Stimulation of HMG-CoA reductase as a consequence of phenobarbital-induced primary stimulation of cholesterol 7α-hydroxylase in rat liver

Elly Sudjana-Sugiaman, Gösta Eggertsen, and Ingemar Björkhem[1]

Department of Clinical Chemistry, Karolinska Institute, Huddinge Hospital, Huddinge, Sweden

Abstract Among nine strains of rat, two were found that responded to phenobarbital treatment with increased activity of hepatic cholesterol 7α-hydroxylase. This effect was maximal after 2–3 days of treatment and was then reduced. Interestingly the increased cholesterol 7α-hydroxylase activity was associated with increased activity of hepatic HMG-CoA reductase in the two responding strains but not in the non-responding strains. In tissues other than the liver, HMG-CoA reductase activity was unaffected in responding rats. Most of the above stimulation occurred at a pretranslatory level and the mRNA levels corresponding to the two enzymes parallelled the activities. The phenobarbital treatment resulted in decreased content of free cholesterol in liver microsomes in a strain of rat that responded with increased cholesterol 7α-hydroxylase activity. It was shown that depletion of cholesterol in the responding strain of rats by lymph fistulation also was associated with a parallel increase in levels of HMG-CoA reductase activity and mRNA. The findings are discussed in relation to the link between HMG-CoA reductase and cholesterol 7α-hydroxylase. A primary up-regulation of the cholesterol 7α-hydroxylase by the cytochrome P450 inducer phenobarbital can be expected to lead to increased consumption of cholesterol substrate. This consumption may result in a compensatory increase in the activity of the HMG-CoA reductase. It is suggested that such a mechanism is responsible for part of the covariation of the two enzyme systems under different conditions.—**Sudjana-Sugiaman, E., G. Eggertsen, and I. Björkhem.** Stimulation of HMG-CoA reductase as a consequence of phenobarbital-induced primary stimulation of cholesterol 7α-hydroxylase in rat liver. *J. Lipid Res.* 1994. **35:** 319–327.

Supplementary key words cholesterol synthesis • bile acid synthesis • HMG-CoA reductase • cytochrome P450

Under most conditions the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase, covariates with 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 is thus almost invariably associated with a compensatory increase in the degradation of cholesterol. Dietary cholesterol, however, causes a marked suppression of HMG-CoA reductase activity and a slight up-regulation of cholesterol 7α-hydroxylase. These effects would help keep the cholesterol content of the hepatocyte as constant as possible.

In order to obtain information about the link between the two enzymes, the most simple approach would be to affect the activity of one of them and then study the effect on the other. Selective inhibitors or selective stimulators must then be used.

To our knowledge no specific inhibitors are known for cholesterol 7α-hydroxylase. Bile acids are potent down-regulators of both cholesterol 7α-hydroxylase and HMG-CoA reductase (1), and it is not known with certainty whether there is a primary effect on only one of the two enzymes.

There are, however, specific HMG-CoA reductase inhibitors. When such inhibitors are used in vivo, there is little or no specific effect on the cholesterol 7α-hydroxylase under normal conditions in rat and humans (4, 5). Some inhibitory effect on cholesterol 7α-hydroxylase by these compounds has, however, been reported when the two enzymes are up-regulated (2, 6, 7). When cholesterol synthesis is stimulated by treatment of rats with mevalonate in vivo, some (8, 9), but not all (10), groups have found a stimulation of cholesterol 7α-hydroxylase. The stimulation may be due to newly synthesized cholesterol or due to an intermediate between mevalonate and cholesterol. The latter stimulation is associated with increased levels of mRNA for cholesterol 7α-hydroxylase (9). According to most work, regulation of cholesterol 7α-hydroxylase activity is mainly controlled at the transcriptional level (7, 11–15). HMG-CoA reductase has been reported to be controlled both at a transcriptional (16) and posttranscriptional level (15, 17).

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl CoA reductase; LDL, low density lipoprotein; HDL, high density lipoprotein.

[1]To whom correspondence should be addressed.

The above studies deal with consequences of a primary effect on cholesterol synthesis or HMG-CoA reductase and there is a need for studies on the consequences of a primary effect on cholesterol 7α-hydroxylase.

In the present work the intention was to produce a primary stimulation of cholesterol 7α-hydroxylase and study whether such a stimulation affects HMG-CoA reductase. It has been reported that at least two specific strains of rats have cholesterol 7α-hydroxylase that is stimulated by treatment with the cytochrome P450 inducer phenobarbital (18–20). Other strains of rats respond to treatment with this cytochrome P450 inducer with a reduced activity of the enzyme (18, 20, 21). Among several strains of rats tested, we found two that respond to phenobarbital treatment with increased cholesterol 7α-hydroxylase activity. There was a simultaneous similar increase in hepatic HMG-CoA reductase activity in both these strains of rats. The increased activity of the two enzymes was associated with increased mRNA levels. The nature of the coupling between the two enzymes is discussed in relation to previous knowledge and possible mechanisms for covariation of the two enzymes.

MATERIALS AND METHODS

Materials

[4-^{14}C]cholesterol and [3-^{14}C]HMG-CoA with specific radioactivities of 56–60 mCi/mmol were obtained from NEN Research Products, Dreieich, Germany. The labeled cholesterol was purified by aluminum oxide chromatography immediately prior to use (22). 7α-[$^{2}H_2$]hydroxycholesterol was prepared as described previously (22).

cDNA probes for rat cholesterol 7α-hydroxylase and hamster HMG-CoA reductase were kindly supplied by Dr. K. Okuda (11) and Dr. P. Hylemon (16), respectively.

