Dexamethasone regulates bile acid synthesis in monolayer cultures of rat hepatocytes by induction of cholesterol 7 α -hydroxylase

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To study the effect of steroid hormones on bile acid synthesis by cultured rat hepatocytes, cells were incubated with various amounts of these compounds during 72 h and conversion of [4-¹⁴C]cholesterol into bile acids was measured. Bile acid synthesis was stimulated in a dose-dependent way by glucocorticoids, but not by sex steroid hormones, pregnenolone or the mineralocorticoid aldosterone in concentrations up to 10 μ M. Dexamethasone proved to be the most efficacious inducer, giving 3-fold and 7-fold increases in bile acid synthesis during the second and third 24 h incubation periods respectively, at a concentration of 50 nM. Mass production of bile acids as measured by g.l.c. during the second day of culture (28–52 h) was 2.2-fold enhanced by 1 μ M-dexamethasone. No change in the ratio of bile acids produced was observed during this period in the presence of dexamethasone. Conversion of [4-¹⁴C]7 α -hydroxycholesterol, an intermediate of the bile acid pathway, to bile acids was not affected by dexamethasone. Measurement of cholesterol 7 α -hydroxylase activity in homogenates of hepatocytes, incubated with 1 μ M-dexamethasone, showed 10-fold and 90-fold increases after 48 and 72 h respectively, as compared with control cells. As with bile acid synthesis from [¹⁴C]cholesterol, no change in enzyme activity was found in hepatocytes cultured in the presence of 10 μ M steroid hormones other than glucocorticoids. Addition of inhibitors of protein and mRNA synthesis lowered bile acid production and cholesterol 7 α -hydroxylase activity and prevented the rise of both parameters with dexamethasone, suggesting regulation at the mRNA level. We conclude that glucocorticoids regulate bile acid synthesis in rat hepatocytes by induction of enzyme activity of cholesterol 7 α -hydroxylase.

INTRODUCTION

The liver plays a central role in the synthesis and catabolism of cholesterol. Formation of bile acids takes place exclusively in the liver and represents the quantitatively most important pathway for removal of cholesterol from the body [1]. Primary monolayer cultures of hepatocytes have been shown to be a valuable model to investigate modulation of bile acid synthesis [2–10]. In this system *in vitro* the effects of bile acids [3,7,10] and the role of cholesterol supply to the hepatocyte by lipoproteins [2,4,9] on bile acid formation have been studied. Moreover, the (side-)effects of several drugs, currently applied in clinical practice, on the synthesis of bile acids can conveniently be determined using monolayer cultures of hepatocytes [2,8]. Until now, however, little research has been performed on the influence of hormones on bile acid synthesis in this system *in vitro*, although experiments using intact animals indicate that hormones of the adrenal cortex, thyroid hormones [1,11,12] and insulin [13] may be involved in regulation.

In this study we concentrate on steroid hormones. From experiments *in vivo*, a possible role of glucocorticoids in the regulation of bile acid synthesis has been suggested. The diurnal rhythm in bile acid synthesis and activity of cholesterol 7 α -hydroxylase parallels the diurnal variation in plasma levels of corticotropin and corticosterone in rat [14–16]. A diurnal rhythm in cholesterol 7 α -hydroxylase (EC 1.14.13.17) activity in newborn rats is not established until the twentieth day after birth,

a stage in postnatal development at which diurnal variation in corticotropin and corticosterone levels is first detectable [11]. In addition, hypophysectomy and adrenalectomy in rats leads to a loss [16] or reduction [17] in the amplitude of diurnal rhythm of cholesterol 7 α -hydroxylase activity, which can be partly restored after administration of glucocorticoids [17]. Recently, Everson demonstrated that dexamethasone stimulated bile acid synthesis and cholesterol 7 α -hydroxylase activity in hybrid cells, created by fusing rat hepatocytes with Reuber H35-FAO hepatoma cells [18]. On the other hand, no effect of cortisol hemisuccinate on the activity of cholesterol 7 α -hydroxylase was observed in suspension cultures of rat hepatocytes [19].

Little is known concerning the effects of other steroid hormones on bile acid synthesis, although there is some evidence that ethinyloestradiol may block bile acid formation in rat by inhibition of cholesterol 7 α -hydroxylase [20,21].

