Differential feedback regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by rat bile acids in primary monolayer cultures of rat hepatocytes

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We have used primary monolayer cultures of rat hepatocytes to study the effects of physiological concentrations of various bile acids, commonly found in bile of normal rats, on the mechanism of regulation of cholesterol 7α-hydroxylase and bile acid synthesis. Addition of taurocholic acid, the most predominant bile acid in rat bile, to the culture medium suppressed cholesterol 7α-hydroxylase activity and mRNA time- and dose-dependently. The decrease in enzyme activity paralleled the changes in mRNA. Maximal suppression of cholesterol 7α-hydroxylase mRNA (−91 %) and enzyme activity (−89 %) was observed after a 16 h incubation period with 50 μM taurocholic acid. The declines in mRNA and enzyme caused by taurocholic acid were tightly coupled and followed first-order kinetics with a half-life of 4 h. Transcriptional activity, as assessed with nuclear run-on assays, was decreased by 44 % at 50 μM taurocholic acid. Mass production of bile acids (chenodeoxycholic acid and β-muricholic acid) was inhibited to a similar extent as the cholesterol 7α-hydroxylase when different concentrations of taurocholic acid were used, giving maximal inhibition (−81 %) at 50 μM taurocholic acid. Glycocholic acid and unconjugated cholic acid were equally effective as taurocholic acid in suppressing cholesterol 7α-hydroxylase mRNA. The more hydrophobic bile acids (chenodeoxycholic acid and deoxycholic acid) showed profound suppression of the cholesterol 7α-hydroxylase mRNA by 85 % and 75 % respectively, whereas the other trihydroxy bile acids in rat bile, α- and β-muricholic acid, were not or only marginally active. We conclude that rat bile acids, in particular the more hydrophobic ones, in concentrations commonly observed in portal blood, exert negative feedback control at the level of cholesterol 7α-hydroxylase mRNA in cultured rat hepatocytes through a direct effect on the hepatocytes, and that down-regulation of transcription is only one of the mechanisms involved in this regulation.

INTRODUCTION

The hepatic conversion of cholesterol into bile acids is a major route for the elimination of cholesterol from the mammalian body [1,2]. According to current concepts, the primary pathway of bile acid biosynthesis in rats and humans is initiated by the 7α-hydroxylation of cholesterol. This reaction is catalysed by cholesterol 7α-hydroxylase, a specific cytochrome *P*-450 isoenzyme located in the smooth endoplasmic reticulum [3,4]. The enzyme has recently been purified to homogeneity, and the molecular cloning of its cDNA has been reported by several research groups [5–10]. Cholesterol 7α-hydroxylase is regulated in a number of ways, the most important of which is considered to be exerted through the enterohepatic circulation of bile acids [1,2]. Interruption of this circulation by biliary diversion or administration of bile acid sequestrants has been shown to lead to a several-fold increase in bile acid biosynthesis and cholesterol 7α-hydroxylase activity [11–15]. Intraduodenal or intravenous infusion of taurocholate returned rates of bile acid synthesis and the enzyme activity in these rats back to normal [13,16,17].

Several observations, however, are not in agreement with the classical concept of negative feedback by bile acids. One of these was the failure to observe a decrease in bile acid biosynthesis in suspended or primary hepatocytes of rat [18–20] and rabbit [21] after addition of bile acids to the culture medium, even at concentrations higher than those found in portal blood. These findings were interpreted to indicate that bile acids do not inhibit bile acid synthesis through a direct effect on the hepatocytes. They prompted several groups to re-evaluate the effects of intravenous and intraduodenal infusions of taurocholate on bile acid synthesis in bile-diverted rats, demonstrating divergent results that both confirmed [22–24] and opposed [25,26] the feedback hypothesis. In addition, there is controversy concerning the mechanism of inhibition. (Tauro)cholate was reported to be not active, whereas (tauro)deoxycholate and lithocholate were inhibitory in rat and rabbit, respectively, suggesting that primary bile acids first have to be converted into secondary bile acids to become regulatory [24,27]. On the other hand, Kwekkeboom et al. [28,29] recently demonstrated that addition of physiological concentrations of bile acids to the culture medium of pig hepatocytes resulted in a strong decrease in bile acid synthesis and cholesterol 7α-hydroxylase activity.

In this study, we have evaluated the effects of bile acids, commonly found in bile of normal rats, on bile acid synthesis and cholesterol 7α-hydroxylase activity in cultured rat hepatocytes. Using a recently isolated cholesterol 7α-hydroxylase cDNA clone, we have studied the mechanism of feedback regulation by determining its mRNA level and by measuring cholesterol 7α-hydroxylase transcription rates using run-on assays. Here we report that addition of various rat bile acids to primary cultures of rat hepatocytes leads to differential effects on cholesterol 7α-hydroxylase activity and mRNA levels through a direct effect on the hepatocyte, and that regulation takes place at both the transcriptional and the post-transcriptional level.

