Suppression of sterol 27-hydroxylase mRNA and transcriptional activity by bile acids in cultured rat hepatocytes

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In previous work we have demonstrated suppression of cholesterol 7 α -hydroxylase by bile acids at the level of mRNA and transcription, resulting in a similar decline in bile acid synthesis in cultured rat hepatocytes [Twisk, Lehmann and Princen (1993) *Biochem. J.* **290**, 685–691]. In view of the substantial contribution of the ‘alternative’ or ‘27-hydroxylase’ route to total bile acid synthesis, as demonstrated in cultured rat hepatocytes and *in vivo* in humans, we here evaluate the effects of various bile acids commonly found in bile of rats on the regulation of sterol 27-hydroxylase in cultured rat hepatocytes. Addition of taurocholic acid, the predominant bile acid in rat bile, to the culture medium of rat hepatocytes resulted in a 72% inhibition of sterol 27-hydroxylase activity. The effect was exerted at the level of sterol 27-hydroxylase mRNA, showing a time- and dose-dependent decline with a maximal suppression (–75%) at 50 μ M taurocholic acid after 24 h of culture. The decline in mRNA followed first-order kinetics with an apparent half-life of 13 h. Under these conditions cholesterol 7 α -hydroxylase mRNA (–91%) and bile acid synthesis (i.e. chenodeoxycholic and β -muricholic acid,

–81%) were also maximally suppressed. In contrast, no change was found in the level of lithocholic acid 6 β -hydroxylase mRNA. Assessment of the transcriptional activity of a number of genes involved in routing of cholesterol towards bile acids showed similar suppressive effects of taurocholate on expression of the sterol 27-hydroxylase and cholesterol 7 α -hydroxylase genes (–43% and –42% respectively), whereas expression of the lithocholic 6 β -hydroxylase gene was not affected. Taurocholic acid and unconjugated cholic acid were equally as effective in suppressing sterol 27-hydroxylase mRNA. The more hydrophobic bile acids, chenodeoxycholic acid and deoxycholic acid, also produced a strong inhibition of 57% and 76% respectively, whereas the hydrophilic β -muricholic acid was not active. We conclude that (1) a number of bile acids, at physiological concentrations, suppress sterol 27-hydroxylase by down-regulation of sterol 27-hydroxylase mRNA and transcriptional activity and (2) co-ordinated suppression of both sterol 27-hydroxylase and cholesterol 7 α -hydroxylase results in inhibition of bile acid synthesis in cultured rat hepatocytes.

INTRODUCTION

Synthesis of bile acids is the most important pathway for the metabolism and excretion of cholesterol from the mammalian body [1]. From investigations in the 1970s, predominantly with rats, it was established that in this sequence of reactions nuclear transformation of cholesterol involving 7 α -hydroxylation by cholesterol 7 α -hydroxylase as an initial step, precedes the side chain degradation, leading to formation of cholic acid and chenodeoxycholic acid. Cholesterol 7 α -hydroxylase is considered to be the major control point in the regulation of bile acid synthesis [1]. However, an alternative pathway may exist, specifically in the formation of chenodeoxycholic acid. Human and rat liver mitochondria are able to convert cholesterol into 27-hydroxycholesterol [2,3]. It has been shown that this intermediate can be further metabolized to 3 β -hydroxy-5-cholenoic acid in rat liver peroxisomes [4] and to 7 α -hydroxy intermediates by human liver microsomes and mitochondria [5,6], ultimately leading to preferential formation of chenodeoxycholic acid in rat and man [7–12]. Introduction of a 27-hydroxy group has been reported to prevent almost completely the subsequent introduction of a 12 α -hydroxy group [1].

