In Vivo and *in Vitro* Studies on the Regulatory Link between 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase and Cholesterol 7α-Hydroxylase in Rat Liver

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The activities of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoA reductase; EC 1.1.1.34), rate-limiting enzyme of cholesterol biosynthesis, and cholesterol 7α-hydroxylase (EC 1.14.13.17), key enzyme of the neutral bile acid synthesis pathway, were measured in the microsomal fraction of rat liver and in rat liver cells to investigate the coordinate regulation of the two pathways.

Both enzyme activities exhibited the same diurnal rhythm and responded in a coordinate fashion to fasting or bile acid-feeding (decrease) and to cholestyramine-feeding (increase). Cholesterol-feeding decreased the activity of HMGCoA reductase, increased that of cholesterol 7α-hydroxylase, and concomitantly increased free cholesterol in microsomes.

In an *ex vivo* setting using primary hepatocytes from animals fed a high cholesterol diet the activity of HMGCoA reductase was initially low and that of cholesterol 7α-hydroxylase was elevated. Release of cholesterol into the medium with ongoing incubation caused HMGCoA reductase activity to increase, and that of cholesterol 7α-hydroxylase to decline. Incubation of hepatocytes with a cholesterol-containing lipoprotein fraction stimulated the activity of cholesterol 7α-hydroxylase, but left HMGCoA reductase activity unaffected.

The results confirm the idea of a joint regulation of the two key enzymes of cholesterol metabolism in response to the levels of substrate and metabolites, and support the notion that with respect to bile acid and cholesterol levels, respectively, regulation of HMGCoA reductase activity may be secondary to that of cholesterol 7α-hydroxylase. The *in vitro* studies supply evidence that the effects of cholesterol and bile acid excess or deficiency are direct and do not involve accessory changes of hormone levels or mediators.

Introduction

Cholesterol exists in two forms in liver, free and esterified. A metabolically active pool of free cholesterol is the source for bile acid synthesis and biliary cholesterol excretion. Several proteins play a role in the regulation of the hepatic free cholesterol pool: HMGCoA reductase regulates *de novo* synthesis of cholesterol (Pandak *et al.*, 1990); cholesterol 7α-hydroxylase initiates the conversion of cholesterol into cholic and chenodeoxycholic acids, and represents a major control point for removal of cholesterol from the body; acyl CoA: cholesterol acyltransferase converts free cholesterol to the esterified or storage form (Suckling and Stange, 1985); neutral cholesterylester hydrolase, located in the cytosol, hydrolyzes cholesterol esters from the liver to form free cholesterol, and the LDL receptor shuttles lipoprotein-associated cholesterol into the cell (cf. Björkhem *et al.*, 1997). It is fair to assume that these proteins act together in concert to fine-tune cholesterol homeostasis in the organism. However, only HMGCoA reductase and cholesterol 7α-hydroxylase, being the rate-limiting enzymes in their respective pathways, play key roles in the synthesis and degradation of cholesterol.

HMGCoA reductase catalyzes the conversion of HMGCoA to mevalonic acid, an early step in the biosynthesis of isoprenoids which is essentially

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irreversible.The enzyme has a wide distribution (see e.g. Boll, 1983; Boll and Kardinal, 1990). In mammalian cells this enzyme activity, located in the microsomes, is rate-limiting for the synthesis of cholesterol (Edwards *et al.*, 1983). Considering the biological significance of cholesterol (e.g., in membrane function) it is evident that both synthesis and activity of the enzyme must be precisely regulated. A number of mechanisms for the regulation have been suggested for hepatic HMGCoA reductase activity (comprehensively reviewed in Björkhem *et al.*, 1997): feedback inhibition by cholesterol; changes in synthesis, degradation or stability of enzyme protein and/or mRNA; regulation of catalytic activity by enzyme-lipid interaction; a phosphorylation cascade; changes in the cellular thiol/disulfide balance; or substrate-dependent modifications of the enzyme as well as changes in substrate availability.

The synthesis of bile acids from cholesterol is initiated by the introduction of a 7α-hydroxyl group into the cholesterol nucleus by cholesterol 7α-hydroxylase, a microsomal, cytochrome P-450-dependent monooxygenase (CYP7; cf. Chiang and Stroup, 1994). Its activity is subject to long-term, mid-term and short-term regulation: bile acid-dependent feedback control represents long-term regulation (Reihnér *et al.*, 1989); the circadian rhythm of enzyme activity is defined as mid-term regulation (Sundseth and Waxman, 1990); finally, cholesterol 7α-hydroxylase is responsive to short-term regulation by hormones, drugs and cholesterol precursors (mevinoline) which can modulate the enzyme activity in a matter of hours (Björkhem *et al.*, 1997). These types of regulation operate primarily at the pre-translational level and are thought to be of greater importance than other mechanisms, e.g., allosteric effects of bile acids, changes in cholesterol availability, or reversible phosphorylation (Sundseth and Waxman 1990).

Metabolic regulation of cholesterol levels in response to varying levels of input is physiologically important and effective unless a genetic defect exists where blood lipoproteins are not able to transport cholesterol appropriately. The purpose of this investigation was to further evaluate factors which might be involved in the coordinate regulation of HMGCoA reductase and cholesterol 7α-hydroxylase activities. A prerequisite for this is to assess both enzyme activities in one and the same animal, a protocol which has not been followed consistently in earlier investigations. Additional studies to support *in vivo* findings were performed using cultured primary hepatocytes.