Animals and animal treatments

Male rats of nine different strains and one strain of gerbils were used. The Sprague-Dawley rats and rats denoted "Wistar" and "Wistar F," were supplied from ALAB Laboratorietjänst, Sollentuna, Sweden. The "Mins Wistar Fu" rats were locally inbred by Dr. G. Jaremko, Huddinge Hospital, Huddinge, Sweden. All the other strains of rats (Wistar Kyoto normotensive, Brown Norwegian, Dahl/Mol, Long Evans, Lewis) were obtained from Möllegaard Ltd, Skensved, Denmark. The gerbils were also obtained from Möllegaard Ltd.

Animals were kept in an controlled environment at 16–18°C on a regular illumination schedule, lights on at 0600 h and off at 1800 h.

Rats or gerbils were injected intraperitoneally daily at 9 AM with phenobarbital, 100 mg/kg body weight, or with saline over a period of 1–4 days. In general, the rats were killed 2 h after the third injection. In some experiments the rats were killed at other days or at other times of the day (See Results). The rats were given free access to a commercial pellet diet and water during the study. There were no significant losses of body weight during the phenobarbital treatment. In accordance with previous work (18–21) the liver weight of the phenobarbital-treated rats increased (up to 10%) compared to the saline-treated rats. In all strains of rats, the phenobarbital treatment resulted in a period of sleeping for 3–6 h after the first injection, with a gradual shortening of the sleeping period after the subsequent injections.

In some experiments Wistar F rats were treated with 2% cholesterol in the diet for 5 days as described previously (23). The control rats were given the same diet except for the cholesterol. In some other experiments Wistar F rats were lymph-fistulated. The thoracic lymph duct was cannulated just proximal to the cisterna magnum through an abdominal approach (23, 24). The proximal part of the lymph duct was ligated and the lymph was drained for 72 h prior to killing. The corresponding control animals were sham-operated but otherwise treated as the lymph-fistula animals.

Preparation of subcellular fractions

Homogenates of rat liver were prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 50 mM NaCl, 1 mM EDTA, and 10 mM DTT (10% homogenate, w/w). 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 a 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 (22). 7α-[^{2}H]hydroxycholesterol was added prior to the extraction steps. The conversion of exogenous [4-^{14}C]cholesterol into 7α-hydroxycholesterol was determined by radioscanning after thin-layer chromatography and the corresponding conversion of endogenous cholesterol was determined by combined gas–liquid chromatography–mass spectrometry as described previously (22). In all experiments, the total conversion of both the endogenous microsomal cholesterol and the exogenous [4-^{14}C]cholesterol were 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 (25). In this assay, [3-^{14}C]HMG-CoA is used as substrate and tritium-labeled mevalonic acid as a marker for the product in connection with a thin-layer chromatography assay.

Assay of microsomal protein

The protein concentration was determined according to the method of Lowry et al. (26).

Assay of cholesterol in liver microsomes

The amount of total and free cholesterol in liver microsomes was measured with isotope dilution mass spectrometry using [$^{2}H_6$]cholesterol as internal standard (24).

RNA isolation and Northern blot analysis

Total cellular RNA was isolated from rat liver slices by the LiCl-urea method (27). Poly A+RNA was prepared from total RNA using Dynabeads Oligo (dT) (Dynal AS, Oslo, Norway) according to the manufacturers' instruction. 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 (28). For the hybridization, cDNA probes for rat cholesterol 7α-hydroxylase, for hamster HMG-CoA reductase (29), and for human glyceraldehyde 3-phosphate dehydrogenase (30) were labeled with ^{32}P using the Pharmacia Oligolabelling kit (Pharmacia, Uppsala, Sweden). Hybridization of the blots with the labeled probes was done according to Gehring et al. (31), 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 28S ribosomal RNA. Semiquantitive analysis of the relative amount of mRNA was estimated by densitometry.

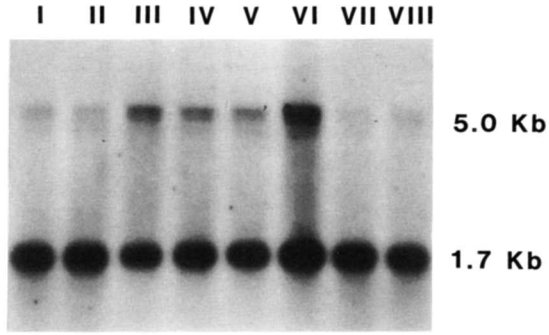

Fig. 1. Northern blot of HMG-CoA reductase mRNA in liver of rats treated with saline (I, II, VII, VIII) or phenobarbital (III–VI). The signal at 5.0 kbs corresponds to HMG-CoA mRNA and that at 1.7 kbs to glyceraldehyde 3-phosphate dehydrogenase mRNA.

Fig. 1 shows a typical Northern blot of HMG-CoA reductase mRNA with a signal at 5.0 kb. The signal at 1.7 kb corresponds to glyceraldehyde 3-phosphate dehydrogenase mRNA. The ratio between the signals was used as a measure of HMG-CoA reductase mRNA.