Evidence is accumulating that, in normal human subjects, both pathways, i.e. the 7 α -hydroxylase or neutral pathway and the 27-hydroxylase or acidic pathway, are involved in biosynthesis of bile acids [11]. The bile acid intermediates 27-hydroxycholesterol and 3 β -hydroxy-5-cholenoic acid are present

in relative high concentrations in human blood [11,13,14]. The 27-hydroxylase pathway becomes the major route to chenodeoxycholic acid in patients with liver disease, in whom the activity of cholesterol 7 α -hydroxylase is low [11]. Recent work by our group supports an important contribution of the 27-hydroxylase pathway to total bile acid synthesis in both rat and human hepatocytes [12]. The immunosuppressive drug cyclosporin A has been shown to selectively inhibit 27-hydroxylation of cholesterol, leading to a large decrease in the production of chenodeoxycholic acid, ultimately resulting in decreased bile acid formation in cultured rat and human hepatocytes [12,15]. In agreement with this finding, a defect in sterol 27-hydroxylase, as is the case in patients suffering from the rare autosomal recessive disease cerebrotendinous xanthomatosis [16], causes reduced levels of bile acids, particularly chenodeoxycholic acid [17].

27-Hydroxylation of cholesterol is catalysed by an ω -hydroxylase in mammalian liver, which is located in the inner mitochondrial membrane and is a member of the cytochrome *P*-450 superfamily. It almost exclusively attacks the C-27 methyl group and is therefore denoted ‘27-hydroxylase’, although it has been referred to as ‘26-hydroxylase’ in the majority of publications on this enzyme. The enzyme from rabbit [18,19], rat [20] and pig [21] liver has been characterized. cDNA sequences of sterol 27-hydroxylase from rabbit [22], rat [23,24] and human [25] have been reported, and mRNA measurements showed that the enzyme is expressed in several organs and tissues other than liver [22].

Abbreviations used: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Thiazolyl Blue); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Little is known about possible regulatory processes affecting 27-hydroxylase. It has been suggested that the liver mitochondrial 27-hydroxylase is of minor importance in the regulation of bile acid synthesis and composition of bile acids formed *in vivo* in rat [26].

We have previously shown that physiological concentrations of bile acids suppress bile acid synthesis and cholesterol 7 α -hydroxylase activity in cultured pig hepatocytes [27,28]. Recently we reported a profound dose-dependent inhibition of bile acid synthesis by bile acids, accompanied by a reduction in cholesterol 7 α -hydroxylase mRNA and transcriptional activity in cultured rat hepatocytes [29,30]. The magnitude of inhibition of bile acid synthesis was found to be similar to that of suppression of cholesterol 7 α -hydroxylase. In view of the significant contribution of the 27-hydroxylase pathway to total bile acid synthesis in these cells [12], it is conceivable that regulation at the level of cholesterol 7 α -hydroxylase alone would result in a less pronounced effect of bile acids on total bile acid synthesis than is actually observed. Therefore we evaluated the effect of various bile acids on bile acid synthesis and sterol 27-hydroxylase activity in cultured rat hepatocytes. Using a recently available rat 27-hydroxylase cDNA clone [22,24], we were able to monitor sterol 27-hydroxylase mRNA levels and to measure transcription rates using nuclear run-off assays. This study shows that, in addition to feedback inhibition of cholesterol 7 α -hydroxylase, bile acids also suppress sterol 27-hydroxylase activity by down-regulation of sterol 27-hydroxylase mRNA and transcriptional activity.

EXPERIMENTAL

Materials

Materials used for isolation and culturing of rat hepatocytes, determination of mass production of bile acids and assaying sterol 27-hydroxylase activity were obtained from sources described previously [31–33]. Taurocholic acid, cholic acid and deoxycholic acid were from Sigma Chemicals (St. Louis, MO, U.S.A.), chenodeoxycholic acid was from Serva (Heidelberg, Germany) and β -muricholic acid 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*. Two 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 [31–33]. 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 units/ml penicillin and 100 μ g/ml streptomycin, and maintained at 37 °C in a 5 % CO $_2$ /95 % air atmosphere [31,32]. After a 4 h attachment period, medium was refreshed with 1.0 ml (six-well plates) or 2.5 ml (dishes) of culture medium with