Abbreviations used: NP-40, Nonidet P-40 (octylphenoxypolyethoxyethanol); PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol (Cleland's reagent); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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EXPERIMENTAL

Materials

Materials used for isolation and culturing of rat hepatocytes, determination of mass production of bile acids and assaying cholesterol 7α-hydroxylase activity were obtained from sources described previously [30–32]. Taurocholic acid, glycocholic acid, unconjugated cholic acid and deoxycholic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), chenodeoxycholic acid was from Serva (Heidelberg, Germany) and lithocholic, α- and β-muricholic acids were obtained from Steraloids (Wilton, NH, U.S.A.). [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (400 Ci/mmol) and [4-^{14}C]cholesterol (60 mCi/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Male Wistar rats weighing 250–350 g were used throughout and were maintained on standard chow and water *ad libitum*; 2 days before isolation of hepatocytes, rats were fed on a diet supplemented with 2% cholestyramine (Questran; Bristol Myers B.V., Weesp, The Netherlands), unless otherwise stated. For preparation of hepatocytes, animals were killed between 09:00 and 10:00 h. Institutional guidelines for animal care were observed in all experiments.

Rat hepatocyte isolation and culture

Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously [30–32]. Viability, as determined by Trypan Blue exclusion, was higher than 90%. The cells were seeded on 60 mm-diameter plastic tissue-culture dishes or six-well cluster plates (Costar, Cambridge, MA, U.S.A.) at a density of 1.5×10^5 cells/cm^2 in Williams E medium supplemented with 10% heat-inactivated fetal-bovine serum, 2 mM L-glutamine, 140 nM insulin, 50 nM dexamethasone, 100 i.u./ml penicillin and 100 μg/ml streptomycin, and maintained at 37 °C in CO_2/air (1:19) [30,31]. After a 4 h attachment period, medium was replaced with 1.0 ml (six-well plates) or 2.5 ml (dishes) of culture medium with hormones as described above. Cells were left to recover for 18 h before being used for further experiments [33]. Bile acids were added to the hepatocytes at various times between 18 and 42 h of culture age, as indicated in the Results section. Cells were harvested at the same time after a 42 h culture period for measurement of cholesterol 7α-hydroxylase activity, mRNA and transcriptional activity.

Quantification of mass production of bile acids

Mass production of bile acids by rat hepatocytes was measured by g.l.c. after a preincubation period of 8 h (from 18 to 26 h of culture age), during the following 24 h culture period from 26 to 50 h as described previously [32], in the presence or absence of bile acids. Rat hepatocytes in monolayer culture synthesize predominantly cholic acid (20–30% of total bile acid synthesis) and β-muricholic acid, together with minor amounts of chenodeoxycholic acid and α-muricholic acid (70–80%) [32,34]. After addition of taurocholic acid as inhibiting agent, synthesis of this bile acid cannot be measured. Values reported are therefore expressed as a percentage of the production of α- and β-muricholic acids and chenodeoxycholic acid.

Assay of cholesterol 7α-hydroxylase

Cholesterol 7α-hydroxylase activity in homogenates of hepatocytes cultured for 42 h was measured as reported previously [31]. Protein and cholesterol were assayed as described by Lowry et al. [35] and Gamble et al. [36] respectively.

Cholesterol 7α-hydroxylase probe synthesis and identification

A probe directed against the cholesterol 7α-hydroxylase mRNA was synthesized by PCR, under conditions as described in [37]. cDNA was synthesized by using Moloney Murine Leukemia Virus RNAseH$^-$Reverse Transcriptase (Superscript; Bethesda Research Laboratories), according to the manufacturer's protocol, with total rat liver RNA as template and 0.1 mM dT_{12-18} as primer.

Oligonucleotides were synthesized by using a DNA synthesizer (Applied Biosystems, model 381A), their sequence being:

oligo1 5′-AGCCGCCAAGTGACATCATCCAGTGTTCGCTTCTTCC-3′
oligo2 5′-ATGATGACTATTTCTTTGATTTGGGGAATTGCCGTG-3′

Sequences of these oligonucleotides were chosen, guided by the rat cholesterol 7α-hydroxylase cDNA sequence as published by Noshiro et al. [8], to amplify the entire coding region of the cholesterol 7α-hydroxylase sequence.