RESULTS

Table 1 shows results from experiments in which nine different strains of rats and one strain of gerbils were treated with phenobarbital under the conditions described

TABLE 1. Effect of phenobarbital treatment for 48 h on hepatic cholesterol 7α-hydroxylase and HMG-CoA reductase activity in male rats of different strains

Strain of Rats		Cholesterol 7α-Hydroxylase Activity	HMG-CoA Reductase Activity
Sprague-Dawley	(n = 5 + 5)	− 15% ($P > 0.05$)	− 30% ($P < 0.01$)
Wistar rats	(n = 4 + 4)	− 45% ($P < 0.01$)	− 58% ($P < 0.01$)
Wistar-F	(n = 10 + 10)	+ 113% ($P < 0.05$)	+ 145% ($P < 0.05$)
Wistar Kyoto normotensive	(n = 4 + 4)	− 3% ($P > 0.05$)	− 12% ($P > 0.05$)
Brown Norwegian	(n = 5 + 5)	+ 29% ($P < 0.01$)	+ 24% ($P < 0.01$)
Dahl/Mol	(n = 4 + 4)	− 24% ($P < 0.01$)	− 41% ($P < 0.01$)
Long-Evans	(n = 4 + 4)	− 11% ($P > 0.05$)	+ 25% ($P < 0.01$)
Lewis	(n = 4 + 4)	+ 70% ($P < 0.01$)	+ 140% ($P < 0.01$)
Gerbils	(n = 5 + 5)	− 14% ($P > 0.05$)	− 5% ($P > 0.05$)
Mins Wistar Fu	(n = 5 + 5)	− 21% ($P < 0.01$)	− 13% ($P > 0.05$)

Male rats of nine different strains and one strain of gerbils were injected daily intraperitoneally with 100 mg/kg body weight phenobarbital or saline and were killed 2 h after the third injection (see Materials and Methods). The difference in specific catalytic activity (pmol conversion/min per mg protein) between phenobarbital-treated and saline-treated rats was calculated. The results shown for each strain are the means from at least 4 + 4 rats. The *P*-value obtained in a statistical evaluation (Student's *t*-test) is given within parentheses. The specific catalytic activity of cholesterol 7α-hydroxylase of the control rats was 23, 17, 29, 30, 17, 15, 18, 32, 12, and 31 pmol/min per mg in the S-D, W, W-F, W-K, B-N, D-M, L.R, L, G, and M W-F rats, respectively. The corresponding figures for HMG-CoA reductase activity were 0.73, 0.55, 0.74, 1.1, 3.8, 0.40, 0.77, 0.94, 0.78, and 0.35 nmol/min per mg, respectively.

in Materials and Methods. Two of the strains, the Wistar F strain and the Lewis strain, responded to phenobarbital with a clear increase of specific activity (about 2-fold) of both cholesterol 7α-hydroxylase and HMG-CoA reductase. The Brown Norwegian strain of rat responded with a slight increase of activity of the two enzymes (about 25%). In the other strains and in the gerbils, these two enzyme activities were decreased or not affected at all by phenobarbital.

The lack of effect of phenobarbital on the cholesterol 7α-hydroxylase in most strains of rats was not coupled to differences in sleeping time. Thus the phenobarbital-induced sleeping time was similar in all the rats as well as in the gerbils. In a separate experiment with rats of the Sprague Dawley and Wistar strain, it was shown that the phenobarbital treatment led to about 2-fold stimulation of microsomal 11-hydroxylation of laurate under the conditions used (results not shown).

The stimulatory effect of phenobarbital on cholesterol 7α-hydroxylase in the Wistar F strain was reproduced in several sets of experiments and was always significant from a statistical point of view. In one typical set of experiments the specific activity of cholesterol 7α-hydroxylase was 62 ± 13 pmol/min per mg protein in Wistar F rats treated with phenobarbital for 48 h and 29 ± 5 pmol/min per mg in the corresponding control rats (mean ± SEM, n = 10, $P < 0.05$, Student's t-test). In one typical set of experiments with Lewis rats, cholesterol 7α-hydroxylase activity was 54 ± 3 pmol/min per mg and 32 ± 2 pmol/min per mg after phenobarbital treatment and saline treatment, respectively (n = 4, $P < 0.01$). The HMG-CoA reductase activity was also significantly increased by phenobarbital in both these experiments.

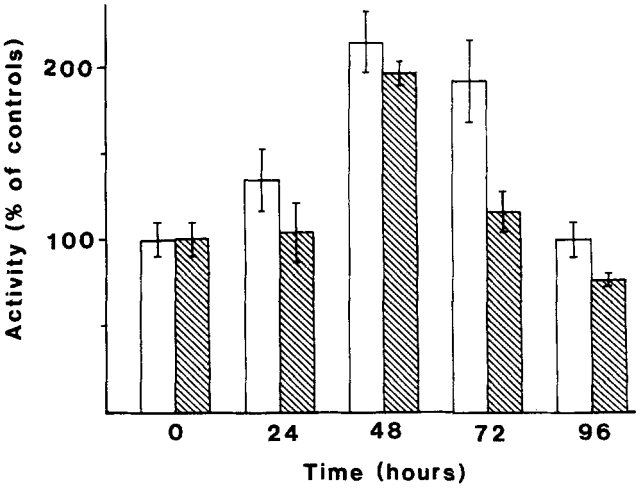

Fig. 2. Effect of phenobarbital on cholesterol 7α-hydroxylase activity (open bars) and HMG-CoA reductase activity (hatched bars). The specific catalytic activity of the two enzymes was compared with one control rat (at time 0) for each phenobarbital-treated rat. The activity of the control rat was set to 100%. The means ± SEM for three different independent experiments are shown.