The aim of the present study was to investigate the role of steroid hormones in the regulation of bile acid synthesis. Experiments were performed using primary monolayer cultures of rat hepatocytes to establish the effects of these compounds directly on hepatocytes in a controlled and chemically defined environment. The results of this study show that glucocorticoids and no other steroid hormones induce bile acid production in cultured hepatocytes by stimulation of 7 α -hydroxylase activity.

Abbreviation used: PCN, pregnenolone 16 α -carbonitrile.

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MATERIALS AND METHODS

Materials used for isolation and culturing of rat hepatocytes, determination of bile acid synthesis from radiolabelled cholesterol and measurement of cholesterol 7 α -hydroxylase activity and other enzyme assays, were obtained from sources described previously [8,22,23].

Steroid hormones were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.) and Steraloids (Wilton, NH, U.S.A.). Reference bile acids β -muricholic acid, cholic acid, chenodeoxycholic acid, murocholic acid, deoxycholic acid and lithocholic acid were from Steraloids. Cycloheximide was purchased from Boehringer (Mannheim, Germany). Actinomycin D and α -amanitin were from Sigma. Hexafluoropropan-2-ol and trifluoroacetic anhydride were obtained from Serva (Heidelberg, Germany). [4-¹⁴C]7 α -Hydroxycholesterol was prepared from [4-¹⁴C]7-oxocholesterol, which is the most abundant impurity in commercially available [4-¹⁴C]cholesterol (60 mCi/mmol) as described [22]. Male Wistar rats (250–350 g) were used throughout, and were housed in an artificially lighted (6:00 to 18:00 h) and temperature-controlled (20 °C) room. They were maintained on a standard pelleted chow RMH-B, containing 22.4 % protein, 6.5 % fat and 58.6 % carbohydrate (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. For preparation of hepatocytes, animals were killed between 9:00 and 10:00 h.

Rat hepatocyte preparation and culture

Rat liver cells were isolated by perfusion with 0.05 % collagenase and 0.005 % trypsin inhibitor as described previously [8,24]. Viability, as determined by Trypan Blue exclusion, was higher than 90 %. The cells were seeded on 6-well cluster plates or on 60 mm diam. plastic tissue culture dishes (Costar, Cambridge, MA, U.S.A.) at a density of 1×10^5 cells/cm² and were maintained in Williams E medium supplemented with 10 % heat-inactivated foetal bovine serum, 2 mM-glutamine, 100 i.u. of penicillin/ml and 100 μ g of streptomycin/ml at 37 °C in a 5 % CO₂/95 % air atmosphere. After a 4-h attachment period, and every 24 h thereafter, medium was refreshed with 1 ml (wells) or 2.5 ml (dishes) of culture medium. Hepatocytes were cultured for 3 days under these conditions and remained viable, as judged by Trypan Blue exclusion (more than 90 % after 76 h) and leakage of the cytoplasmic enzyme lactate dehydrogenase to the culture medium [8].

[4-¹⁴C]Cholesterol (0.15 μ Ci) or [4-¹⁴C]7 α -hydroxycholesterol (6000 d.p.m.; sp. radioactivity 2.6 mCi/mmol) was added, solubilized in foetal bovine serum. Steroid hormones were supplied to the medium in ethanol, giving a final concentration of 0.1 % ethanol, with an equal volume of ethanol added to control cultures. This concentration of ethanol did not affect viability of cells or bile acid synthesis.

Determination of bile acid synthesis from radiolabelled cholesterol and 7 α -hydroxycholesterol

Synthesis of bile acids in primary cultures of hepatocytes was determined by measuring conversion of 0.15 μ Ci of [4-¹⁴C]cholesterol per 10 cm² of cells into bile acids, accumulated during 24-h periods after 28, 52 or 76 h incubation. After harvest of cells and media and extraction according to Bligh & Dyer [25], bile acids were extracted using a reverse-phase cartridge (Sep-Pak C-18;

Waters Associates, Milford, MA, U.S.A.) with [G-³H]-taurocholic acid as recovery standard [8]. Bile acids were deconjugated using cholyglycine hydrolase, solvolyzed and separated on a thin layer of silica. The plates were developed in toluene/dioxane/glacial acetic acid (20:10:2, by vol.) using radioactive or unlabelled bile acids as markers and autoradiographed. Unlabelled reference bile acids on vertical edges were revealed by heating after spraying with H₂SO₄. Areas containing bile acids were scraped off as reported before [23] and counted using a double-label program.