PCR was performed in a 100 μl reaction mixture containing 4 μl of cDNA mixture, 20 μl of a 5× buffer (250 mM KCl, 100 mM Tris/HCl, pH 8.4, 15 mM $MgCl_2$, 0.005% gelatin), and 10 μl of each primer (8 ng/μl). After denaturing at 95 °C for 7 min, 10 μl of dNTPs and 1 unit of *Taq* polymerase (Cetus Corp., Emeryville, CA, U.S.A.) were added. The reaction mixture was covered with 70 μl of mineral oil to prevent evaporation. The actual PCR was then carried out by denaturing the RNA–cDNA hybrid at 95 °C for 1 min, annealing the primers for 30 s at 58 °C, and extending the primers at 70 °C for 5 min. This cycle was repeated 30 times by using a programmable heat block manufactured by Cetus. After the final cycle, the temperature of the reaction mixture was kept at 70 °C for several minutes to allow re-annealing of the amplification products, and the mixture was chilled. To ascertain that the PCR probe was indeed of cholesterol 7α-hydroxylase origin, it was analysed by restriction-enzyme analysis (enzymes from Bethesda Research Laboratories). After cloning the probe via TA-cloning procedures (Invitrogen Corp.), it was further analysed by sequencing of the first and last 200 base-pairs of the probe, with oligo1 and oligo2 as sequencing primers (TaqTrack; Promega).

RNA isolation, blotting and hybridization procedures

Total RNA was isolated from cultured rat hepatocytes by the isolation procedure of Chomczynski and Sacchi [38]. After washing the RNA pellets with 70% ethanol, they were dissolved in water and concentrations were determined spectrophotometrically, by assuming that $A_{260} = 1$ at 40 μg/ml RNA. Equal amounts of total RNA from different incubations were fractionated by electrophoresis on a 0.8%-agarose gel containing 1 M formaldehyde, and transferred to Hybond-N filter (Amersham) in accordance with the manufacturers' instructions. For slot-blotting of total RNA, samples were diluted to appropriate concentrations in a buffer containing 1 M NaCl, 50 mM sodium phosphate, pH 7.0, and 6% (v/v) formaldehyde and applied on to a filter by using the Minifold II slot-blotting apparatus (Schleicher and Schuell), as described by Krawczyk and Wu [39].

After both procedures, filters were cross-linked with u.v. light for 5 min and then hybridized with different probes at 65 °C in

0.5 M sodium phosphate buffer, pH 7.5, containing 7% (w/v) SDS and 1 mM EDTA. DNA fragments used as probes were isolated from low-melting agarose (Bio-Rad) [40]. Each blot was hybridized with 25 ng of probe, labelled by the random-primer method (Multi-prime; Amersham) to approx. 6×10^8 c.p.m./μg of DNA. After hybridization, blots were washed twice with 2 × SSC/1% SDS (30 min at 65 °C) (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The filters were exposed to Hyperfilm MP (Amersham) together with an intensifying screen (Eastman Kodak Co.) for 48–120 h at −80 °C. To quantify the relative amounts of mRNA, the autoradiographs were scanned with a Shimadzu (Kyoto, Japan) CS 910 chromatograph scanner, and areas under the curves were integrated by using a data processor (Shimadzu). The mRNA levels were quantified by using three different amounts of total RNA, giving a linear relation between the specific mRNA signal and the amount of RNA applied.

The following DNA fragments were used as probes in hybridization experiments: a 1.2 kb *Pst*I fragment of hamster actin cDNA, kindly provided by Dr. W. J. Quax [41], and the 1.6 kb PCR-synthesized fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region (see above). For both probes, a linear relationship between areas under the curves and mRNA concentration was shown on an autoradiograph, by using concentrations between 2 and 8 μg of total RNA. Actin was used as an internal standard to correct for differences in the amount of total RNA applied on to the gel or filter.

Nuclear run-on studies

These were conducted essentially as described by Groudine et al. [42], with minor modifications.

Isolation of nuclei

Cells were washed, scraped with a rubber policeman, and collected by centrifugation at 500 *g* at 4 °C for 5 min. They were resuspended in NP40 lysis buffer (10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 1 mM PMSF, 1 mM DTT) and, after being left on ice for 5 min, homogenized in a Potter–Elvehjem tube with pestle B for 15 strokes at 4 °C. Resulting nuclei were again centrifuged at 500 *g* for 5 min and resuspended in NP40-lysis buffer. This procedure was repeated until the nuclei were free of cellular debris. They were then taken up in glycerol storage buffer (50 mM Tris/HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 5 mM DTT), counted, and divided into batches of approx. 2×10^7/200 μl before being frozen at −80 °C.