Methods

Animals and experimental conditions

Female Sprague-Dawley rats (150–200 g) from our own husbandry were used. The animals were fed a standard stock diet (diet #1324, Altromin International, Lage, FRG; (for composition see Boll *et al.*, 1996) and routinely housed in a temperature- (25±2 °C) and humidity-controlled room on a 12h light and dark cycle: lights on 6 a.m. to 6 p.m. for the diurnal rhythm study (Fig. 1), and 3 p.m. to 3 a.m. for all other experiments. With the changed lighting cycle (light-reversed animals) maximum enzyme activities occurred during regular working hours, i.e., around 10 a.m. (see also section on circadian rhythm). The animals had free access to feed and water.

Diets containing cholesterol, cholestyramine or bile acids were prepared by adding the substances, dissolved in a suitable solvent, to the standard stock diet which had been powdered before. The mixture was then pasted with 2 parts water, pellets were formed and dried at 25 °C for 3 days.

Preparation of liver microsomes

Unless stated otherwise livers were removed at 9–10 a.m. (diurnal maximum of enzyme activity in reversed animals) under ether anesthesia and rapidly chilled on ice. Livers were washed once in homogenization buffer (0.1 M potassium phosphate, pH 7.4, 0.3 M sucrose, 10 mM β-mercaptoethanol, 1 mM EDTA) and homogenized in the same buffer (1 g liver /2 ml) with a Potter homogenizer (10 strokes). Homogenates were centrifuged for 20 min. at 18,000×*g*, the resulting supernatants then centrifuged for 60 min at 105,000×*g*. All procedures were carried out at 4 °C. The microsomal pellets were stored in liquid nitrogen. For enzyme assays pellets were resuspended in 0.1 M KH_2PO_4 buffer pH 7.4, 2 mM dithioerythritol (DTE), at a protein concentration of approx. 60 mg/ml.

Enzyme assays

Cholesterol 7α-hydroxylase (EC 1.14.13.17) activity was determined as described in detail by

Nimmannit and Porter (1980) with some modifications: a) Tween 80 was used instead of Emulgen 911; b) 10 µl of unlabeled 7α-cholesterol (50 µg) in 2 ml ethanol was added as carrier to the assay mixture to terminate the reaction; c) unmodified substrate was separated from 4-[^{14}C] 7α-cholesterol formed during the reaction by TLC using silica gel plates 60 F_{264} (Merck) instead of silica gel G plates. 4-[^{14}C] 7α-cholesterol was identified by exposure to iodine vapor.

Assay mixtures contained, in a total volume of 0.9 ml, up to 0.3 mg microsomal protein and 5.5 KBq 4-[^{14}C] cholesterol. The substrate was prepared by removing the solvent from the 4-[^{14}C] cholesterol stock solution with a stream of nitrogen and suspending the tracer in a small amount of ethanol. The substrate was added to a solution of 0.1 M KH_2PO_4, pH 7.4, containing 2 mM DTE and 0.52 mg Tween 80 per ml, and resuspended by sonication.

HMGCoA reductase (EC 1.1.1.34) activity was assayed according to Jenke *et al.,* (1981) using 0.1 M KH_2PO_4 (pH 7.2), 10 mM EDTA, 10 mM DTE, 40 mM glucose-6-phosphate, 4 mM NADP, 6 units of glucose-6-phosphate dehydrogenase, 0.1 mg bovine serum albumin, 77 µM DL-3-hydroxymethyl 3-[^{14}C] glutaryl Coenzyme A , 1 mM DL-HMGCoA, and enzyme solution in a total volume of 0.25 ml.

Aryl hydrocarbon hydroxylase (EC 1.14.14.1) activity was assayed as described by Nebert and Gelboin (1968) with some modifications: 3-methylcholanthrene (124 mg/Kg in vegetable oil) was injected i.p. into rats. Enzyme activity was assayed 36h later. The incubation mixture contained, in a total volume of 1 ml, 0.05 M Tris [tris(hydroxymethyl)aminomethane] HCl, pH 7.5, 0.36 µmole NADPH, 3 µmol $MgCl_2$ and microsomal fraction, containing 250–800 µg protein. The reaction was started with 80 nmol of benzopyrene in 0.05 ml acetone and the mixture incubated immediately for 30 min with shaking. The reaction was stopped by addition of 4 ml propanol:hexane (1:3, v/v), and the mixture was shaken vigorously for 10 sec. 2 ml aliquots of the organic phase were shaken with 2 ml 1 N NaOH for 20 sec. After 5 min centrifugation at low speed the two phases were separated. The concentration of extracted hydroxylated benzopyrene was determined by fluorescence spectrophotometry in the aqueous phase (activation at 396 nm, emission at 520 nm). A blank was used where the substrate had been omitted.

All enzyme activities were measured in triplicate at 37 °C. The specific activity of cholesterol 7α-hydroxylase was expressed as pmol 7α-hydroxycholesterol formed per min and mg protein, that of HMGCoA reductase as nmol mevalonic acid formed per min and mg protein, that of aryl hydrocarbon hydroxylase as nmol hydroxybenzopyrene formed per min and mg protein. Protein was determined with the method of Lowry *et al.,* (1951), using bovine serum albumin as standard.