hormones as described above. Bile acids were added to the hepatocytes at various times between 18 and 48 h after the start of culture as indicated in the Results section. Cells were harvested at the same time for measurement of sterol 27-hydroxylase activity, mRNA and transcriptional activity. Cell viability, after culture with various bile acids, was assessed by ATP measurements [34] and assays with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [35]. The latter is dependent on the cellular reduction of MTT (Sigma) by the mitochondrial dehydrogenase of viable cells, to a blue formazan product which can be measured spectrophotometrically. The assay was performed essentially as described by De Vries et al. [35]. In short, parallel with the various incubations, cells on 12-well plates (5×10^5 cells/well) in 0.5 ml of medium were incubated with bile acids. At the end of the incubation period, 55 μ l of MTT solution (5 mg of MTT/ml of PBS) was added to each well for 2 h. The medium was aspirated, and 1 ml of 100 % dimethyl sulphoxide was added to solubilize the formazan crystals. Absorbance at 545 nm was measured immediately.

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 after the start of culture), during the following 24 h culture period from 26 to 50 h as described previously [33], 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 and α -muricholic acids (70–80 %) [33,36]. After addition of taurocholic acid as inhibiting agent, synthesis of cholic acid cannot be measured. Values reported are therefore expressed as the percentage of the production of α - and β -muricholic acids and chenodeoxycholic acid.

Assay of sterol 27-hydroxylase

Sterol 27-hydroxylase activity in homogenates of cultured hepatocytes was measured as described previously [37]. 14 C-labelled products were analysed by t.l.c., and the amount of 27-hydroxy[14 C]cholesterol was quantified by scraping off and counting the spots containing this product, using the [14 C]-cholesterol input as a recovery standard. Blank values, determined by running parallel incubations without an NADPH-generating system, were subtracted before calculating enzyme activity. Protein and cholesterol were assayed as previously described [32].

RNA isolation, blotting and hybridization procedures

Isolation of total RNA and subsequent electrophoresis, blotting and hybridization techniques were performed as described previously [29,38]. mRNA was quantified using slot-blotting techniques [39].

The following DNA fragments were used as probes in hybridization experiments: a 1.6 kb *HindIII*–*XbaI* fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. J. Strauss [24]; the 1.6 kb PCR-synthesized fragment of rat cholesterol 7 α -hydroxylase cDNA, spanning the entire coding region [29]; a 0.7 kb *EcoRI* fragment of pFR29-3 containing the cDNA for hamster lithocholic acid 6 β -hydroxylase (CYP3A10), kindly provided by Dr. G. Gil [40]. Sterol 27-hydroxylase cDNA was isolated from a rat liver cDNA library [24] using rabbit sterol 27-hydroxylase cDNA, previously isolated by the group of Russell, as a probe [22]. As controls a 1.2 kb *PstI* fragment of hamster actin cDNA [41], a 1.2 kb *PstI* fragment of glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) cDNA [42] and a 0.8 kb *Pst*I–*Bam*HI fragment of rat cyclophilin cDNA [43] were used. For all probes used in slot-blotting experiments, a linear relationship between areas under the curves and mRNA concentration was shown on an autoradiograph, using concentrations between 2 and 8 μ g of total RNA. The hamster actin cDNA was used as an internal standard to correct for differences in the amount of total RNA applied to the gel or filter.

Nuclear run-off studies

Nuclear run-off studies were conducted essentially as described by Groudine et al. [44], with minor modifications [29].

Hybridization

Target DNA, 5 μ g of plasmid material, containing cDNA sequences of rat sterol 27-hydroxylase, rat cholesterol 7 α -hydroxylase, hamster lithocholic acid 6 β -hydroxylase, hamster actin, rat GAPDH (see above) and the empty vector pUC18 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 [29], and hybridized with the labelled RNA for 36 h in the same buffer. [³²P]UTP had been incorporated into nascent RNA, using isolated nuclei from cells that had been cultured with or without bile acids for 24 h, at between 18 and 42 h of culture time. After hybridization, the various filters were washed once for 5 min and twice for 30 min in 2 $\times$ SSC/1 % SDS (where 1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65 °C, and exposed to Hyperfilm MP (Amersham) for 2–5 days. Relative amounts of mRNA were determined as described previously [29].