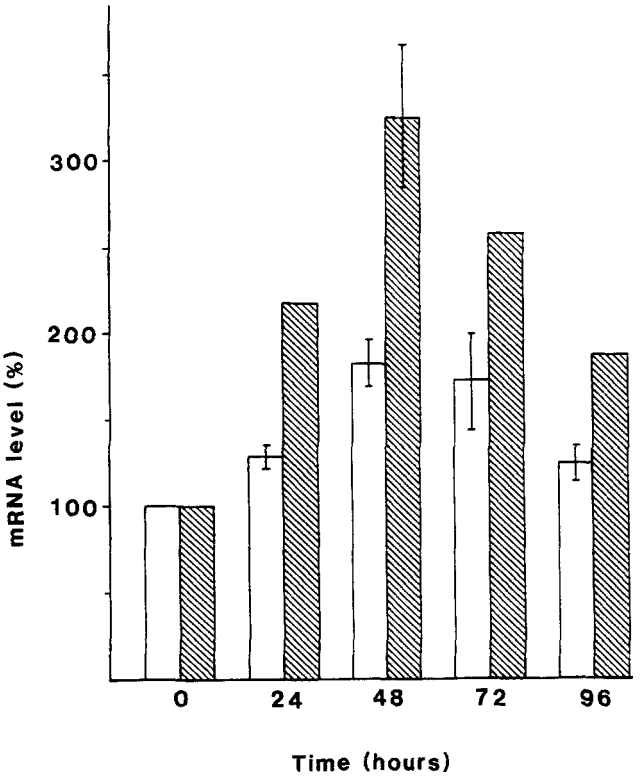

Fig. 3. Effect of phenobarbital on cholesterol 7α-hydroxylase mRNA (open bars) and HMG-CoA reductase mRNA (hatched bars). The mRNA level of the two enzymes was compared with one control rat at time 0 for each phenobarbital-treated rat (Northern blot, see Materials and Methods). The level of the control was set to 100%. The means ± SEM for three different independent experiments are shown or the means of two experiments (HMG-CoA reductase mRNA at 24, 72, and 96 h).

The phenobarbital-induced stimulation was highest in the Wistar F rats and this strain of rats was therefore used in all the subsequent experiments. In this strain the degree of stimulation of cholesterol 7α-hydroxylase obtained after 48 h of treatment varied in different experiments between 80% and 200% whereas the degree of stimulation of HMG-CoA reductase varied between 40% and 200%.

Fig. 2 shows the effect of phenobarbital on the two enzyme activities in relation to the duration of the treatment. The maximal effect on both cholesterol 7α-hydroxylase and HMG-CoA reductase was obtained after 48 h. The stimulation of cholesterol 7α-hydroxylase decreased slowly with time whereas a significant stimulatory effect on HMG-CoA reductase was only seen after 24 and 48 h (in the experiments shown in Fig. 2, only after 48 h).

Most of the stimulatory effect of the phenobarbital treatment appeared to be at a pretranslation level, at least in case of cholesterol 7α-hydroxylase. As shown in **Fig. 3**, cholesterol 7α-hydroxylase mRNA levels thus parallelled the activity of the enzyme. The increase of HMG-CoA reductase mRNA was somewhat higher than that of the corresponding activity.

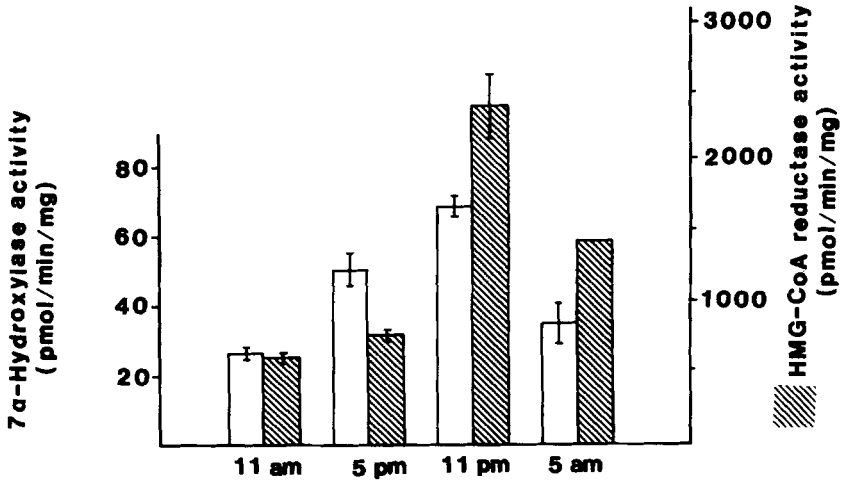

Fig. 4. Diurnal variation of cholesterol 7α-hydroxylase activity (open bars) and HMG-CoA reductase activity (hatched bars) in rats treated with phenobarbital for 48 h. The specific catalytic activity for the two enzymatic activities are shown as means ± SEM of four independent experiments.

The possibility was investigated that the effect seen on cholesterol 7α-hydroxylase and HMG-CoA reductase was due to phenobarbital-induced change in diurnal rhythm of the two enzymes. As shown in **Fig. 4,** the two enzyme activities showed the normal diurnal rhythm with a maximum at 11 PM also in phenobarbital-treated rats. There was a tendency to a delay in the HMG-CoA reductase activity as the relative increase of HMG-CoA reductase was slightly lower than that of cholesterol 7α-hydroxylase at 5 PM and slightly higher at 5 AM (Fig. 4). On the other hand, there was not such an effect on HMG-CoA reductase mRNA in relation to cholesterol 7α-hydroxylase mRNA at 5 PM (**Fig. 5**). With the exception of the situation at 5 PM the mRNA levels corresponding to the two enzymes parallelled the enzyme activities (Fig. 5).