RNA labelling and isolation

A portion of frozen nuclei was added to 200 μl of transcription buffer (10 mM Tris/HCl, pH 7.9, 140 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dGTP, 1 mM dATP, 1 mM dCTP, 0.1 mM *S*-adenosyl-L-methionine, 14 mM β-mercaptoethanol, 1 mg/ml heparin sulphate, 1.7 mM spermidine, 10 mM phosphocreatine, 40 μg/ml creatine kinase, 25% glycerol and 100 μCi of [α^{32}P]UTP), and incubated with shaking at 30 °C for 30 min. Then 600 μl of a buffer containing 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris/HCl, pH 7.4 and 200 units/ml DNAase I (Bethesda Research Laboratories) was added, and the mixture was incubated for an additional 5 min at 30 °C. Then 200 μl of SDS/Tris (5% SDS, 0.5 M Tris/HCl, pH 7.4, 0.125 M EDTA) with 200 μg/ml Proteinase K (Boehringer Mannheim) was added, and the mixture was incubated for 30 min at 42 °C. RNA was extracted with 1 vol. of phenol/chloroform/3-methylbutan-1-ol (50:49:1, v/v), precipitated with 2.5 vol. of ethanol and 10 μg/ml tRNA, washed, and taken up in 50 μl of Tris/EDTA (10 mM Tris/HCl, pH 7.4, 1 mM EDTA). Labelled RNA was separated from free nucleotides by passage over a Sephadex G50 (fine) column (Boehringer Mannheim). The RNA was mildly degraded by incubation for 10 min on ice in 0.25 M NaOH, and the mixture was neutralized by addition of 0.5 vol. of 1 M Hepes (free acid) and precipitated with 2 vol. of ethanol and 0.1 vol. of 3 M sodium acetate. Incorporation of label was measured by liquid-scintillation counting, and equal amounts of labelled RNA were added to the filters.

Hybridization

Target DNA, which was 5 μg of plasmid material containing cDNA sequences of rat cholesterol 7α-hydroxylase and hamster actin (see above), and rat GAPDH [43] were slot-blotted on to strips of Hybond-N^+ filter (Amersham), and cross-linked with 0.4 M NaOH for 30 min. The filters were preincubated for 30 min at 65 °C in a sodium phosphate buffer as described above, and hybridized with the labelled RNA for 36 h in the same buffer. The various filters were washed once for 5 min and then for 2 × 30 min in 2 × SSC/1% SDS at 65 °C, and exposed to Hyperfilm MP (Amersham) for 2–5 days. Relative amounts of mRNA were quantified as described above.

Statistical analysis

Data were analysed statistically by using Student's paired *t* test with the level of significance selected to be $P < 0.05$. In Figure 1 inset (see below), the lines were fitted to the points by the method of least squares. Values are expressed as means ± S.D.

RESULTS

Time course of the effect of taurocholic acid on cholesterol 7α-hydroxylase activity and mRNA

Addition of 50 μM taurocholic acid, the most predominant bile acid in rat bile, to the culture medium of rat hepatocytes between 18 and 42 h of culture resulted in an 89 ± 6% inhibition of cholesterol 7α-hydroxylase activity. Figure 1(a) shows that maximal inhibition is reached after an incubation period of 16 h. The relationship between the logarithm of the enzyme activity and incubation time was linear, indicating a decline of enzyme activity following first-order kinetics with an apparent half-life of 4 h (Figure 1 inset).

Detection of cholesterol 7α-hydroxylase mRNA was done with the PCR probe directed against it. Northern-blot hybridization of total RNA isolated from cultured hepatocytes revealed the existence of at least three distinct messengers (Figure 2), of which the two most abundant are approx. 2.1 and 4.0 kb in length. These results on the relative amounts and lengths of cholesterol 7α-hydroxylase mRNAs in cells correspond well with previous findings observed in the rat liver *in vivo* [9,10]. Quantification of the amount of mRNA by slot-blotting experiments (Figure 1b) in total RNA from cells treated with taurocholate showed that cholesterol 7α-hydroxylase mRNA levels follow the same pattern as the enzyme activity, i.e. 91 ± 8% inhibition after 24 h of incubation with 50 μM taurocholate (Figure 1a). The apparent half-life of the mRNA is estimated to be 4 h (Figure 1 inset). The actin mRNA, used as a control, exhibited no significant changes in level when taurocholic acid