Statistical analysis

Data were analysed statistically using Student's paired *t* test with the level of significance selected to be $P < 0.05$. Values are expressed as means $\pm$ S.D.

RESULTS

Effect of taurocholic acid on bile acid synthesis

Incubation of rat hepatocyte cultures with taurocholic acid, the most predominant bile acid in rat bile, resulted in a dose-dependent decline in bile acid synthesis, as determined by mass production of bile acids. Figure 1 shows a significant reduction in bile acid synthesis of 31 % at 10 μ M taurocholic acid, reaching 81 $\pm$ 15 % inhibition after a 24 h incubation period at a concentration of 50 μ M.

Effect of taurocholic acid on sterol 27-hydroxylase activity and mRNA

To assess the possible role of sterol 27-hydroxylase in down-regulation of bile acid biosynthesis by bile acids, sterol 27-hydroxylase activity towards cholesterol and concentration of its mRNA were measured. Sterol 27-hydroxylase activity amounted to 73 $\pm$ 29 pmol/h per mg of cell protein in control cells, and was significantly suppressed ($-72 \pm 20\%$) on addition of 50 μ M taurocholic acid to the culture medium for 24 h. Northern-blot analysis of total RNA isolated from cultured hepatocytes revealed the existence of a single 2.4 kb mRNA for this enzyme (Figure 2), in agreement with the previously reported size of the 27-hydroxylase mRNA in rat liver *in vivo* [24]. Figure 2 also shows a strong suppression of the sterol 27-hydroxylase mRNA in the presence of 50 μ M taurocholic acid, concomitant with a

Figure 1 Effect of taurocholic acid on mass production of bile acids

Rat hepatocytes were incubated for 24 h with various amounts (0–50 μ M) of taurocholic acid. Bile acid synthesis was measured after an 8 h preincubation period (18–26 h), from 26 to 50 h of culture time, as described in the Experimental section, in the presence or absence of taurocholic acid. Values are expressed as means $\pm$ S.D. of independent experiments using hepatocytes from five to six rats. The absolute value for synthesis of chenodeoxycholic acid and β -muricholic acid was 2.02 ± 0.66 μ g/24 h per mg of cell protein. *Significant difference ($P < 0.05$) compared with control values.

Figure 2 Effect of taurocholic acid on mRNA of enzymes involved in bile acid synthesis

Total RNA from cells exposed to 50 μ M taurocholic acid for 24 h, between 18 and 42 h of culture (lane 2), and from untreated cells (lane 1) was electrophoresed in a 0.8% agarose/1 M formaldehyde gel, transferred to Hybond-N⁺ and subsequently hybridized with probes for sterol 27-hydroxylase, cholesterol 7 α -hydroxylase and lithocholic acid 6 β -hydroxylase, and GAPDH, actin and cyclophilin mRNA, as described in the Experimental section; 10 μ g of total RNA was applied in each lane.

profound decrease in cholesterol 7 α -hydroxylase mRNA under these circumstances. In contrast, lithocholic acid 6 β -hydroxylase mRNA did not change significantly on addition of 50 μ M taurocholic acid. Taurocholic acid therefore does not show similar effects on all mRNAs coding for cytochrome *P*-450 enzymes involved in bile acid biosynthesis, and inhibition by the compound is thus not a reflection of a general effect on these messengers. Taurocholic acid had no effect on the mRNAs of

Table 1 Effect of increasing concentrations of taurocholic acid on mitochondrial dehydrogenase activity and cellular ATP levels

Rat hepatocytes were incubated for 24 h, from 18 to 42 h, with various amounts (0–70 μ M) of taurocholic acid to assess the effect on cell viability. After 42 h of culture, reduction of MTT by mitochondrial dehydrogenase and ATP content of these cells were measured as described in the Experimental section. Data are expressed as a percentage of control (no bile acids added), and are means $\pm$ S.D. of independent experiments using hepatocytes from four to six rats. The absolute value for cellular ATP content was 20.1 ± 4.3 nmol/mg of cell protein. *Significant difference ($P < 0.05$) compared with control values.