Determination of cytochrome P450

The content of cytochrome P450 in microsomes was determined according to Omura and Sato (1964).

Cholesterol determination

Cholesterol was determined according to Röschlau *et al.,* (1974). The method was found suitable also for microsomal suspensions. Control determinations of microsomal cholesterol with the method of Weiss and Barth (1980) gave identical results.

Preparation of primary hepatocytes

Hepatocytes were prepared at 9–10 a.m. (time of maximal enzyme activity) according to Spencer and Pitot (1982). Viability of the cells was tested with 0.4% trypan blue. Cells were plated on Petri dishes (8.5 cm diameter) in a hormone- and nutrient-enriched (optimized) medium as follows: Ham's F12 nutrient mixture (10.72 g/l), $NaHCO_3$ (2.24 g/l), oleic and linoleic acids (5 mg/l each), L-alanine (11.2 mg/l), L-serine (12.8 mg/l), L-asparagine (24 mg/l), δ-aminolevulinic acid (1 µM), bovine insulin (8 mg/l), DL-α-tocopherol acetate (5 mg/l), D-thyroxine (10 µM), testosterone (1 µM), glucagon (50 nM), hydrocortisone 21-acetate (50 µM), 17 β-estradiol (1 µM), penicillin (100 mg/l), kanamycin (50 mg/l), 10% calf serum, and 5% fetal calf serum. Cell density at the time of plating was 8.6×10^4 cells/ml. Each dish received 7.5 ml of the cell suspension and was kept at 37 °C in an atmosphere of 95% O_2 and 5% CO_2. Cell cultures were incubated up to 48 h. Media were renewed

after 4, 17 and 27 h, respectively, but fetal calf serum was not added to the last two changes.

To harvest the hepatocytes the incubation medium was removed and cells were rinsed with the extraction buffer used for the preparation of liver microsomes (see above). Cells were scraped off the dishes, sonicated for 30 sec at 4 °C and subsequently centrifuged at 105,000×*g* for 60 min. The pellets were resuspended (5 mg protein/ml) in a small volume of buffer (0.1 M KH_2PO_4, pH 7.4 and 2 mM DTE) and used as enzyme source.

In the experiments with a cholesterol-rich lipoprotein fraction freshly prepared hepatocytes were incubated (15 ml optimized medium as above, containing 8.6×10^4 cells/ml) with the addition of either 2 ml vehicle, delipidized lipoprotein, or lipoprotein fraction (see below), at 37 °C for 5 h under 95% O_2, 5% CO_2. Thereafter cells were collected and microsomal fractions were prepared as described above.

Lipoproteins

Human blood plasma (German Red Cross Blood Bank, Munich, FRG) was incubated at 56 °C for 30 min. The heat inactivated serum was then centrifuged for 20 min at 18,000×*g*, 4 °C. The clear supernatant was subsequently brought to a density of 1.215 g/ml by addition of NaBr (Hatch and Lees, 1968), and centrifuged for 48 h at 105,000×*g*, 0 °C. The floating total lipoprotein fraction was collected, dialyzed for 24 h against 0.15 M NaCl, freeze-dried and then reconstituted at 3-fold concentration. The concentration of free cholesterol in this solution was 12 mM. This fraction was used for the experiments.

Delipidation was performed by extracting 1 volume lipoprotein fraction with 2 volumes of a 4:6 butanol: diisopropylether mixture for 3 h at 37 °C. After centrifugation at 2,000×*g* for 15 min the upper phase was discarded and the lower phase was freed of the organic solvent by use of an evaporator. This phase was essentially free of cholesterol but showed no loss in protein content. It was freeze-dried and reconstituted at 3-fold concentration for use as delipidized fraction in the experiments with the cholesterol-rich lipoprotein fraction.

Suppliers of biochemicals

DL-3-hydroxy-3-methyl [glutaryl-3-^{14}C] Coenzyme A (spec. act. 2.1 GBq/mmol) and 4-[^{14}C] cholesterol (spec. act. 1.84 GBq/mmol) were obtained from New England Nuclear, Dreieich, FRG. DL-3-hydroxy-3-methylglutaryl (HMG) Coenzyme A, HMG acid, 7α-hydroxycholesterol (5-cholesten-3β,7α-diol), mevalonolactone, bile acids, benzo(a)pyrene (3,4-benzpyrene), hormones and nutrients for the optimized medium, and glucose-6-phosphate dehydrogenase were supplied by Sigma Chemical Company, Deisenhofen, FRG. Cholestyramine was a product of Fluka, Buchs, Switzerland. All chemicals used were of analytical grade.

Statistical analysis

Statistical significance was determined by ANOVA, followed by the Tukey-Kramer multiple comparison Test.