Conversion of [4-¹⁴C]7 α -hydroxycholesterol into bile acids was measured as previously described [8].

Quantification of mass production of bile acids

Mass production of bile acids by rat hepatocytes was measured during the second 24-h culture period from 28 to 52 h. Cells were cultured under the same conditions as with determination of bile acid synthesis using [4-¹⁴C]cholesterol, i.e. in Williams E medium containing 10 % foetal bovine serum. Bile acids in cells and medium from two dishes were quantified, essentially as described by Davis *et al.* [2] with the following modifications. Bile acids were extracted using a Sep-Pak C-18 cartridge after addition of 1 μ g of deoxycholic acid as recovery standard, and deconjugated and solvolyzed. The bile acid-containing residue was dissolved in 2 mM-NaOH, extracted with hexane and after acidification to pH 2.8 extracted in dichloromethane. After evaporation of the solvent, bile acids were derivatized with trifluoroacetic anhydride and hexafluoropropan-2-ol for 1 h at 60 °C in 0.5 ml Reactivials (Pierce Chemical Company, Rockford, IL, U.S.A.). The solvent was evaporated and the residue was dissolved in 5–10 μ l of hexane. Samples of 1 μ l were subjected to g.l.c. separation using a Chrompack-Packard 438 S gas chromatograph, equipped with a CP Sil-19(CB) column (25 m $\times$ 0.32 mm internal diameter) and flame ionization detector. Hydrogen was used as carrier gas at a flow rate of 7 ml/min, and samples were split with a 1:10 ratio. The injector temperature was initially 205 °C, and oven temperature was raised after 0.1 min by 1 °C/min and after 20 min by 20 °C/min to 290 °C, which temperature was maintained for 10 min. The retention time of deoxycholic acid was 10.56 min. Chenodeoxycholic, murocholic, cholic and β -muricholic acids had relative retention times of 1.23, 1.28, 1.43 and 1.56 respectively compared with deoxycholate. Bile acids were quantified using peak area ratios. Relationships between weight and peak area were similar for all bile acids synthesized by rat hepatocytes. Recovery of deoxycholate was 75–85 % throughout the entire procedure. Bile acid synthesis was calculated as the amount in medium and cells corrected for the amount of bile acids in foetal bovine serum (0.36 μ g of cholic acid and 1.52 μ g of chenodeoxycholic acid per ml), and minus the amount in cells 24 h earlier. Since most of the chenodeoxycholic acid was converted to β -muricholic acid [2,6,23], these two bile acids were added before corrections were made.

Cholesterol 7 α -hydroxylase assay

Cholesterol 7 α -hydroxylase activity was measured in homogenates of cultured hepatocytes as has been described [22].

Protein and cholesterol determinations

Protein and cholesterol were assayed according to Lowry *et al.* [26] and Gamble *et al.* [27] respectively.

Fig. 1. Effect of steroids on induction of bile acid synthesis in cultured rat hepatocytes

Hepatocytes were isolated and cultured as described in the Materials and methods section. After a 4-h attachment period and 24 h thereafter cells were refreshed with medium containing one of the indicated steroids. At 28 h of culture age hepatocytes received, besides steroids, $0.15 \mu\text{Ci}$ of $[4\text{-}^{14}\text{C}]$ cholesterol per 10 cm^2 well. After a further 24 h, cells and media were harvested, cells were washed four times after removal of medium and intracellular and extracellular bile acids were determined. Conversion of $[^{14}\text{C}]$ cholesterol into bile acids by control hepatocytes (100% value) was $9300 \pm 4080 \text{ d.p.m./24 h}$ per mg during this 24-h incubation from 28 to 52 h after plating. Values shown are means ($\pm \text{s.d.}$) of duplicate incubation with hepatocytes from between three and six rats. * indicates a significant difference ($P < 0.05$) between control and steroid-treated cells.