Extracellular taurocholate concn. (μ M)	MTT assay (% of control)	Cellular ATP (% of control)
0	100	100
30	98 ± 5	92 ± 13
50	96 ± 14	96 ± 12
70	$84 \pm 5^*$	97 ± 10

Figure 3 Time course of suppression of sterol 27-hydroxylase mRNA by taurocholic acid

Cells were exposed to taurocholic acid (50 μ M) for different lengths of time, between 18 and 48 h of culture, and were harvested simultaneously at 48 h of culture time. The amount of sterol 27-hydroxylase mRNA was assessed by slot-blotting and densitometric scanning of the resulting autoradiographs (inset), using actin mRNA as an internal standard to correct for differences in the amount of RNA applied. Values shown are expressed as percentages of control values (incubations without taurocholic acid) and have been plotted semilogarithmically, as means $\pm$ S.D. of incubations using hepatocytes from three to eight rats. *Significant difference ($P < 0.05$) compared with control values.

the housekeeping genes actin and GAPDH, nor on cyclophilin mRNA, used as internal standards (Figure 2).

In addition, MTT and ATP measurements showed that taurocholic acid did not have adverse effects on cell viability, up to the 50 μ M concentrations used in this study (Table 1).

Figure 4 Dose-dependence of suppression of sterol 27-hydroxylase and cholesterol 7 α -hydroxylase mRNA by taurocholic acid

Rat hepatocytes were incubated for 24 h, from 18 to 42 h of culture, with various amounts (0–50 μ M) of taurocholic acid. Cells were harvested after 42 h of culture, for determination of sterol 27-hydroxylase (●) and cholesterol 7 α -hydroxylase (○) mRNA; the latter is shown for comparison. The amount of sterol 27-hydroxylase and cholesterol 7 α -hydroxylase mRNA was assessed by slot-blotting and densitometric scanning of the resulting autoradiographs, using actin mRNA as an internal standard to correct for differences in the amount of total RNA applied to the filter. For details see the Experimental section. Data are expressed as percentages of control values (no bile acids added) and are means $\pm$ S.D. of independent experiments using hepatocytes from three to eight rats. *Significant difference ($P < 0.05$) compared with control values.

Time- and dose-dependence of the effect of taurocholic acid on sterol 27-hydroxylase mRNA level

Quantification of the amount of mRNA by slot-blotting experiments using total RNA from cells treated with 50 μ M taurocholic acid showed a time-dependent decline, reaching maximal inhibition ($-75 \pm 16\%$) after 24 h of incubation with taurocholic acid (Figure 3). The apparent half-life of the mRNA is estimated to be 13 h. Actin mRNA, used as a control in these experiments, exhibited no significant changes in level when taurocholic acid was present in the medium (inset). The reduction in sterol 27-hydroxylase mRNA fully explains the decline in enzyme activity (-75% and -72% respectively at 50 μ M taurocholate).

Suppression of sterol 27-hydroxylase mRNA was found to be a dose-dependent process (Figure 4). Maximal reduction was reached at an extracellular concentration of 30 μ M taurocholic acid ($-74 \pm 14\%$), which also resulted in profound suppression of cholesterol 7 α -hydroxylase mRNA ($-88 \pm 12\%$) and bile acid synthesis ($-67 \pm 18\%$; Figure 1). The concentrations of bile acids applied are all well within the physiological range as detected in portal blood of rats [45,46].

Effect of taurocholic acid on bile acid synthesis and sterol 27-hydroxylase activity and mRNA in hepatocytes from rats fed on normal chow

To exclude the possibility that the effect of taurocholic acid on sterol 27-hydroxylase activity and mRNA results from a suppression of derepressed bile acid synthesis after supplementation of the diet of the intact animal with 2% cholestyramine before isolation of the hepatocytes, some of the experiments described above were repeated using hepatocytes from rats fed on normal chow. Bile acid synthesis (i.e. chenodeoxycholic acid and β -muricholic acid synthesis) in these hepatocytes (1.03 ± 0.51 μ g/24 h per mg of cell protein) was suppressed by $67 \pm 12\%$ ($n = 3$) after incubation with 50 μ M taurocholic acid.