Results and Discussion

HMGCoA reductase and cholesterol 7α-hydroxylase in liver microsomes

Circadian rhythm

Hepatic HMGCoA reductase and cholesterol 7α-hydroxylase activities display a circadian rhythm (Björkhem *et al.*, 1997). In the present study variations of both enzyme activities were tested in the same animal in order to obtain a reliable reference basis. On a normal lighting schedule (lights on: 6 a.m. to 6 p.m.) both activities varied 6- to 10-fold between nadir (around 1 p.m.) and apex (around 1 a.m.) (Fig. 1). The increase began just before the onset, and maximal activity occurred around the middle of the dark period. The 7-fold diurnal variation in the activity of cholesterol 7α-hydroxylase observed here was considerably greater than the 3-fold variation described by Sundseth and Waxman (1990). Diurnal variations of HMGCoA reductase activity found here also were higher than previously reported values (Easom and Zammit, 1984). As can be seen from Fig. 4c (control) microsomal cholesterol levels also displayed diurnal differences varying up to 75% between nadir and apex of enzyme activities. 92.3% of microsomal cholesterol were free, and 7.7% were esterified, while in serum 76% of the cholesterol were esterified, but only 24% were free. No diurnal changes of the serum cholesterol levels were observed.

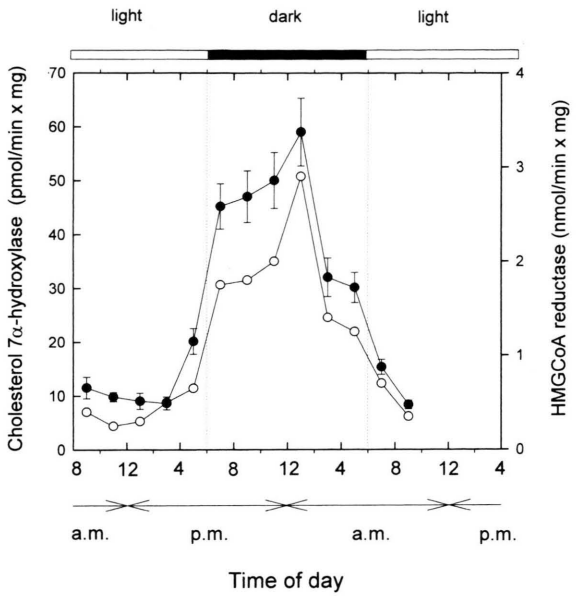

Fig. 1. Diurnal variations of HMGCoA reductase and cholesterol 7α-hydroxylase activities in rat liver microsomes.
HMGCoA reductase (*open circles*); cholesterol 7α-hydroxylase (*full circles*). Rats were kept on the standard stock diet on a normal lighting cycle (lights on: 6 a.m. to 6 p.m.). Values are means of 4 animals. Standard error (SEM) shown for cholesterol 7α-hydroxylase only; SEMs for HMGCoA reductase were of comparable magnitude.

Regulation of the diurnal variation of the enzyme activities occurs most likely at the pre-translational level (Sundseth and Waxman, 1990); in the case of cholesterol 7α-hydroxylase this is achieved by the cyclic release of the transcription factor DBP (albumin promotor D-site binding protein; Lee *et al.*, 1994). A possible role of hypophyseal hormones in diurnal control is unclear as hypophysectomy does not abolish circadian variation (cf. Björkhem *et al.*, 1997). Rats are nocturnally active and feed during dark hours, and consequently the circadian regulation of these enzyme activities is related to nutritional intake (Ogawa *et al.*, 1997). However, at least in the case of HMGCoA reductase this increase cannot be attributed solely to dietary conditions as some increase of activity during the dark phase occurs even when animals are trained to feed during day time (Gregory *et al.*, 1972).

Since the activities of both enzymes exhibit peak activities around midnight as shown in Fig. 1, the light-dark cycle was changed for all subsequent experiments (see Method section). This allowed a more convenient performance of work, with apex enzyme activities now occurring around 10 a.m. while nadir was at 1 a.m. After shifting the light-dark phase a new stable rhythm of enzyme activities was established within 10 days.

Fasting and cholestyramine feeding (depletion of resources)

Table I summarizes changes of enzyme activities elicited by nutritional stimuli. Fasting decreased the activity of both enzymes, whereas feeding cholestyramine, a resin which sequesters cholesterol metabolites (bile acids) in the intestine and thus interrupts their enterohepatic circulation, led to an increase of both activities. The increase could be observed within 2 days of cholestyramine feeding and attained its maximum after several days. This may reflect the time interval required to deplete endogenous bile acid stores. In the case of cholesterol 7α-hydroxylase it had been shown that the induction process required 4–6 days to reach a maximum at 9-fold normal levels, involving *de novo* synthesis of both mRNA and protein (Sundseth and Waxman, 1990).

HMGCoA reductase activity responded to stimuli more pronounced then cholesterol 7α-hydroxylase activity. The response of both enzyme activities to cholestyramine feeding was as expected - depletion of nutritional or endogenous sources triggered increases in synthesis of cholesterol and bile acids. With fasting one would likewise expect an increase of activities to make up for the missing cholesterol intake. The decrease of activities observed here must be interpreted as a measure of the organism to conserve energy. The underlying regulatory mechanism is not known.

Bile acid feeding (excess intake)

Feeding rats with diets which contain bile acids caused the activities of both enzymes to decline (Table I). One-half percent of either cholic acid, deoxycholic acid, chenodeoxycholic acid or taurocholic acid in the diet caused a 55–70% inhibition of both enzyme activities. The extent of inhibition was about the same for both enzyme activities, with chenodeoxycholic acid exhibiting a slightly stronger effect than the other bile acids (Table I).

