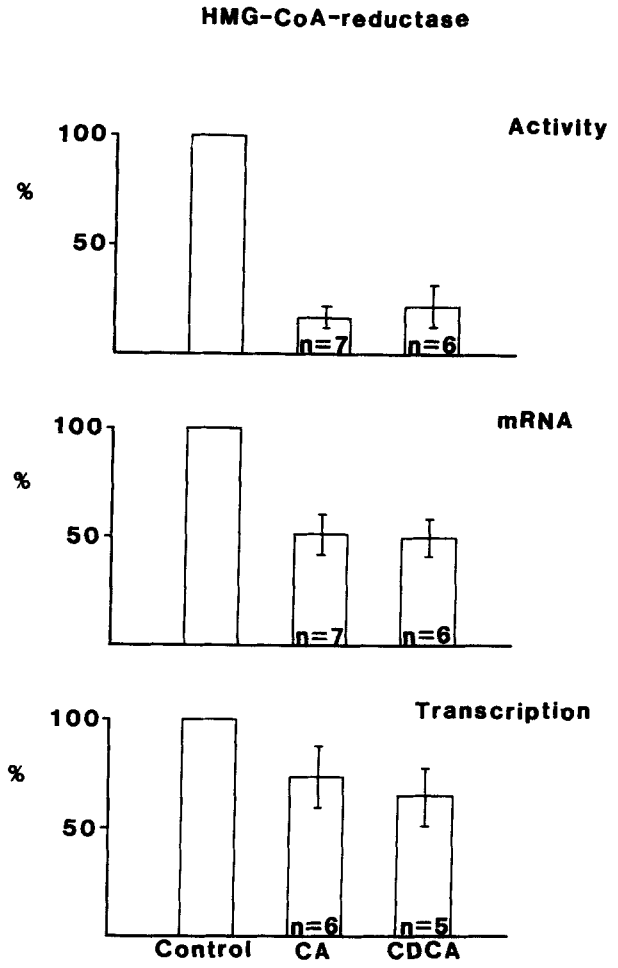

Fig. 2. Effect of feeding 1% cholic acid (CA) and 1% chenodeoxycholic acid (CDCA) on HMG-CoA reductase activity, mRNA levels, and transcription rates in lymph-fistula rats. Values shown are mean ± SEM from five to seven independent experiments. The results obtained from each individual rat were compared to the results obtained from a corresponding control rat operated on at the same time and fed the control diet without addition of bile acid. For further details, see Materials and Methods.