Table 2 Effect of different bile acids on sterol 27-hydroxylase mRNA in rat hepatocytes

Rat hepatocytes were incubated with different bile acids (50 μ M) from 18 to 42 h of culture. Cells were harvested at 42 h, and the sterol 27-hydroxylase mRNA level was determined as described in the Experimental section. Data are expressed as a percentage of the control (no bile acids added) and are means $\pm$ S.D. of independent experiments using hepatocytes from three to eight rats. The amount of sterol 27-hydroxylase mRNA was assessed by slot-blotting and densitometric scanning of resulting autoradiographs, using actin mRNA as an internal standard to correct for differences in the amount of total RNA applied to the filter. *Significant difference ($P < 0.05$) between control and treated cells.

Added bile acid	Sterol 27-hydroxylase mRNA (% of control)
No bile acid	100
Taurocholic acid	25 $\pm$ 16*
Cholic acid	28 $\pm$ 8*
Deoxycholic acid	24 $\pm$ 12*
Chenodeoxycholic acid	43 $\pm$ 13*
β -Muricholic acid	86 $\pm$ 24

Addition of 50 μ M taurocholic acid to the medium of non-induced hepatocytes for a period of 24 h led to a strong suppression of both cholesterol 7 α -hydroxylase and sterol 27-hydroxylase activities and mRNA levels ($-85 \pm 11\%$ and $-81 \pm 3\%$ for cholesterol 7 α -hydroxylase activity and mRNA respectively and $-69 \pm 16\%$ and $-74 \pm 7\%$ for sterol 27-hydroxylase activity and mRNA respectively). These values are comparable to those obtained with hepatocytes from cholestyramine-treated rats, indicating that taurocholic acid also inhibits sterol 27-hydroxylase activity and mRNA in hepatocytes from rats fed on normal chow.

Effect of different bile acids on sterol 27-hydroxylase mRNA in cultured rat hepatocytes

In previous work, we and others [28,29,47,48] have shown that different bile acids have a different effect on suppression of cholesterol 7 α -hydroxylase activity and mRNA. To assess whether the mRNA level of sterol 27-hydroxylase is also differentially affected by various bile acids, hepatocytes were incubated with different bile acids commonly found in bile of normal rats (all at 50 μ M), for a period of 24 h (from 18 to 42 h of culture). No differences were found in the uptake of the various bile acids by the hepatocytes, as determined by measurement of bile acids using g.l.c.

Table 2 shows that the more hydrophobic bile acids, chenodeoxycholic acid and deoxycholic acid, were as effective as taurocholic acid in suppressing sterol 27-hydroxylase mRNA levels, whereas the hydrophilic β -muricholic acid did not change the mRNA level significantly. No difference was found between taurocholic acid and unconjugated cholic acid.

Effect of taurocholic acid on the transcriptional activity of the sterol 27-hydroxylase gene in cultured rat hepatocytes

To examine further the mechanism of suppression of sterol 27-hydroxylase activity and mRNA levels, nuclear run-off studies were conducted using nuclei isolated from hepatocytes that had been incubated with 50 μ M taurocholic acid for 24 h, between 18 and 42 h of culture time. α - 32 P-labelled total RNA was hybridized to rat sterol 27-hydroxylase cDNA, rat cholesterol 7 α -hydroxylase cDNA, hamster lithocholic acid 6 β -hydroxylase cDNA,

Figure 5 Transcriptional activity of the sterol 27-hydroxylase gene in response to taurocholic acid