Statistical analysis

Statistical significance of differences was calculated using Student's *t* test for paired data with the level of significance selected to be $P < 0.05$. Values are expressed as means $\pm \text{s.d.}$

RESULTS

Effects of steroid hormones on bile acid synthesis

To measure bile acid synthesis, we first determined conversion of $[4\text{-}^{14}\text{C}]$ cholesterol to methanol/water-soluble materials, which have previously been shown to be conjugated bile acids [6,23]. To exclude the possibility that the added compounds might influence uptake of cholesterol by the hepatocytes, we measured $[4\text{-}^{14}\text{C}]$ cholesterol absorption by the cells during the 24-h incubation period. No differences in $[^{14}\text{C}]$ cholesterol uptake were found with the amounts of hormones used. Furthermore, viability of cells as determined by Trypan Blue exclusion ($> 90\%$) and by leakage of the cytoplasmic enzyme lactate dehydrogenase to the culture medium was not affected by the steroids. Fig. 1 shows the effects of various steroids on bile acid synthesis. Both synthetic (dexamethasone) and natural (cortisol and corticosterone) glucocorticoids increased bile acid formation in a dose-dependent manner. Dexamethasone proved to be the most potent inducer, giving maximal stimulation at 50 nM, whereas much higher concentrations of the natural corticosteroids were required to obtain induction. In contrast, representatives of each of the other steroid classes (oestrogen, androgen, progesterone, mineralocorticoid) and pregnenolone at concentrations of 1 and $10 \mu\text{M}$ failed to promote conversion of $[^{14}\text{C}]$ cholesterol into bile acids.

Time course of induction of bile acid synthesis by dexamethasone

Unless otherwise stated, a concentration of $1 \mu\text{M}$ -dexamethasone was employed in further experiments. A time-course of bile acid synthesis over the 72-h incubation period (4–76 h after plating) in control and dexamethasone-treated cells is depicted in Fig. 2(a). In control cells, bile acid formation during the first 24-h period was significantly lower than during the second day of incubation and declined slightly in hepatocytes incubated during the third day. A similar pattern of bile acid synthesis has been observed by other investigators, measuring mass production of bile acids [2,6]. During the 24-h interval between 4 and 28 h of culture age, the hepatocytes were refractory to induction of bile acid production by dexamethasone. On continued exposure of the monolayers to medium containing $1 \mu\text{M}$ -dexamethasone, the rates of bile acid synthesis increased steadily, reaching values 3 and 7 times higher than the rates in control cultures during the second and third 24-h periods respectively (Fig. 2b). The amount of induction of bile acid production was not dependent on either the length of time of exposure of cultures to dexamethasone or the time at which dexamethasone was first administered. A similar magnitude of stimulation after 52 and 76 h of culture age was attained with only one addition of $1 \mu\text{M}$ -dexamethasone at 28 or 52 h (Fig. 2b) or in the presence of 50 nM-dexamethasone (results not shown).

In these experiments, no change was observed in the proportions of individual bile acids produced in cultures with or without addition of $1 \mu\text{M}$ -dexamethasone during the first 52 h of culture age (Table 1). In contrast, there was a significant difference between formation of β -

Fig. 2. Time course of induction of bile acid synthesis by dexamethasone

After a 4-h attachment period and every 24 h thereafter, cells were renewed with control medium or medium with 1 μ M-dexamethasone, both containing 0.15 μ Ci of [4- 14 C]cholesterol. Bile acid synthesis was measured as described in the legend to Fig. 1 during the consecutive periods from 4–28, 28–52 and 52–76 h. (a) Sum of accumulated radioactivity (d.p.m.) in intracellular and extracellular bile acids in cultures in the absence (●, control) or continuous presence (■) of 1 μ M-dexamethasone during the three culture periods. (b) Effect of age of culture on the magnitude of induction of bile acid synthesis by dexamethasone (Dex). At the indicated times cultures were transferred to medium containing 1 μ M-dexamethasone. Values shown are means ($\pm$ S.D.) of duplicate incubations with hepatocytes of six [a and b (Dex added at 4 h)] or two rats [b (Dex added at 28 and 52 h)]. Differences between control and dexamethasone-treated cells after 52 h and 76 h were significant ($P < 0.05$) in all experiments.

muricholic acid and polar [23] and monohydroxy bile acids in the presence (55 ± 2 , 12 ± 4 and 7 ± 2 % of total intracellular and extracellular bile acids respectively) and in the absence (39 ± 6 , 19 ± 4 and 13 ± 3 % of total bile acid synthesis respectively) of 1 μ M-dexamethasone during the third 24-h incubation period.