Table I. Response of liver microsomal HMGCoA reductase and cholesterol 7α-hydroxylase activities to different dietary manipulations.

Treatment	Duration	HMGCoA reductase (nmol/min × mg)	Cholesterol 7α-hydroxylase (pmol/min × mg)
Control		1.77 ± 0.02	48.5 ± 5.1
Fasting	3d	0.26 ± 0.03	24.9 ± 2.7
Cholestyramine, 3%	2d	3.58 ± 0.04	66.1 ± 7.2
Cholic acid, 0.5%	1d	0.61 ± 0.07	20.7 ± 2.4
Deoxycholic acid, 0.5%	1d	0.72 ± 0.08	21.4 ± 2.3
Chenodeoxycholic acid, 0.5%	1d	0.54 ± 0.06	17.1 ± 1.85
Taurocholic acid, 0.5%	1d	0.81 ± 0.09	20.8 ± 2.15

Animals received the stock diet (controls), were fasted, or received the stock diet containing cholestyramine or bile acids starting at the diurnal maximum of activity. Values are means of 4 animals ± SEM. Significance: Values of bile acid feeding are significant versus control ($p < 0.001$); taurocholic acid versus other bile acids ($p < 0.05$) with HMGCoA reductase.

The time course of this inhibition, shown in Fig. 2A for cholic acid, was the same for both enzyme activities. The low points of enzyme activities were reached within 2 days of cholic acid feeding. Inhibition depended on the amount of cholic acid in the feed, with a near maximum effect occurring at 0.2% (Fig. 2B). A time course of the changes of enzyme activities during the first 12 h of bile acid feeding is shown in Fig. 3. During the initial phase of the diurnal rhythm cholesterol 7α-hydroxylase activity increased slightly faster in the presence of dietary cholic acid than HMGCoA reductase activity. However, cholesterol 7α-hydroxylase apparently was more sensitive to the inhibitory action of the bile acids: at the expected maximum of the diurnal rhythm, i.e., 6–8h after onset of bile acid feeding, the activity of cholesterol 7α-hydroxylase was already depressed, whereas HMGCoA reductase activity was still high, exhibiting the normal diurnal changes (Fig. 3).

Bile acid synthesis is under negative feedback control by bile acids in the enterohepatic circulation. The acids act on cholesterol 7α-hydroxylase and even as far back as HMGCoA reductase activity (Björkhem *et al.*, 1993). Pandak *et al.* (1991) demonstrated that the down-regulation of cholesterol 7α-hydroxylase activity by bile acids affected the rate of transcription and mRNA levels. Hydrophobic bile acids inhibited both cholesterol 7α-hydroxylase and HMGCoA reductase (Pandak *et al.*, 1995). It appears that position and orientation of the hydroxyl groups are more important for this effect than degree of hydrophobicity (cf. Björkhem *et al.*, 1997) with chenodeoxycholic acid being most effective (Reihnér *et al.*, 1989). Hydrophilic bile acids, however, were ineffective (Heuman *et al.*, 1989). The hydrophobic bile acids act with the help of hepatocyte nuclear factors on bile acid-responsive elements in the promoter of cholesterol 7α-hydroxylase (Chiang and Stroup, 1994). The enzyme activity is also subject to post-translational regulation (Björkhem *et al.*, 1993).

The mechanism of HMGCoA reductase control, although very similar to that of cholesterol 7α-hydoxylase in many respects, is much less clear. The cholesterol 7α-hydroxylase promotor contains bile acid responsive elements and is regulated by hepatic nuclear factors, a family of transcription factors that belong to the nuclear hormone receptor family (Björkhem *et al.*, 1997) but despite the response of HMGCoA reductase to cholesterol, bile acids and oxosterol levels, no corresponding elements have been identified in its promotor. Duckworth *et al.* (1991) maintain that HMGCoA reductase activity is controlled only at the post-transcriptional level. Shefer *et al.* (1992) even suggest that HMGCoA reductase is controlled by taurocholate flux, whereas the control of cholesterol 7α-hydroxylase involves cholesterol supply and, therefore, might be secondary to that of HMGCoA reductase (Pandak *et al.*, 1992). In contrast, Björkhem *et al.* (1997) make a strong point for HMGCoA reductase activity control being secondary to the bile acid-controlled regulation of cholesterol 7α-hydroxylase activity. Indeed our own data in Fig. 3 support this idea, as cholesterol