Autoradiograph of run-off transcripts for sterol 27-hydroxylase, cholesterol 7 α -hydroxylase, lithocholic acid 6 β -hydroxylase, actin and GAPDH isolated from nuclei from rat hepatocytes incubated with (lane 2) or without (lane 1) 50 μ M taurocholic acid for 24 h. 32 P-labelled total RNA was hybridized to immobilized cDNAs as indicated in the Experimental section, and the resulting blot was exposed to Hyperfilm for 48–120 h. Non-specific hybridization was checked using the empty vector pUC18. The relative amounts of transcribed [32 P]mRNA were assessed by densitometric scanning, as described, using actin mRNA as an internal standard.

hamster actin and rat GAPDH cDNA. The latter two served as transcriptional activity controls between the different samples, and specific transcriptional activity of the genes is expressed relative to that of actin. The empty vector pUC18 shows that there is no non-specific hybridization of the gene transcripts (Figure 5).

Addition of 50 μ M taurocholic acid lowered the rate of transcription of sterol 27-hydroxylase by $43 \pm 9\%$ ($n = 4$), as was the case for the rate of transcription of cholesterol 7 α -hydroxylase ($-42 \pm 9\%$; $n = 4$), indicating that the decreased sterol 27-hydroxylase mRNA level results in part from reduced transcriptional activity. There was no effect on transcription of the lithocholic acid 6 β -hydroxylase gene nor on expression of the GAPDH gene.

DISCUSSION

This study shows, to our knowledge for the first time, that sterol 27-hydroxylase in cultured rat hepatocytes is down-regulated by physiological concentrations of bile acids. The reduction in enzyme activity towards cholesterol was accompanied by a comparable decrease in sterol 27-hydroxylase mRNA level and transcriptional activity of the corresponding gene.

This may seem surprising in view of the scarcity of reports showing regulation of sterol 27-hydroxylase *in vivo* [26]. However, it is important to note that most studies addressing the contribution of the 27-hydroxylase pathway to chenodeoxycholic acid synthesis and regulation of the sterol 27-hydroxylase have been performed using rats, often after stimulation of bile acid synthesis by feeding cholestyramine or by bile diversion. The absence of a gallbladder in addition to an unusually long small intestine in this species results in a high bile acid-biosynthetic capacity [49]. As a consequence of these anatomical differences a major portion of available cholesterol in the rat is probably routed via the cholesterol 7 α -hydroxylase or 'neutral' pathway in contrast with the situation in man [11].

The concentration of 27-hydroxycholesterol is considerably lower in the circulation of rat than in man [26], suggesting that the pathway involving initial 27-hydroxylation of cholesterol is of less importance in the former species. This contention is supported by the fact that cholic acid is the predominant bile acid in bile of rats [50]. Stimulation of bile acid synthesis via

interruption of the enterohepatic circulation will further increase the contribution of the 7α -hydroxylase pathway.

Nevertheless, there are indications that sterol 27-hydroxylase is subject to regulation in the rat. Preliminary data reported a 30–60% reduction in the amount of the 27-hydroxylase enzyme and catalytic activity in cholic acid-fed and starved rats [51]. In addition, we found an approx. 1.5–2-fold increment in sterol 27-hydroxylase activity, mRNA and transcriptional activity in livers from cholestyramine-treated rats, as compared with control animals (J. Twisk and H. M. G. Princen, unpublished work). In line with these findings, it has been shown in hypercholesterolaemic patients that treatment with cholestyramine results in normal or increased levels of 27-hydroxycholesterol detectable in plasma [52]. Additional support is obtained from the heterogeneous localization of bile acid synthesis in the liver acinus of the rat. The presence of a strong bile acid gradient over the liver lobule results in a 6-fold higher bile acid concentration in the periportal zone [53,54]. As a consequence, bile acid synthesis is 4-fold higher in the pericentral zone [36], concomitant with a pericentral expression of both cholesterol 7α -hydroxylase and sterol 27-hydroxylase [55].