In contrast to the situation in the liver, phenobarbital treatment did not stimulate HMG-CoA reductase in kidneys (**Table 2**). In the experiments shown in Table 2, HMG-CoA reductase activity was even lower in the phenobarbital-treated rats than in the controls. Also adrenal HMG-CoA reductase activity seemed to be unaffected or decreased by phenobarbital treatment.

The possibility must be considered that the increased HMG-CoA reductase activity in the liver may be due to reduced levels of cholesterol as a consequence of increased cholesterol 7α-hydroxylase activity. In accordance with this, liver microsomes from phenobarbital-treated Wistar F rats had a significantly lower cholesterol content than saline-treated controls (**Table 3**). In contrast, liver microsomes from phenobarbital-treated Sprague-Dawley rats (non-responders) had cholesterol levels similar to those of saline-treated controls.

If cholesterol depletion in the liver leads to increased HMG-CoA reductase, this may occur at a transcriptional or posttranscriptional level. According to recent studies (15, 17), regulation of HMG-CoA reductase occurs mainly at a posttranscriptional level in rats. In view of this, it was considered important to establish that HMG-CoA reductase activity can also be regulated by pretranslation mechanisms other than by phenobarbital treatment. Lymphatic drainage is known to result in increased HMG-CoA reductase activity in the liver (23), presumably due to loss of cholesterol-rich chylomicrons. As shown in **Table 4,** lymphatic drainage resulted in a marked stimulation of HMG-CoA reductase in the liver of the Wistar F rats with a parallel increase in HMG-CoA reductase mRNA. The cholesterol content of the liver microsomes of the rats subjected to lymphatic drainage was lower than

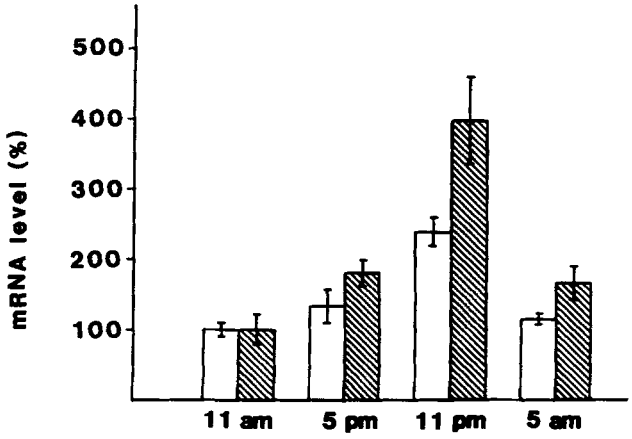

Fig. 5. Diurnal variation of cholesterol 7α-hydroxylase mRNA (open bars) and HMG-CoA reductase mRNA (hatched bars) in rats treated with phenobarbital for 48 h. The mRNA level at each time point was compared with that of a corresponding control at 11 AM, which was set to 100%. The means ± SEM of four independent experiments are shown.

TABLE 2. Activity of cholesterol 7α-hydroxylase in liver and HMG-CoA reductase in liver and kidney of Wistar F rats after treatment with phenobarbital for 48 h

Enzyme Activity	Control Rats	Phenolbarbital-Treated Rats	Difference
	pmol/min/mg		%
Hepatic cholesterol 7α-hydroxylase	35 ± 3	64 ± 5	+83[a]
Hepatic HMG-CoA reductase	744 ± 36	1083 ± 35	+39[a]
Renal HMG-CoA reductase	59 ± 2	37 ± 2	−37[a]
Adrenal HMG-CoA reductase	38	29	−24

Male rats of the Wistar F strain were injected daily intraperitoneally with 100 mg/kg body weight phenobarbital (n = 5) or saline (n = 5) and were killed 2 h after the third injection (see Materials and Methods). The results shown are the means ± SEM with the exception of the experiments with adrenals, in which the mean of two independent experiments is shown.

[a] $P < 0.01$, Student's *t*-test.

that of the control animals (Table 4). For reasons of comparison, Wistar F rats were also treated with 2% cholesterol for 5 days. This treatment resulted in a markedly reduced level of both HMG-CoA reductase activity and HMG-CoA reductase mRNA (results not shown).

DISCUSSION

In spite of considerable efforts, the nature of the link between the two important rate-limiting enzymes in cholesterol homeostasis, HMG-CoA reductase and cholesterol 7α-hydroxylase, is still unknown. It is evident, however, that there may be interactions between these two enzymes at several different levels. A basis for all possible regulatory models must be the well-established down-regulation of cholesterol 7α-hydroxylase by bile acids and the down-regulation of HMG-CoA reductase by cholesterol.

We have previously discussed the possibility that the regulation of HMG-CoA reductase by bile acids may be secondary to the effect of bile acids on cholesterol 7α-hydroxylase (32). If bile acids are the primary regulators of cholesterol 7α-hydroxylase, the activity of the latter enzyme may be of importance for the cholesterol content in the hepatocyte. If HMG-CoA reductase is primarily regulated by the size of a critical cholesterol pool in the cell, and this pool is also a substrate for cholesterol 7α-hydroxylase, this would explain at least part of the covariation of the two enzymes under most conditions.