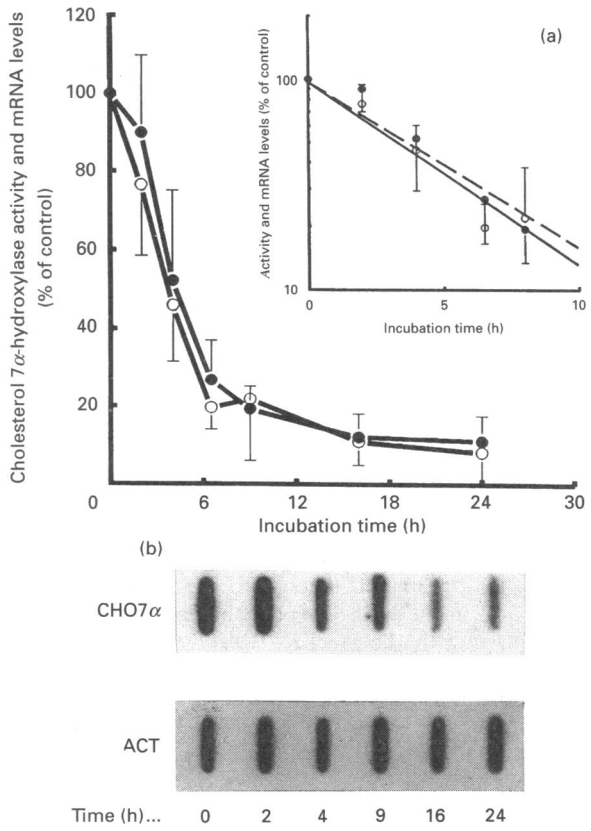

Figure 1 Time course of inhibition of cholesterol 7α-hydroxylase activity and mRNA by taurocholic acid

(**a**) Cells were exposed to taurocholic acid (50 μM) for different lengths of time, between 18 and 42 h of culture, and were harvested simultaneously at 42 h of culture time. Cholesterol 7α-hydroxylase activity (●) and mRNA (○) were assessed as described in the Experimental section. The absolute values for cholesterol 7α-hydroxylase activity were 343±130 pmol/h per mg of cell protein. Values shown are expressed as percentages of those incubations without taurocholic acid, and are means±S.D. of duplicate incubations, using hepatocytes from 3–12 rats. Inset: same data plotted semi-logarithmically (●——●, cholesterol 7α-hydroxylase; ○——○, cholesterol 7α-hydroxylase mRNA). The lines were fitted to the points by the method of least squares. (**b**) The amount of cholesterol 7α-hydroxylase mRNA (CHO7α) was assessed by slot-blotting and densitometric scanning of resulting autoradiographs, the actin mRNA (ACT) being used as an internal standard to correct for differences in the amount of RNA applied.

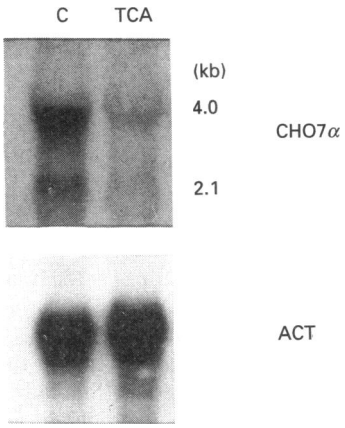

Figure 2 Northern-blot hybridization of cholesterol 7α-hydroxylase mRNA

Total RNA of cells exposed to 50 μM taurocholic acid (TCA) for 24 h, between 18 and 42 h of culture, as opposed to untreated cells (C), was electrophoresed in a 0.8% agarose/1 M formaldehyde gel, transferred to Hybond-N and subsequently hybridized with probes for cholesterol 7α-hydroxylase (CHO7α) and actin mRNA (ACT), as described in the Experimental section; 10 μg of total RNA was applied in both lanes.

Table 1 Dose-dependency of suppression of cholesterol 7α-hydroxylase activity, mRNA and bile acid synthesis by taurocholic acid

Rat hepatocytes were incubated for 24 h, from 18 to 42 h of culture, with various amounts of taurocholic acid (0–70 μM). Cells were harvested after 42 h of culture, for determination of cholesterol 7α-hydroxylase activity and mRNA. Bile acid synthesis was measured after an 8 h preincubation period, from 26 to 50 h of culture time, as described in the Experimental section. Data are expressed as a percentage of control (no bile acids added) and are means±S.D. of independent experiments using hepatocytes from the numbers of rats shown in parentheses. The amount of cholesterol 7α-mRNA was assessed by slot-blotting and densitometric scanning of resulting autoradiographs, by using the actin mRNA as an internal standard to correct for differences in the amount of total RNA applied to the filter. The absolute values for cholesterol 7α-hydroxylase activity were 343±130 pmol/h per mg of cell protein, and for bile acid synthesis 2.72±0.90 μg/24 h per mg of cell protein; * indicates a significant difference ($P < 0.05$) compared with control values.