In cultured rat hepatocytes a substantial part of the bile biosynthesis has been reported to proceed via the 27-hydroxylase route [12], as may also be the case in cultured pig hepatocytes [56]. Since we found that bile acid synthesis in rat hepatocytes is down-regulated to the same extent as cholesterol 7α -hydroxylase by hydrophobic bile acids [29], we wondered whether the first enzyme in the alternative pathway was also affected. The results show that sterol 27-hydroxylase activity towards cholesterol can be down-regulated by taurocholic acid (–72%), similar to the 88% suppression of cholesterol 7α -hydroxylase activity as reported previously [29]. Taken together, this parallel decline in the two enzyme activities can explain the concomitant decrease (–81%) in mass production of bile acids (β -muricholic acid and chenodeoxycholic acid) under these conditions (Figure 1).

The effect of taurocholic acid is exerted at the level of sterol 27-hydroxylase mRNA and gene transcription. The mRNA showed an apparent half-life of approx. 13 hours, indicative of a much slower decline than is the case for cholesterol 7α -hydroxylase (4 h, [29]). On the basis of our previous findings showing that the suppression of steady-state levels of cholesterol 7α -hydroxylase mRNA by taurocholate is considerably larger than the suppression of transcriptional activity of the gene as determined using nuclear run-off [29] and transient transfection assays [30], we have suggested that feedback regulation of this enzyme takes place both at the transcriptional and post-transcriptional level, e.g. by stability of the mRNAs. Whether post-transcriptional regulation is a mechanism in modulation of sterol 27-hydroxylase remains to be determined. The sterol 27-hydroxylase mRNA contains a short 5' sequence coding for a mitochondrial signal sequence, and only a relatively small 3'-non-coding region. However, the multiple AU-rich sequences present in the 3'-non-coding region of the cholesterol 7α -hydroxylase messenger, which may be a target for this particular mode of regulation [57,58], are virtually absent from the sterol 27-hydroxylase mRNA [24], potentially providing an explanation for the difference in apparent half-life between the two mRNAs. We have recently shown, using transient transfection experiments with promoter-reporter constructs, that a putative bile acid-regulated element in the cholesterol 7α -hydroxylase promoter may be located in the region between nucleotides –47 and –79 [30]. Comparison of these sequences with DNA sequences of the human sterol 27-hydroxylase promoter [59] – the sequence of the rat 27-hydroxylase promoter is still unknown – revealed a high degree of homology in the region between nucleotides –254 and –280,

suggesting that similar elements in the cholesterol 7α -hydroxylase and sterol 27-hydroxylase promoter may be involved in negative feedback regulation by bile acids.

Considerable differences between bile acids most common to rat were observed with respect to their inhibitory effects. In general, the potency of suppression of a given bile acid is linked to its hydrophobicity, as has been reported for inhibition of cholesterol 7α -hydroxylase [28,29,47,48], although there may be exceptions [28,29], since cholic acid, especially conjugated cholic acid, does not entirely fit the model.

Interestingly, it has been reported that the abundance of the sterol 27-hydroxylase mRNA in organs and tissues correlates positively with the cholesterol-biosynthetic capacity of the tissues that were assayed [22]. This finding, together with results showing down-regulation of cholesterol supply genes by 27-hydroxycholesterol and other oxysterols [60], has led to the suggestion that sterol 27-hydroxylase plays a role in maintaining cholesterol homeostasis in multiple tissues [60,61]. We have shown in the present paper that sterol 27-hydroxylase is down-regulated by bile acids in cultured hepatocytes. The finding that the enzyme is subject to regulation would support its involvement in the regulation of cholesterol homeostasis, at least in the liver. The cultured rat hepatocyte is an attractive model for further investigation of factors that may affect sterol 27-hydroxylase and the mechanism of regulation.

We thank Professor Jerome Strauss III for providing us with the PCMV-4-derived plasmid containing sterol 27-hydroxylase cDNA and Dr. Gregorio Gil for providing the pFR29-3 clone containing lithocholic acid 6β -hydroxylase cDNA. Miss Marianka van der Tol is gratefully acknowledged for excellent technical assistance, and Miss Marisa Horsting for typing the manuscript. This work was supported by a grant from the Netherlands Heart Foundation (grant 89.079).

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