In the present work the latter hypothesis was tested, taking advantage of the fact that it is possible to obtain a stimulation of cholesterol 7α-hydroxylase activity by the cytochrome P450 inducer phenobarbital in some strains of rats. We show here that at least two specific strains of rats have a cholesterol 7α-hydroxylase system that is induced by phenobarbital. The phenobarbital treatment also caused an increased HMG-CoA reductase activity in these rats. Such stimulation was not seen in the strains of rats that have cholesterol 7α-hydroxylase that does not respond to phenobarbital. The stimulatory effect of

TABLE 3. Effect of phenobarbital treatment for 48 h on liver and kidney content of cholesterol in Wistar F rats and Sprague-Dawley rats

Source	Cholesterol Content	
	Unesterified	Total
	μg/mg protein	
Wistar F rats		
Liver microsomes from control rats	17.2 ± 1.1	22.2 ± 1.7
Liver microsomes from phenobarbital-treated rats	13.3 ± 0.7[a]	18.0 ± 0.5[a]
Kidney microsomes from control rats	52.3 ± 1.2	60.1 ± 1.4
Kidney microsomes from phenobarbital-treated rats	48.7 ± 2.1[b]	57.0 ± 3.9[b]
Sprague-Dawley rats		
Liver microsomes from control rats	17.7 ± 1.0	23.8 ± 1.4
Liver microsomes from phenobarbital-treated rats	16.5 ± 0.7[b]	20.6 ± 1.3[b]

Male rats of two different strains were injected daily intraperitioneally with 100 mg/kg body weight phenobarbital (n = 5) or saline (n = 5) and were killed 2 h after the third injection (see Materials and Methods). The total and free cholesterol in the liver microsomes were measured with a mass spectrometric method with use of a deuterium-labeled internal standard (24). The results shown are the means ± SEM.

[a] $P < 0.05$.

[b] $P > 0.05$.

TABLE 4. Effect of lymphatic drainage on HMG-CoA reductase activity, HMG-CoA reductase mRNA, and content of cholesterol in liver microsomes of Wistar F rats

	Lymph-Fistula Rats	Sham-Operated Rats
HMG-CoA reductase activity (pmol/min/mg)	1037 ± 109[a]	378 ± 51
HMG-CoA reductase mRNA (% of control)	229 ± 24[a]	100 ± 40
Total cholesterol content in liver microsomes (μg/mg/protein)	22.2 ± 2.8[b]	30.0 ± 0.8
Free cholesterol content in liver (μg/mg/protein)	20.7 ± 2.5[b]	27.9 ± 1.5

Wistar F rats were treated with a lymph fistula for 72 h (n = 5), using the conditions described in reference 23. The control animals were sham-operated at the same time as the lymph fistulation and were otherwise treated as the fistulated animals. The results shown are the means ± SEM.

[a] $P < 0.01$.

[b] $P < 0.05$.

phenobarbital on HMG-CoA reductase could only be demonstrated in the liver, and the corresponding activity in the kidneys was slightly reduced. These results are in accord with the contention that the increased HMG-CoA reductase activity is secondary to the increased cholesterol 7α-hydroxylase activity. If so, the size of the pool of cholesterol that regulates hepatic HMG-CoA reductase activity could be the link between the two enzymes. Alternatively, the regulation of HMG-CoA reductase may be mediated by an oxysterol derived from the above pool of cholesterol. In accordance with the hypothesis, the content of free cholesterol in the liver microsomes of the phenobarbital-treated rats was lower than that of the controls. Phenobarbital-treated rats that had a cholesterol 7α-hydroxylase system that was not stimulated by the treatment were shown to have normal levels of free cholesterol in the liver microsomes. It should be emphasized that the critical pool of cholesterol controlling HMG-CoA reductase may be small in relation to the bulk of cholesterol. Thus, no marked changes in the cholesterol content would be expected under different conditions.

The possibility that HMG-CoA reductase is primarily stimulated by phenobarbital seems highly unlikely in view of the fact that a stimulatory effect on this enzyme was seen only in the liver and in the presence of a stimulated cholesterol 7α-hydroxylase system. It is known that in addition to hydroxylases phenobarbital also stimulates cytochrome P450 reductase, epoxide hydroxylase, and glutathione S-transferase. The latter enzymes have properties very different from those of HMG-CoA reductase, however.

It has been reported in at least one previous study that phenobarbital treatment of rats is associated with reduced cholesterol content in the liver (33). It has also been reported that treatment of humans with phenobarbital is associated with reduced circulating levels of LDL and a reduced LDL/HDL ratio (34, 35). Such changes are likely to occur in a state with increased degradation of cholesterol to bile acids, and are thus in accordance with the results of the present study. In another study, however, phenobarbital was found to increase plasma cholesterol and triglycerides in three of four healthy humans (36). According to a report by Coyne et al. (37) both HMG-CoA reductase and cholesterol 7α-hydroxylase activities are at least slightly increased in human liver in response to phenobarbital treatment, and the situation seems to be similar in hamsters (38). The possibility that increased HMG-CoA reductase activity could be secondary to increased cholesterol 7α-hydroxylase activity was not, however, discussed by these authors.

The exact mechanism by which cytochrome P450 enzymes are induced by phenobarbital is unknown. The species difference in the response of cholesterol 7α-hydroxylase towards phenobarbital may be due to differences in expression of transcription factors or structural differences in the promotor region of the gene coding for cholesterol 7α-hydroxylase. Attempts to correlate the gene structure of cholesterol 7α-hydroxylase in different species with the specific ability to respond to phenobarbital treatment have been initiated in our laboratory.

It is of interest to note that the phenobarbital-induced increase in HMG-CoA reductase activity was associated with increased levels of the corresponding mRNA. Such an increase in mRNA levels would not be expected if the activity is only posttranscriptionally controlled, as recently suggested by some authors (15, 17). Since we could show that cholesterol depletion by lymphatic drainage caused increased mRNA levels and that cholesterol feeding caused a decrease in such levels, it is evident that HMG-CoA reductase is, at least to some extent, controlled by transcription or stability of mRNA in the specific strain of rats studied here. The different mechanisms for regulation of HMG-CoA reductase that have been reported in rats may be due to species differences. In a recent study it was well documented that there are different mechanisms for down-regulation of HMG-CoA reductase by dietary cholesterol in two different strains of mice (39). It seems likely that much of the controversy with respect to different mechanisms for regulation of cholesterol homeostasis in rats and mice may be due to strain differences.