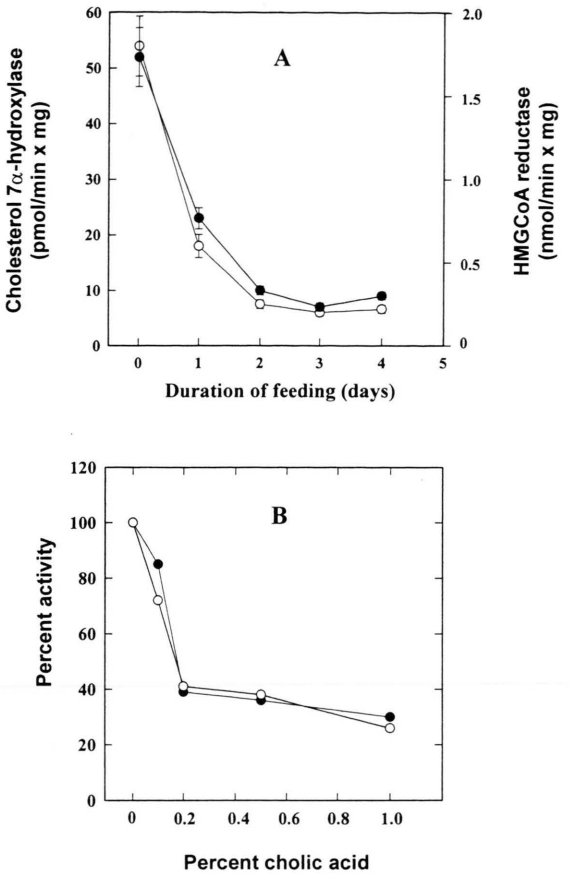

Fig. 2. Effect of dietary cholic acid on the activities of HMGCoA reductase and cholesterol 7α-hydroxylase in rat liver microsomes.
HMGCoA reductase (*open circles*); cholesterol 7α-hydroxylase (*full circles*). Values are percent activity, means of 4 animals ± SEM. **Panel A**: Kinetics of bile acid action. Experimental animals received a diet containing 1 percent cholic acid starting at time zero (10 a.m., diurnal maximum in light-reversed animals) and activities were determined at the indicated times at the respective diurnal maximum. **Panel B:** Effect of bile acid concentration. Enzyme activities were determined at the diurnal maximum 1 day after initiation of cholic acid feeding. Values are percent of controls fed a cholic acid-free standard diet.

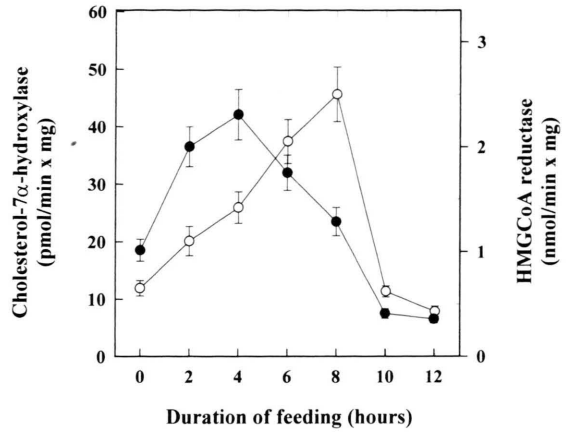

Fig. 3. Kinetics of the effect of dietary cholic acid on the activities of HMGCoA reductase and cholesterol 7α-hydroxylase in rat liver microsomes.
HMGCoA reductase (*open circles*); cholesterol 7α-hydroxylase (*full circles*). Experimental animals received a diet containing 1% cholic acid starting at time zero (3 a.m., beginning of dark phase of diurnal rhythm in light-reversed animals). Values are means of 4 animals ± SEM.

7α-hydroxylase activity responded earlier to bile acid feeding than HMGCoA reductase.

Regulation of cholesterol synthesis and degradation

Feeding a diet containing 3% cholesterol changed the specific activity of the two enzymes in opposite directions: the activity of HMGCoA reductase declined (Fig. 4 A), and the activity of cholesterol 7α-hydroxylase increased (Fig. 4 B). The diurnal increase of HMGCoA reductase activity in the liver of cholesterol-fed animals was significantly smaller than in controls (A). In contrast, cholesterol 7α-hydroxylase activity reached higher levels in cholesterol-fed animals (B). After the end of the first dark cycle HMGCoA reductase activity in cholesterol-fed animals remained at low levels, with no indication of recurrence of a diurnal rhythm (Fig. 4 A). In contrast cholesterol 7α-hydroxylase activity in the cholesterol-fed animals continued to display diurnal variations at higher level than the controls (B). Enzyme activities determined at 52h and 74h, respectively, corresponded to the maximum of the circadian rhythm. Feeding of cholesterol-containing diets resulted in a doubling of the cholesterol content of microsomes (Fig. 4 C).

Cholesterol regulates the activity of HMGCoA reductase opposite to that of cholesterol 7α-hydroxylase. It emerges from Fig. 4 A and B that high cholesterol intake suppresses HMGCoA reductase activity and concomitantly increases cholesterol 7α-hydroxylase activity such as to drain cholesterol towards bile acids. It has been sug-