Extracellular taurocholate (μM)	Cholesterol 7α-hydroxylase		Bile acid synthesis (% of control)
	Activity (% of control)	mRNA (% of control)	
0	100 (12)	100 (12)	100 (5)
5	97±6 (3)		
10	61±14* (6)	45±5* (3)	69±27* (5)
20	48±10* (3)		
30	26±8* (4)	12±6* (3)	33±18* (4)
50	12±6* (12)	9±8* (12)	19±15* (4)
70	11±8* (4)		

was present in the medium. Northern-blot hybridization of these same samples demonstrated that all three detectable cholesterol 7α-hydroxylase mRNAs were susceptible to inhibition (Figure 2).

Dose-dependency of the effect of taurocholic acid on the cholesterol 7α-hydroxylase activity and mRNA and on bile acid synthesis

Suppression of cholesterol 7α-hydroxylase activity, mRNA levels and bile acid synthesis by taurocholic acid was found to be a dose-dependent process (Table 1). A maximal decrease was reached at an extracellular concentration of 50 μM taurocholic acid, whereas 10 μM is already sufficient to ensure significant inhibition at the level of enzyme activity (−39%), mRNA (−55%) and bile acid synthesis (−31%). The suppression of bile acid synthesis paralleled the decrease in cholesterol 7α-hydroxylase. The concentrations of bile acids applied are all well within the physiological range as detected in portal blood of rats [44,45]. ATP measurements showed that taurocholate did not have adverse effects on cell viability, up to concentrations of 70 μM (results not shown).

Effect of taurocholic acid on the transcription rate of cholesterol 7α-hydroxylase mRNA in cultured rat hepatocytes

Nuclear run-on studies were conducted with nuclei isolated from hepatocytes which had been incubated with 50 μM taurocholic acid for 24 h, between 18 and 42 h of culture time. α-^{32}P-labelled total RNA was hybridized to rat cholesterol 7α-hydroxylase cDNA, and to hamster actin cDNA and rat GAPDH cDNA. The last two served as transcriptional activity controls between

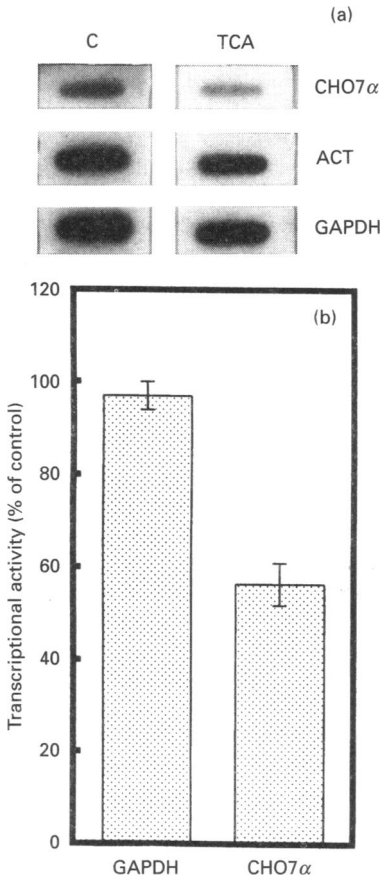

Figure 3 Transcriptional activity of the cholesterol 7α-hydroxylase gene in nuclei isolated from hepatocytes incubated with taurocholic acid

Cells were exposed to 50 μM taurocholic acid (TCA) for 24 h, between 18 and 42 h of culture, and were harvested simultaneously with untreated cells (C) after this period. (**a**) ^{32}P-labelled total RNA was synthesized and isolated from nuclei, and hybridized to 5 μg of cholesterol 7α-hydroxylase cDNA (CHO7α), actin cDNA (ACT) and GAPDH cDNA (GAPDH), as described in the Experimental section. (**b**) The amount of ^{32}P-labelled cholesterol 7α-hydroxylase mRNA and GAPDH mRNA was assessed by densitometric scanning of resulting autoradiographs, the actin mRNA signal being used as a transcriptional control. Data are presented as transcriptional activity relative to that of actin, and are means ± S.D. of three independent experiments.

Table 2 Effect of different bile acids on cholesterol 7α-hydroxylase mRNA in rat hepatocytes

Rat hepatocytes were incubated with different bile acids (50 μM) from 18 to 42 h of culture. Cells were harvested at 42 h, and the cholesterol 7α-hydroxylase mRNA level was determined. Data are expressed as a percentage of control (no bile acids added) and are means ± S.D. of independent experiments using hepatocytes from the numbers of rats shown in parentheses: * indicates a significant difference ($P < 0.05$) between control and treated cells. The amount of cholesterol 7α-hydroxylase mRNA was assessed by slot-blotting and densitometric scanning of resulting autoradiographs, by using the actin mRNA as an internal standard to correct for differences in the amount of total RNA applied to the filter.