It should be noted that the effect of phenobarbital on the two enzymes could be observed only during the first few days of treatment. Whether this is due to increased metabolism of phenobarbital or due to compensatory effects on the key enzymes in cholesterol homeostasis was not investigated.

To summarize, our results suggest that a primary upregulation of cholesterol 7α-hydroxylase may lead to a secondary increase in HMG-CoA reductase. In accordance with this we recently demonstrated that transfection of COS-cells with a cDNA corresponding to human cholesterol 7α-hydroxylase leads to increased activity of HMG-CoA reductase activity in the cells (E. Sudjana-Sugiaman, G. Eggertsen, and I. Björkhem, unpublished observation). As we found a reduced content of free cholesterol in the liver microsomes of the phenobarbital-treated rats and as cholesterol depletion is likely to result in a compensatory increase in HMG-CoA reductase activity, it appears likely that utilization of cholesterol by cholesterol 7α-hydroxylase is an important link between the two enzymes. It is tempting to suggest that the above mechanism is responsible for part of the covariation between cholesterol 7α-hydroxylase and HMG-CoA reductase not only in connection with phenobarbital treatment but also under other conditions.

The authors are grateful to Ulla Andersson for help with lymph fistulation of rats and to Manfred Held for help with synthesis and cholesterol measurements. We are further grateful to Professor K. Okuda and Professor P. Hylemon for the generous gift of the cDNA used in the study. This work was supported by a grant from The Swedish Medical Research Council (Project 03X-3141).

Manuscript received 6 July 1993 and in revised form 27 September 1993.

REFERENCES

1. Björkhem, I. 1985. Mechanism of bile acid biosynthesis. *In* Sterols and Bile Acids. Comprehensive Biochemistry. Vol. 12. H. Danielsson and J. Sjövall, editors. Elsevier Science Publishers, Amsterdam. 231–278.
2. Vlahcevic, Z. R., D. M. Heuman, and P. B. Hylemon. 1991. Regulation of bile acid biosynthesis. *Hepatology.* **13:** 590–600.
3. Russel, D. W., and K. D. R. Setchell. 1992. Bile acid biosynthesis. *Biochemistry.* **31:** 4737–4749.
4. Björkhem, I. 1986. Effects of mevinolin in rat liver: evidence for a lack of coupling between synthesis of HMG-CoA reductase and cholesterol 7α-hydroxylase activity. *Biochim. Biophys. Acta.* **877:** 43–49.
5. Reihnér, E., M. Rudling, D. Ståhlberg, L. Berglund, S. Ewerth, I. Björkhem, and K. Einarsson. 1990. Influence of pravastatin, a specific inhibitor of HMG-CoA reductase, on hepatic metabolism of 17-cholesterol. *N. Engl. J. Med.* **323:** 224–228.
6. Pandak, W. M., D. M. Heuman, P. B. Hylemon, and Z. R. Vlahcevic. 1990. Regulation of bile acid biosynthesis. IV. Interrelationship between cholesterol and bile biosynthesis. *J. Lipid Res.* **31:** 79–90.
7. Pandak, W. M., Z. R. Vlahcevic, J. Y. L. Chiang, D. M. Heuman, and P. B. Hylemon. 1992. Bile acid synthesis. VI. Regulation of cholesterol 7α-hydroxylase by taurocholate and mevalonate. *J. Lipid Res.* **33:** 659–668.
8. Mitropoulos, K. A., S. Balasubramaniam, S. Venkatesan, and B. E. A. Reeves. 1978. On the mechanism for the regulation of HMG-CoA reductase, of cholesterol 7α-hydroxylase and of ACAT by free cholesterol. *Biochim. Biophys. Acta.* **530:** 99–111.
9. Sundseth, S. S., and D. J. Waxman. 1990. Hepatic P-450 cholesterol 7α-hydroxylase. Regulation in vivo at the protein and mRNA level in response to mevalonate, diurnal rhythm and bile acid feedback. *J. Biol. Chem.* **265:** 15090–15095.
10. Björkhem, I., and J-E. Åkerlund. 1988. Studies on the link between HMG-CoA reductase and cholesterol 7α-hydroxylase in rat liver. *J. Lipid Res.* **29:** 136–143.
11. Noshiro, M., M. Nishimoto, K. Morohashi, and K. Okuda. 1992. Molecular cloning of cDNA for 7α-hydroxylase from rat liver microsomes. *FEBS Lett.* **257:** 97–100.
12. Li, Y. C., D. P. Wang, and J. Y. L. Chiang. 1990. Regulation of cholesterol 7α-hydroxylase in rat liver. *J. Biol. Chem.* **265:** 12012–12019.
13. Jelinek, D. F., S. Andersson, C. A. Slaughter, and D. W. Russel. 1990. Cloning and regulation of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. *J. Biol. Chem.* **265:** 8190–8197.
14. Pandak, W. W. M., Y. C. Li, J. Y. L. Chiang, E. J. Studer, E. C. Gurley, D. M. Heuman, Z. R. Vlahcevic, and P. B. Hylemon. 1991. Regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by taurocholate in the chronically biliary diverted rat. *J. Biol. Chem.* **266:** 3416–3421.
15. Spady, D. K., and J. A. Cuthbert. 1992. Regulation of *hepatic sterol metabolism.* Parallel regulation of activity and mRNA for 7α-hydroxylase but not for HMG-CoA reductase or LDL-receptor. *J. Biol. Chem.* **267:** 5584–5591.
16. Duckworth, P. F., Z. R. Vlahcevic, E. J. Studer, E. C. Gurley, D. M. Heuman, Z. H. Beg, and P. B. Hylemon. 1991. Effect of hydrophobic bile acids on HMG-CoA reductase activity and mRNA levels in the rat. *J. Biol. Chem.* **266:** 9413–9418.
17. Ness, G. C., R. K. Keller, and L. C. Pendleton. 1991. Feedback regulation of hepatic HMG-CoA reductase activity by dietary cholesterol is not due to altered mRNA levels. *J. Biol. Chem.* **266:** 14854–14857.
18. Shefer, S., S. Hauser, and E. H. Mosbach. 1972. Stimulation of cholesterol 7α-hydroxylase by phenobarbital in two different strains of rats. *J. Lipid Res.* **13:** 69–70.
19. Wada, F., K. Hirata, K. Nakao, and Y. Sakamoto. 1968. Participation of P-450 in 7α-hydroxylase of cholesterol. *J. Biochem. (Tokyo)* **64:** 415–417.
20. Cohen, B. I. 1975. Effects of phenobarbital upon bile acid synthesis in two strains of rats. *Lipids.* **10:** 168–174.
21. Einarsson, K., and G. Johansson. 1968. Effect of phenobarbital on hydroxylation of bile acids by rat liver microsomes. *Eur. J. Biochem.* **6:** 293–298.
22. Björkhem, I., and A. Kallner. 1976. Hepatic 7α-hydroxylation of cholesterol in ascorbate-deficient and ascorbate-supplemented guinea pigs. *J. Lipid Res.* **17:** 360–365.
23. Björkhem, I., G. Eggertsen, and U. Andersson. 1991. On the mechanism of stimulation of cholesterol 7α-hydroxylase by dietary cholesterol. *Biochim. Biophys. Acta.* **1085:** 329–335.
24. Björkhem, I., R. Blomstrand, and L. Svensson. 1974. Serum cholesterol determination by mass fragmentogra-