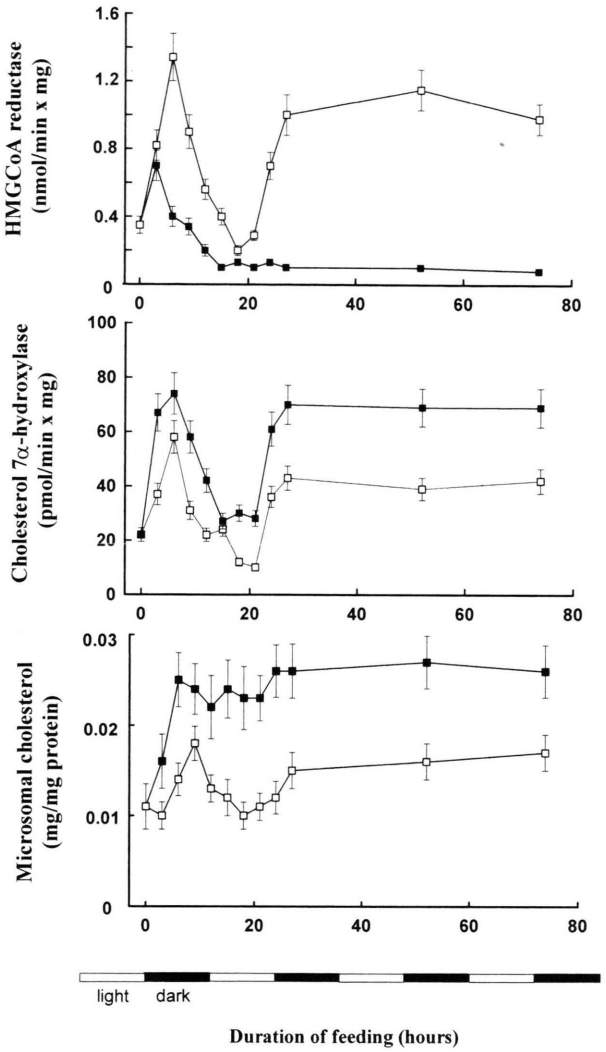

Fig. 4. Effect of dietary cholesterol on the activity of HMGCoA reductase, cholesterol 7α-hydroxylase and on the cholesterol content in rat liver microsomes.
Panel A: HMGCoA reductase; **Panel B:** cholesterol 7α-hydroxylase; **Panel C:** microsomal free cholesterol. *Open symbols*: control; *full symbols*: with dietary cholesterol. Experimental animals received a stock diet containing 3% cholesterol starting at time zero (see legend to Fig. 3). Values are means of 4 animals ± SEM.

gested that there is a critical size of the cholesterol pool in liver which is regulated via HMGCoA reductase activity (Mitropoulos, 1983). Cholesterol acts as or on a repressor directly at the level of transcription to regulate HMGCoA reductase synthesis (Chambers and Ness, 1998). There is a direct correlation between the microsomal level of cholesterol and HMGCoA reductase activity, as an increase of free cholesterol in the microsomes causes a decrease of HMGCoA reductase (negative regulation; Jenke *et al.*, 1983).

By the same token the size of the cholesterol pool could exert a positive control on cholesterol 7α-hydroxylase activity. The data presented in Fig. 4 do not contradict the notion of HMGCoA reductase activity being controlled secondary to that of cholesterol 7α-hydroxylase. There is no doubt that both cholesterol and bile acids can repress and/or activate transcriptional events, but the mediators have not been identified, nor is it clear if cholesterol and bile acids compete for one mediator or act through different mediators. High concentrations of cholesterol affect membrane fluidity and the activity of HMGCoA reductase (Davis and Poznansky, 1987), an effect which likely is mediated by a membrane-associated receptor system (Jackson *et al.*, 1997), but does not affect cholesterol 7α-hydroxylase (Sipat and Sabine, 1981).

In vitro effects on HMGCoA reductase and cholesterol 7α-hydroxylase activities

In microsomes isolated from primary hepatocytes specific activities of the two enzymes were lower than in microsomes isolated from intact liver: cholesterol 7α-hydroxylase: 0.022 vs. 0.048 nmol/min × mg, HMGCoA reductase 0.21 vs. 1.77 nmol/min × mg. Activities in liver microsomes were determined at the diurnal maximum (9 a.m.) and hepatocytes were procured at this time point. Enzyme activities in hepatocytes depended on the composition of the incubation medium and were highest in the optimized medium (cf. Method section).

The activity of cholesterol 7α-hydroxylase activity in hepatocytes decreased slowly with time of incubation (Fig. 5 A), as cytochrome P450 enzymes commonly do in cultured cells. The activity of aryl hydrocarbon hydroxylase, used here as a control typical of P450 enzymes, declined 55% during a 6h incubation (not shown). Cytochrome P450 itself was only slightly reduced (15% in 6h). Thus, the decrease of cholesterol 7α-hydroxylase activity apparently was not related to a loss of cytochrome P450. Contrary to cholesterol 7α-hydroxylase, HMGCoA reductase activity increased during incubation (Fig. 5 A). Obviously this was a response to the release of cholesterol from the hepatocytes into the medium (see below).