Added bile acid	Cholesterol 7α-hydroxylase mRNA (% of control)
Taurocholic acid	9 ± 8* (12)
Glycocholic acid	14 ± 5* (3)
Cholic acid	22 ± 10* (3)
Deoxycholic acid	25 ± 16* (4)
Chenodeoxycholic acid	15 ± 8* (3)
α-Muricholic acid	103 ± 22 (3)
β-Muricholic acid	86 ± 14 (3)

the different samples, and specific transcriptional activity for cholesterol 7α-hydroxylase is expressed relative to that for actin.

Figure 3 shows that addition of 50 μM taurocholic acid lowers the transcription rate of cholesterol 7α-hydroxylase of cells by 44 ± 5%, whereas it has no effect on the transcription of the GAPDH gene.

Effect of different bile acids on cholesterol 7α-hydroxylase mRNA in cultured rat hepatocytes

Hepatocytes were incubated with 50 μM of different bile acids, commonly found in bile of normal rats, for a period of 24 h (from 18 to 42 h of culture). Uptake of the various bile acids by the hepatocytes, in terms of intracellular accumulation as determined by g.l.c., was similar for each bile acid used.

Glycocholic acid and unconjugated cholic acid were as effective as taurocholic acid in suppressing cholesterol 7α-hydroxylase mRNA levels (Table 2). The more hydrophobic bile acids, chenodeoxycholic acid and deoxycholic acid, also showed a strong inhibitory effect on cholesterol 7α-hydroxylase mRNA of 85% and 75% respectively, whereas the other trihydroxy bile acids, α- and β-muricholic acid, were not or only marginally active.

DISCUSSION

The present study shows that bile acid synthesis in cultured rat hepatocytes is subject to negative feedback regulation by bile acids through a direct effect of bile acids on the hepatocytes. Failure to observe such suppression of bile acid synthesis *in vitro* by exogenously added bile acids, as has been reported in hepatocytes of both rat [18–20] and rabbit [21], has challenged the idea that bile acids inhibit their own synthesis directly when returning to the liver via the enterohepatic circulation. It has led to the suggestion that an extrahepatic repressor, possibly originating from the intestine in the presence of bile acids, is responsible for the actual inhibition [46]. Our results show unequivocally that a possible repressor system must originate in the hepatocyte. These data are in good agreement with those obtained by Kwekkeboom et al. [28,29], using cultured pig hepatocytes, indicating that this regulation is not a species-specific effect.

The reason that we find negative feedback regulation in our systems *in vitro* is most probably due to the fact that the hepatocytes of both species were cultured under conditions that have been shown to maintain bile acid biosynthetic capacity and cholesterol 7α-hydroxylase activity during culture [29,47,48]. Elevation of the initial levels of cholesterol 7α-hydroxylase activity and mRNA by feeding rats with chow supplemented with 2% cholestyramine, before isolation of the hepatocytes, was not found to be obligatory to observe feedback regulation. Although bile acid synthesis was 2.3-fold lower in hepatocytes from rats fed on control chow (1.17 ± 0.62 μg/24 h per mg of cell protein [49] versus 2.72 ± 0.90 μg/24 h per mg of cell protein, in the present study), similar results to those reported in this paper were obtained with rats fed on control chow (results not shown). We conclude that rat hepatocytes are a suitable model for studies on negative feedback regulation by bile acids, provided that a proper maintenance of cholesterol 7α-hydroxylase is ensured during culture.

A previous suggestion that rat hepatocytes do not show feedback regulation of bile acid synthesis *in vitro* as a result of loss of capacity to take up exogenous bile acids from the medium [29] cannot be upheld. The hepatocytes are still able to take up sufficient bile acids to be able to down-regulate bile acid synthesis

as well as cholesterol 7α-hydroxylase, notwithstanding the gradual loss of capacity to accumulate bile acids intracellularly during culture [45]. Even low concentrations (10 μM) of bile acids are already sufficient to cause a marked decrease in cholesterol 7α-hydroxylase activity and mRNA levels. The magnitude of inhibition is comparable with that reported for cultured pig hepatocytes [29].