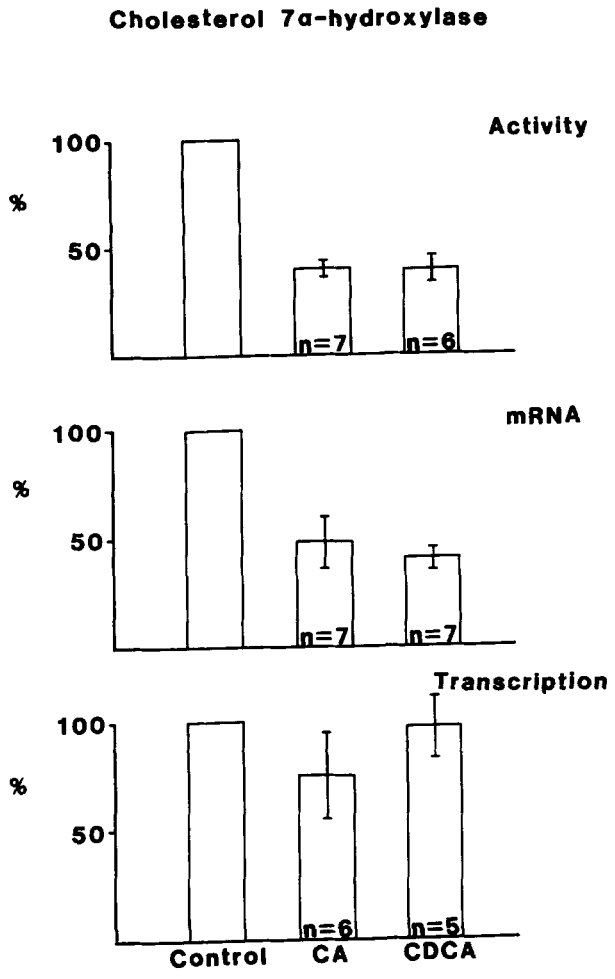

Fig. 3. Effect of feeding 1% cholic acid (CA) and 1% chenodeoxycholic acid (CDCA) on cholesterol 7α-hydroxylase activity, mRNA levels, and transcription rates in lymph-fistula rats. Values shown are mean ± SEM from five to seven independent experiments. The results obtained from each individual rat were compared to the results obtained from a corresponding control rat operated on at the same time and fed the control diet without addition of bile acids. For further details, see Materials and Methods.

The enzymatic activity was reduced by 60% by both cholic acid and chenodeoxycholic acid. The mRNA levels were reduced by 52% and 59%, respectively. The transcriptional rate was reduced by 24% and 3%, respectively.

Chenodeoxycholic acid is metabolized into α- and β-muricholic acid in rats. Analysis of the fecal bile acids by gas chromatography showed that about half of the chenodeoxycholic acid had been transformed into muricholic acid with a ratio of about 1:1 between the α- and β-epimers.

Treatment with chenodeoxycholic acid may cause toxic effects on the liver. Under the conditions used, with 1% of chenodeoxycholic acid in the diet, there were no histological changes in the livers of the treated animals.

DISCUSSION

Limitations with different animal models used for studies on enzymes involved in cholesterol homeostasis

Studies on the link between cholesterol biosynthesis and degradation in intact animals are difficult due the multitude of variables that can occur at different levels. Bile acids have repeatedly been reported to down-regulate both cholesterol 7α-hydroxylase and HMG-CoA reductase in different animal models (1–3). Part of the down-regulation of HMG-CoA reductase by bile acids in intact animals may be due to the need for bile acids for absorption of cholesterol, which is a potent repressor of HMG-CoA reductase. In order to study the effect of bile acids per se on HMG-CoA reductase, use of lymph-fistula rats is therefore an attractive model. In such animals there is no flux of cholesterol from the intestine to the liver. In previous studies on the effect of cholesterol on the enzyme, 2% cholesterol was given in the diet. We have, however, shown that feeding this amount of cholesterol may result in a malabsorption of bile acids, resulting in up-regulation of cholesterol 7α-hydroxylase (8). As a consequence of this, one may obtain multiple effects on HMG-CoA reductase in such an animal model, i.e., a suppressive effect by the cholesterol load and a stimulatory effect by the loss of bile acids. This may explain the previous controversy in the literature with respect to mechanism of suppression of HMG-CoA reductase by dietary cholesterol (12–15, 25–27). Bile-fistula animals have been used in many experiments designed to study effects of bile acids and cholesterol on the two rate-limiting enzymes. In such animals HMG-CoA reductase is up-regulated due to the reduced flux of both cholesterol and bile acids to the liver whereas cholesterol 7α-hydroxylase is up-regulated by the reduced flux of bile acids. As administration of bile acids to such animals will increase the flux of cholesterol from the intestine to the liver, mixed effects may also be obtained here. Due to all this it is very difficult to draw conclusions from experiments in which both bile acids and cholesterol have been fed.

In lymph-fistula animals there is a marked up-regulation of HMG-CoA reductase due to the cholesterol depletion (11). There is also a slight up-regulation of cholesterol 7α-hydroxylase (11). There is some evidence that the latter up-regulation may be due to a lymphatic factor. As this lymphatic factor seems to require an intact enterohepatic circulation for its effect (11), lymph-fistula animals are probably not optimal for studies on the effect of bile acids on cholesterol 7α-hydroxylase.