Effect of dexamethasone on mass production of bile acids

In the above-mentioned experiments, bile acid synthesis was determined by measuring conversion of pre-existent radiolabelled cholesterol, shown to be predominantly associated with lipoproteins [7], into bile acids. To check the possibility that dexamethasone may change the

amount of cholesterol available for bile acid formation, and consequently bile acid synthesis, mass production of bile acids was determined. The mass of bile acids synthesized by control hepatocytes in the three consecutive 24-h periods was 0.20 ± 0.05 , 0.76 ± 0.22 and 0.59 ± 0.19 μ g/24 h per mg of cell protein ($n = 3$) respectively, showing a similar pattern to bile acid synthesis from [4- 14 C]cholesterol. Hepatocytes synthesized predominantly β -muricholic acid and cholic acid, which comprised 65, 67 and 76 %, and 30, 28 and 22 % respectively of total bile acid synthesis in the three periods, in agreement with previous reports [2,5,6]. A small amount of murocholic acid (0–1 %) and chenodeoxycholic acid (2–4 %) was detected, which latter

Table 1. Effect of dexamethasone on the proportions of individual bile acids formed from [14 C]cholesterol

Hepatocytes were cultured as described in the legend to Fig. 2 and bile acids were determined as described in the Materials and methods section. Values are expressed as percentages of total intracellular and extracellular bile acids, formed during the appropriate 24-h period, and are means ($\pm$ S.D.) of duplicate incubations with hepatocytes from six rats. A significant difference ($P < 0.05$) between dexamethasone-treated and control cells in the same 24-h period is indicated by *.

Bile acid(s)	Addition of dexamethasone	Incubation time (h)...	% of total bile acids		
			4–28	28–52	52–76
Polar bile acids	–		15 $\pm$ 5	10 $\pm$ 2	19 $\pm$ 4*
	+		16 $\pm$ 6	11 $\pm$ 3	12 $\pm$ 4
Cholic acid	–		15 $\pm$ 2	15 $\pm$ 4	12 $\pm$ 2
	+		15 $\pm$ 2	15 $\pm$ 4	12 $\pm$ 2
β -Muricholic acid	–		48 $\pm$ 7	55 $\pm$ 3	39 $\pm$ 6*
	+		48 $\pm$ 7	56 $\pm$ 3	55 $\pm$ 2
Dihydroxy bile acids	–		13 $\pm$ 2	11 $\pm$ 2	17 $\pm$ 2
	+		12 $\pm$ 1	12 $\pm$ 1	14 $\pm$ 2
Monohydroxy bile acids	–		9 $\pm$ 2	9 $\pm$ 1	13 $\pm$ 3*
	+		9 $\pm$ 3	6 $\pm$ 1	7 $\pm$ 2

Table 2. Effect of dexamethasone on mass production of bile acids in rat hepatocytes

Hepatocytes were cultured as described in the legend to Fig. 1. Bile acid synthesis in the period from 28–52 h was determined in cells and media as described in the Materials and methods section. Value shown are means ($\pm$ S.D.) of duplicate incubations with hepatocytes from three rats. * indicates a significant difference ($P < 0.05$) between control and dexamethasone-treated cells.

Bile acid(s)	Bile acid production ($\mu\text{g}/24 \text{ h per mg}$)	
	Control	Dexamethasone
Murocholic acid	0.01 ± 0.01	0.01 ± 0.01
Chenodeoxycholic + β -muricholic acids	0.54 ± 0.15	$1.30 \pm 0.39^*$
Cholic acid	0.21 ± 0.07	$0.37 \pm 0.08^*$
Total	0.76 ± 0.22	$1.68 \pm 0.41^*$

Fig. 3. Effect of dexamethasone on induction of cholesterol 7 α -hydroxylase activity in cultured rat hepatocytes

After isolation, a portion of the hepatocytes was washed and frozen in liquid N_2 ($t = 0 \text{ h}$ samples), and the other portion of the cells was seeded on 60 mm diam. tissue culture dishes. For each time point, five dishes were plated. After a 4-h attachment period and every 24 h thereafter, cells were refreshed with control medium or medium containing $1 \mu\text{M}$ -dexamethasone. At 8 h before harvesting, monolayers received a final medium change. Hepatocytes were scraped at the indicated times and cholesterol 7 α -hydroxylase activity was determined as described under Materials and methods. Values shown are means ($\pm$ S.D.) of experiments with hepatocytes from six rats. A significant difference ($P < 0.05$) between control (open bars) and dexamethasone-treated cells (hatched bars) of similar culture age is indicated by *.

bile acid was added to β -muricholic acid before correction for bile acids present in foetal bovine serum (see the Materials and methods section).