phy. *Clin. Chim. Acta.* **54:** 185–193.
25. Brown, M. S., J. L. Goldstein, and J. M. Dietschy. 1979. Active and inactive forms of HMG-CoA reductase in liver of rat. Comparison with the rate of cholesterol synthesis in different physiological states. *J. Biol. Chem.* **254:** 5144–5149.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
27. Auffrey, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* **107:** 303–314.
28. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
29. Chin, D. J., G. Gil, J. R. Faust, J. L. Goldstein, M. S. Brown, and K. L. Luskey. 1985. Sterols accelerate degradation of hamster HMG-CoA reductase encoded by a constitutively expressed cDNA. *Mol. Cell Biol.* **5:** 634–641.
30. Tso, J. Y., X. H. Sun, T. Kao, S. Reece, and R. Wu. 1985. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs. *Nucleic Acids Res.* **13:** 2485–2502.
31. Gehring, M. R., B. R. Shiels, W. Northeman, M. H. L. deBruijn, C. C. Kan, A. C. Chain, D. J. Noonan, and G. H. Fey. 1987. Sequence of rat liver α-$_2$-macroglobulin and acute phase control of its messenger. *J. Biol. Chem.* **262:** 446–454.
32. Åkerlund, J-E., I. Björkhem, and U. Andersson. 1990. Studies on the regulation of cholesterol 7α-hydroxylase and HMG-CoA reductase in rat liver: effects of lymphatic drainage and ligation of the lymph duct. *J. Lipid Res.* **31:** 2159–2166.
33. Hayes, E. P., P. Scala, and G. Wilz. 1986. Lipid composition of liver microsomes from rats treated with acetone. *Toxicol Lett.* **31:** 139–145.
34. Luoma, P. V., E. A. Solaniemi, R. O. Pelkonen, and H. I. Pirttiaho. 1985. Serum low-density lipoprotein and high-density lipoprotein cholesterol, and liver size in subjects on drugs inducing hepatic microsomal enzyme. *Eur. J. Clin. Pharmacol.* **28:** 615–618.
35. Yamamoto, T., S. Takahashi, H. Nakaoko, M. Nakazalo, M. Suda, Y. Moriwaki, T. Hada, M. Yanagishsawa, and K. Higashino. 1989. The effect of phenobarbital on human plasma lipids. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **27:** 463–466.
36. Miller, N. E., and P. J. Nestel. 1973. Altered bile acid metabolism during treatment with phenobarbitone. *Clin. Sci. Mol. Med.* **45:** 257–262.
37. Coyne, M. J., G. G. Bonorris, L. I. Goldstein, and L. J. Schoenfield. 1976. Effect of chenodeoxycholic acid and phenobarbital on the rate-limiting enzymes of hepatic cholesterol and bile acid. *J. Lab. Clin. Med.* **87:** 281–291.
38. Schoenfield, L. J., G. G. Bonorris, and P. Ganz. 1973. Induced alterations in the rate-limiting enzymes of hepatic cholesterol and bile acid synthesis in the hamster. *J. Lab. Clin. Med.* **82:** 858–868.
39. Hwa, J. J., S. Zollman, C. H. Warden, B. A. Taylor, P. A. Edwards, A. M. Fogelman, and A. J. Lusis. 1992. Genetic and dietary interactions in the regulation of HMG-CoA reductase gene expression. *J. Lipid Res.* **33:** 711–725.