Cholesterol depletion

An increase of dietary cholesterol resulted in a decrease of HMGCoA reductase and a concomitant increase of cholesterol 7α-hydroxylase activity (cf. Fig. 4). An opposite situation, i.e., decrease or lack of cellular cholesterol, should

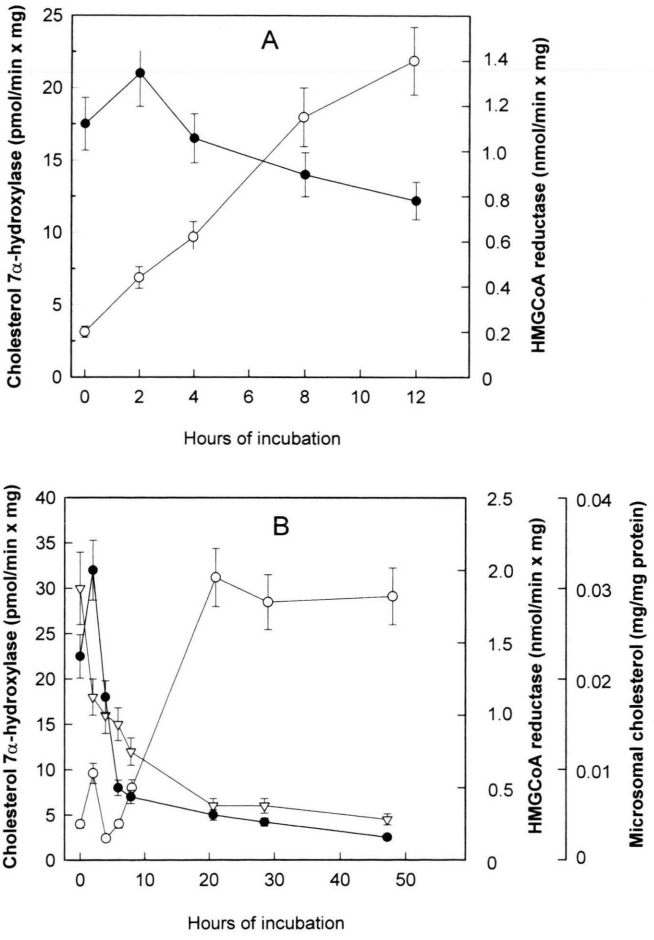

Fig. 5. Activities of HMGCoA reductase, cholesterol 7α-hydroxylase and content of microsomal free cholesterol in rat primary hepatocytes.
HMGCoA reductase (*open circles*); cholesterol 7α-hydroxylase (*full circles*); microsomal cholesterol (*triangles*). Hepatocytes were procured at the time of diurnal maximum of enzyme activity from animals on a reversed light cycle and incubated in optimized medium (see Method section). **Panel A**: Hepatocytes from rats previously fed a stock diet. **Panel B**: Hepatocytes from rats fed 3% cholesterol in the diet for 5 days. Values are means of 4 replicate incubations ± SEM.

have opposite effects on the two enzyme activities. This was modeled in an *in vivo / ex vivo* setting, i.e., primary hepatocytes were obtained from rats which had been fed for 5 days a diet containing 3% cholesterol. As expected, the activity of HMGCoA reductase was initially low, and that of the 7α-hydroxylase high (Fig. 5 B). Since hepatocytes release cholesterol into the medium during incubation (Fig. 5 B, cf. Daniels *et al.*, 1981), intracellular cholesterol stores were depleted and the respective enzyme activities reverted, resulting in high HMGCoA reductase and marginal cholesterol 7α-hydroxylase activities (Fig. 5 B). The rather rapid decline of cholesterol 7α-hydroxylase probably reflected two concurrent processes, viz., the release of cholesterol from the hepatocytes plus the incubation-dependent decrease. The initial transient rise of both enzyme activities might be the result of transferring the hepatocytes into the optimized medium. The increase of HMGCoA reductase activity seen in Fig. 5 A indicated that hepatocytes from rats fed a standard diet still release enough cholesterol to produce these activity changes.

A situation comparable to the one described here was produced in rats *in vivo* by Sudjana-Sugiaman *et al.* (1994) who found cholesterol 7α-hydroxylase activity in liver to be induced after phenobarbital treatment. The resulting enzyme induction raised cholesterol consumption and diminished the cholesterol pool which, in turn, caused a compensatory increase of HMGCoA reductase activity.

Excess cholesterol

The low activity of cholesterol 7α-hydroxylase which resulted from low cholesterol levels in the hepatocytes (Fig. 5 B) was reversed by excess of cholesterol. When freshly prepared hepatocytes were incubated for 5 h with a cholesterol-rich lipoprotein fraction (see Method section) activity of cholesterol 7α-hydroxylase increased almost 80 percent. A delipidized lipoprotein fraction was ineffective. The activity of HMGCoA reductase was not affected by incubation with a cholesterol-rich lipoprotein fraction.

The effect of cholesterol on the 7α-hydroxylase takes place at the transcriptional level and involves an LDL-sensitive liver-specific gene enhancer (Ramirez *et al.*, 1994). The activity of HMGCoA reductase is normally also affected *in vitro* by lipoproteins, albeit in a more complicated fashion, viz., LDL suppresses its activity, HDL enhances it significantly, VLDL has no effect (Stange *et al.*, 1982). According to a more recent study a subfraction of the latter, β-VLDL, suppresses HMGCoA reductase activity, and enhances that of cholesterol 7α-hydroxylase (Yu and Mamo, 1997). Since in the present study the lipoproteins were not fractionated, the results may reflect a cancellation of HDL and LDL effects on HMGCoA reductase. In addition, the fact that HMGCoA reductase activity responded more slowly to external stimuli than cholesterol 7α-hydroxylase activity can be discussed in terms of a 5 hr cell culture experiment being too short to elicit the effect on both enzymes, or, once again, as an indication of the regulation of HMGCoA reductase activity being secondary to that of cholesterol 7α-hydroxylase.

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