Addition of $1 \mu\text{M}$ -dexamethasone significantly increased synthesis of chenodeoxycholic + β -muricholic acids and cholic acid by rat hepatocytes, giving a 2.2-fold rise in total bile acid production during the second day of culture (Table 2). This value, and the finding that there was no change in the ratio of individual bile acids during the second culture period compared well with the data obtained from the [^{14}C]cholesterol conversion measurement.

Conversion of 7 α -hydroxycholesterol into bile acids under the influence of dexamethasone

Since cholesterol 7 α -hydroxylase is the first and rate-limiting enzyme in the bile acid synthesis pathway [1,11,12], the effect of dexamethasone on conversion of its product, 7 α -hydroxycholesterol, into bile acids by rat hepatocyte monolayers was studied. It has been previously shown that exogenous 7 α -hydroxycholesterol is metabolized to bile acids [6,8]. Experiments were performed during various time intervals (from 15 min to 6 h) in the last 6 h of the second 24-h incubation period (46–52 h of culture age), in which bile acid synthesis was induced 2–3-fold by dexamethasone. Addition of $1 \mu\text{M}$ -dexamethasone did not affect conversion of $1 \mu\text{M}$ -[^{14}C]7 α -hydroxycholesterol into bile acids at any time point. After a 6-h incubation, 80 % of the added 7 α -hydroxycholesterol was converted into bile acids by both control and dexamethasone-treated cells.

Effect of dexamethasone on cholesterol 7 α -hydroxylase activity in cultured hepatocytes

The findings that (i) there was no change in the proportions of individual bile acids synthesized by hepatocytes between 28 and 52 h, whereas total bile acid formation was increased with dexamethasone, and that (ii) there was no influence of dexamethasone on the rate of conversion of 7 α -hydroxycholesterol into bile acids, strongly suggest that induction of bile acid synthesis by dexamethasone takes place at the level of cholesterol 7 α -hydroxylase. To confirm these results, cholesterol 7 α -hydroxylase activity was measured in cultured hepatocytes [22] incubated in the presence or absence of $1 \mu\text{M}$ -dexamethasone. In 28-h-old monolayer cultures in the absence of dexamethasone, enzyme activity had declined to 50 % of the initial level in freshly isolated hepatocytes; it then remained constant until 52 h in culture and finally decreased to the limit of detection after 76 h (Fig. 3).

When hepatocytes were exposed to medium containing $1 \mu\text{M}$ -dexamethasone, cholesterol 7 α -hydroxylase activity did not differ from that in control cells after the first 24 h (culture age 28 h). This observation is consistent with the insensitivity of bile acid synthesis to dexamethasone during this period (Fig. 2). On prolonged exposure of the cells to the glucocorticoid, enzyme activity increased briskly, reaching values 10 and 90 times higher than cholesterol 7 α -hydroxylase activity in incubated control cultures after 52 and 76 h respectively. A similar magnitude of induction was obtained after a single addition of $1 \mu\text{M}$ -dexamethasone and with administration of a concentration of 50 nM-dexamethasone to the hepatocyte monolayer cultures, whereas cholesterol 7 α -hydroxylase activity remained unchanged upon addition of up to $10 \mu\text{M}$ -dexamethasone directly into the assay mixture

Table 3. The influence of inhibitors of translation and transcription on induction of bile acid synthesis and cholesterol 7 α -hydroxylase activity

After a 4-h attachment period and 24 h thereafter, hepatocytes were refreshed with control medium or medium containing 1 μ M-dexamethasone. At 28 h of culture age, inhibitors were added in concentrations as indicated. After a further 24 h, cells and medium were harvested. Values shown are means ($\pm$ s.d.) of duplicate incubations with hepatocytes from three rats. * $P < 0.05$ with respect to control incubations without inhibitors; ** $P < 0.05$ with respect to hepatocyte cultures containing 1 μ M-dexamethasone in the absence of inhibitors. N.D., not determined.