The inhibition of bile acid synthesis by bile acids is a dose-dependent process. The extracellular concentration of 50 μM taurocholic acid, giving maximal suppression, is within the range of bile acid concentrations normally found in portal blood of rat (30–200 μM) [44,45]. As has been demonstrated in this paper and reported previously by Heuman et al. [50] and Kwekkeboom et al. [29], the different bile acids are not equally effective suppressors. Approx. 70% of the bile acids present in the bile acid pool of normal rats [51] have suppressing properties. These bile acids, i.e. cholic, chenodeoxycholic and deoxycholic, have comparable potencies in suppressing cholesterol 7α-hydroxylase (Table 2). The concentrations of inhibitory bile acids in portal blood of rat will therefore vary between 20 and 140 μM. However, as a consequence of the presence of a strong bile acid gradient over the liver lobule, giving rise to a 6-fold higher bile acid concentration in the periportal zone [52,53], only the hepatocytes in the periportal area of the acinus are exposed to this high concentration of inhibitory bile acids. Thus, at normal bile acid load, cholesterol 7α-hydroxylase will be suppressed predominantly in these cells. Corresponding to this, we found a 7.9-fold higher enzyme activity in perivenous hepatocytes as compared with periportal hepatocytes from rats fed on normal chow [34].

The apparent half-life of the enzyme and its mRNA (4 h), as calculated from the time course of inhibition by taurocholic acid, is in agreement with reported values for cholesterol 7α-hydroxylase activity *in vivo* in rat [54,55]. This relatively short half-life is considered to be a general property of rate-limiting enzymes. It is also consistent with the finding that the cholesterol 7α-hydroxylase mRNA contains multiple AU-rich sequences in its 3′-non-coding region [56], which have been linked to rapid degradation of mRNA [57]. The half-life of cholesterol 7α-hydroxylase in the rat is considerably shorter than that reported for the pig (10 h) [29], whereas the magnitude and dose-dependency of the suppression are similar. The reason for this species difference is as yet unknown. It cannot be attributed to limitation of bile acid uptake by pig hepatocytes, as the capacity to do so is even greater in pig than in rat hepatocytes. We suggest that it may be linked to the presence or absence of a gallbladder. In pig, a large quantity of bile acids is released from the gallbladder to aid in the proper degradation and uptake of lipids and lipophilic nutrients in the intestine. In the absence of a gallbladder, as in the rat, the use of bile acids during and after feeding needs to be compensated for by synthesis. Thus the latter has to be susceptible to a form of short-term regulation, whereas in the pig the larger bile acid pool can act as a buffer.

Our data clearly demonstrate that bile acids regulate cholesterol 7α-hydroxylase at the mRNA level, in agreement with observations made *in vivo* by others [9,10,58]. We found a 91% suppression of steady-state mRNA levels, whereas transcriptional activity was lowered by 44%. *In vivo* in cholestyramine-fed rats we have also observed a considerably larger increase in cholesterol 7α-hydroxylase mRNA than in transcriptional activity (results not shown). These results suggest that, although there is a substantial effect of bile acids on transcription rates of the cholesterol 7α-hydroxylase gene, the mRNA levels may be determined for a major part at the post-transcriptional level, e.g. by stability of the mRNAs. It is tempting to speculate that the multiple AU-rich sequences in the 3′-non-coding region of cholesterol 7α-hydroxylase mRNA are somehow involved in this regulation. Our data correspond in broad outline to those reported by Pandak et al. [58], who also found in the rat *in vivo* that cholesterol 7α-hydroxylase is regulated at the level of mRNA, but, in contrast with our results, predominantly at the level of transcription.

Considerable differences between the bile acids most common to the rat, with respect to their inhibitory effect, were observed. Both primary (cholic and chenodeoxycholic acid) and secondary (deoxycholic acid) bile acids had a profound effect on cholesterol 7α-hydroxylase. This finding is in contrast with results *in vivo*, in which a strong inhibition of bile acid synthesis was found only after intravenous infusions of (tauro)deoxycholic acid [24], or lithocholic acid or 3β-hydroxy-5-cholenoic acid [27], but not when taurocholic acid was used. It was postulated that primary bile acids had to be converted into secondary ones in order to become active. Judging from our results, this is not necessary. The trihydroxy bile acids α- and β-muricholic acid, which account for 30% of the total bile acid pool size in the rat *in vivo* [51], had only marginal effects. The potency of suppression was shown to be dependent on the bile acid structure and linked to hydrophobicity of the particular bile acid, as has been postulated by Heuman et al. [50]. Cholic acid does not entirely fit this model, however, as it is less hydrophobic than deoxy- or chenodeoxycholic acid, but nonetheless just as active an inhibitor. Additionally, there were no differences between the conjugated and unconjugated forms of cholic acid with respect to inhibitory action, whereas there are major differences in hydrophobicity of these bile acids [50]. Apparently conjugation is of no importance for regulating potency, but much more so is the structure of the free bile acid. The cultured rat hepatocyte is an attractive model to elucidate further structure–function relationships with respect to differential effects of bile acids on bile acid synthesis.

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