In view of all this it appears suitable to study specific effects of bile acids on HMG-CoA reductase in lymph-fistula rats and specific effects of bile acids on cholesterol 7α-hydroxylase in bile-fistula rats.

Effect of bile acids on HMG-CoA reductase and cholesterol 7α-hydroxylase in lymph-fistula rats

The effect of dietary cholic acid and chenodeoxycholic acid on cholesterol 7α-hydroxylase activity and HMG-CoA reductase activity in the lymph fistula rats was similar to that obtained in previous studies with intact rats (12, 28).

Interestingly, feeding cholic and chenodeoxycholic acids had relatively smaller effects on the transcription rate and levels of mRNA than on enzyme activity. Also, in the previous study on the effect of bile acids in intact animals, cholic acid and chenodeoxycholic acid suppressed the activity of this enzyme more than the mRNA levels (12). In that study the transcription rate was significantly reduced by cholic acid but not by chenodeoxycholic acid. The differences in results between the present and the previous study may be explained by the fact that there was no flux of cholesterol to the liver in the present animal model. In any case it is evident from the present study that bile acids are able to regulate hepatic HMG-CoA reductase by a mechanism independent of their effect on cholesterol absorption.

As discussed above, lymph-fistula animals may not be optimal for studies on the effect of bile acids on hepatic cholesterol 7α-hydroxylase. The parallel suppression of activity and mRNA that was obtained by cholic and chenodeoxycholic acids is, however, in accord with results of previous work with bile-fistula rats (4–7). The transcription rate was found to be less affected. The difference as compared to the results of the previous investigation may be that rats with only a slightly up-regulated cholesterol 7α-hydroxylase were used in the present study.

Coordinate regulation of cholesterol 7α-hydroxylase and HMG-CoA reductase

It is evident that the two rate-limiting enzymes studied here are regulated by complex mechanisms at both transcriptional and post-transcriptional levels. It is of interest that the levels of mRNA corresponding to the two enzymes were suppressed to about the same degree by the two bile acids, whereas significant differences were observed at the other levels.

The marked suppression of HMG-CoA reductase activity by cholic acid, mainly by a post-transcriptional mechanism, is interesting. This pattern is similar to that reported in recent reports in which it was shown that most of the feedback regulation of hepatic HMG-CoA reductase gene expression occurs at a post-transcriptional level in intact rats exposed to dietary cholesterol (13–15). It is difficult, however, to draw conclusions from results with other animal models in other laboratories. In a recent work by Hwa et al. (29), it was shown that two strains of mice had different responses to dietary cholesterol and that the mRNA levels were markedly reduced in one of the strains but not in the other. Similar genetic differences may exist also in different strains of rats. It is evident, however, that the mechanism of regulation of HMG-CoA reductase by cholesterol in the present strain of rat involves both transcriptional and post-transcriptional mechanisms. Thus lymphatic drainage, which should result in cholesterol depletion, caused an increase of both enzymatic levels and mRNA levels. Treatment with dietary cholesterol caused depression of both HMG-CoA reductase activity and mRNA levels, with more marked depression of the enzymatic activity.

The possibility has been discussed that there is a small pool of cholesterol in the liver, the size of which is of regulatory importance for the HMG-CoA reductase (10). If such a pool of cholesterol exists and if it is available as a substrate for cholesterol 7α-hydroxylase, bile acids may regulate the HMG-CoA reductase via effects on the cholesterol 7α-hydroxylase. Although the results of the present investigation would fit with such a mechanism, several alternative explanations can be offered, including a more direct effect of bile acids on the HMG-CoA reductase gene. Further studies on the coordinate regulation of the enzymes will now be performed in cultured hepatocytes, thus avoiding some of the limitations of the different in vivo models discussed here. Suitable conditions for such studies were recently reported from one of our laboratories (30).

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