Addition	Bile acid synthesis† (d.p.m./24 h per mg)		Cholesterol 7 α -hydroxylase‡ (pmol/h per mg)	
	Control	Dexamethasone	Control	Dexamethasone
None	8830 $\pm$ 3010	32320 $\pm$ 12820	104 $\pm$ 63	1239 $\pm$ 403
5 μ M-Cycloheximide	6090 $\pm$ 1970*	9370 $\pm$ 2260**	20 $\pm$ 7*	26 $\pm$ 23**
1 μ g of α -amanitin/ml	4420 $\pm$ 1410*	4710 $\pm$ 1590**	N.D.	N.D.
1 μ M-Actinomycin D	5920 $\pm$ 1680*	4210 $\pm$ 1430**	23 $\pm$ 8*	38 $\pm$ 19**

† Bile acid synthesis was determined during the 24-h incubation period from 28 to 52 h after plating.

‡ Cholesterol 7 α -hydroxylase activity was measured in cells harvested at $t = 52$ h.

(results not shown). Since the amount of free cholesterol in hepatocytes maintained in the presence of 1 μ M-dexamethasone (16.0 $\pm$ 2.0 μ g/mg of cell protein) did not differ significantly from that in control cells (13.6 $\pm$ 2.6 μ g/mg of cell protein), and since free cholesterol from cells comprised only 33% of total free cholesterol in the enzyme assay [22], the rise in enzyme activity cannot be attributed to differences in substrate availability for the cholesterol 7 α -hydroxylase.

In agreement with the results on bile acid formation from [14 C]cholesterol, no change in cholesterol 7 α -hydroxylase activity at 52 h was detected in hepatocytes cultured from 4 to 52 h in the presence of 10 μ M of each of the steroid hormones other than glucocorticoids, mentioned in Fig. 1 (results not shown).

Effect of inhibitors of protein and RNA synthesis on induction of bile acid synthesis and cholesterol 7 α -hydroxylase activity

To determine whether protein and RNA synthesis were required for the maintenance and the stimulation of bile acid synthesis and cholesterol 7 α -hydroxylase activity, hepatocytes were incubated with inhibitors of protein (cycloheximide) and RNA (actinomycin D and α -amanitin) synthesis. Addition of 5 μ M-cycloheximide, or 1 μ g of α -amanitin/ml or 1 μ M-actinomycin D, significantly reduced bile acid synthesis in control cells to 69, 50 and 67% respectively of the control value without inhibitors (Table 3, first column). Cholesterol 7 α -hydroxylase activity in control hepatocytes incubated with inhibitors was even more potently inhibited (Table 3, third column). However, one should realize that bile acid synthesis is a cumulative process over a 24-h period, whereas measurement of the enzyme activity gives an instantaneous picture at the end of this 24-h period.

Furthermore, the induction of bile acid formation and cholesterol 7 α -hydroxylase activity by dexamethasone was completely prevented after addition of these inhibitors (Table 3, second and fourth columns). These data demonstrate that for maintenance and stimulation of bile acid synthetic capacity and enzyme activity in hepatocytes cultured between 28 and 52 h, translation and transcription were obligatory.

DISCUSSION

The data presented in this paper demonstrate hormonal regulation of bile acid synthesis by modulation of cholesterol 7 α -hydroxylase activity in cultured rat hepatocytes. Bile acid production was measured by (i) determination of conversion of pre-existing radiolabelled cholesterol and (ii) measurement of mass production of bile acids. With both methods a stimulation of bile acid synthesis by dexamethasone was observed, which correlated well with the induction of cholesterol 7 α -hydroxylase activity. The observation that the increase in enzyme activity in the presence of dexamethasone was higher than the rise in bile acid synthesis and the finding that inhibitors of protein and RNA synthesis blocked the 7 α -hydroxylase activity more potently than bile acid production may be explained by the fact that bile acid synthesis was measured over a 24-h period, whereas determination of cholesterol 7 α -hydroxylase activity gives only a reflection of the situation at the end of this incubation period. However, the possibility that in this system *in vitro* part of the bile acid synthesis may take place via alternative pathways, other than via initial 7 α -hydroxylation of cholesterol, as has been reported for rats [28,29], cannot be excluded. This last hypothesis is also supported by the finding that the cholesterol 7 α -hydroxylase activity in control cells at 52 and 76 h is lower than at 0 and 28 h, whereas bile acid synthesis in the third culture period is higher than during the first day, suggesting that the enzyme may not always be rate-limiting. Another explanation in conditions of stimulation might be that other enzymes in the bile acid synthetic pathway or supply of substrate cholesterol may become rate-limiting.

Previously, Graham *et al.* [30] reported an increase in taurocholate production in organ cultures of foetal rat liver after administration of cortisol. Although specific induction of bile acid synthesis cannot be excluded, this incremental rise in taurocholate synthesis might be a result of acceleration of differentiation and general stimulation of maturation in foetal liver cells by cortisol [30,31]. In contrast, no increase in protein synthesis or yield of total cell protein was found in our experiments with adult hepatocytes.

The present results are in good agreement with findings of Everson in immortalized rat liver cells [18]. However, no effect on cholesterol 7 α -hydroxylase activity of cortisol hemisuccinate was observed by Botham & Boyd [19] in suspension cultures of adult rat hepatocytes. The absence of responsiveness to glucocorticoids might be due to the fact that the culture period was too short to detect stimulation, or that hepatocytes were refractory to induction of bile acid synthesis and cholesterol 7 α -hydroxylase activity during the first 28 h of culture (Figs. 2 and 3). Poor hormonal inductions of enzymes including mono-oxygenases [32] in cells, together with catabolic protein turnover and decreased ATP concentration, have been reported as a consequence of membrane damage during hepatocyte isolation [33,34]. This damage, which is almost completely repaired after culturing of cells in monolayers for 24 h [33,34], might explain the absence of induction of bile acid synthesis and cholesterol 7 α -hydroxylase activity in the first 24-h culture period.

Hepatocytes lost cholesterol 7 α -hydroxylase activity (50 %) during culturing for 28 h. The remaining enzyme activity was preserved for the following 24-h culture period, but decreased afterwards in the absence of dexamethasone. Loss of total cytochrome *P*-450 has been well established as one of the phenotypic changes associated with adaptation of hepatocytes to conditions of monolayer culture [34,35]. Since cholesterol 7 α -hydroxylase is a cytochrome *P*-450-dependent enzyme [1,11,12], a similar phenomenon may occur with this enzyme as has already been reported for monolayer [6] and suspension [19] cultures.

The issue has to be addressed of whether the effect of glucocorticoids on total bile acid production and cholesterol 7 α -hydroxylase activity was general in character, since involvement of this class of steroid hormones has been described in maintaining cytochrome *P*-450 content [34,35]. Two lines of evidence argue against this hypothesis. First, cholesterol 7 α -hydroxylase activity is induced significantly above the initial enzyme activity in freshly isolated hepatocytes. Secondly, addition of cycloheximide, which has also been reported to stabilize the mono-oxygenase system *in vitro* [34,35], prevented induction of both parameters by dexamethasone. On the other hand, maintenance and induction of bile acid hydroxylations by glucocorticoids have been demonstrated by Lambiotte & Thierry in rat hepatocytes and rat hepatoma cells [36].

Dexamethasone and pregnenolone 16 α -carbonitrile (PCN) have been shown by Guzelian and co-workers to induce a specific form of cytochrome *P*-450, designated cytochrome *P*-450_{PCN}, *in vivo* and *in vitro* [37,38]. The question arises of whether cholesterol 7 α -hydroxylase is related to this PCN- and dexamethasone-inducible cytochrome *P*-450, or is the enzyme activity regulated via a process involving the classical glucocorticoid receptor pathway [31]? Several findings argue in favour of the latter possibility. First, the concentration of dexamethasone required to achieve half-maximal induction (approx. 10 nM, Fig. 1) agreed closely with the reported dexamethasone concentration that half-saturates binding to the glucocorticoid receptor in rat liver cytosol [39]. Much higher concentrations were required for induction of *P*-450_{PCN} [38]. Furthermore, the time course of induction of cholesterol 7 α -hydroxylase activity (Fig. 3) is divergent from that of cytochrome *P*-450_{PCN} [38]. Finally, in experiments *in vivo* using rats, treatment with PCN

showed an equal or decreased microsomal cholesterol 7 α -hydroxylase activity [40]. We therefore believe that induction of cholesterol 7 α -hydroxylase activity is mediated through the classic glucocorticoid receptor.

In contrast to experiments *in vivo* with ethinyl-oestradiol [20,21], no effect of this steroid was observed on bile acid synthesis or cholesterol 7 α -hydroxylase activity in cultured hepatocytes. The reason for this discrepancy is unknown at this moment.

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