Characterization of the inhibitory effects of bile acids on very-low-density lipoprotein secretion by rat hepatocytes in primary culture

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In this study the effects of bile acids and other organic anions on the secretion of very-low-density lipoproteins (VLDLs) were evaluated in rat hepatocytes in primary culture. Incubation of cells with portal blood concentrations (10–200 μ M) of bile acids resulted in dose-dependent suppression of secretion of VLDL-associated [3 H]triglyceride (TG) formed from [3 H]glycerol, and also of TG mass. The degree of the inhibition was highly correlated with intracellular bile acid concentration. Prolonged incubation with 100 μ M extracellular taurocholic acid (TC) decreased the secretion of [3 H]TG and TG mass to 35% and 50% of the controls respectively. Cellular content of mass and of [3 H]TG during prolonged incubation with TC were about 20% and 60% higher than the controls respectively. The inhibitory effect remained for at least 24 h in the presence of TC without altering VLDL-lipid and VLDL-apolipoprotein compositions or the size distribution of the particles. Secretion of apoB-100 and

of apoB-48 was inhibited to a similar extent. Cells largely lost their capacity to accumulate bile acids intracellularly after 48 h in culture. In these cells TC was unable to exert its suppressive effects. Taurine and glycine conjugates of all common bile acids were capable of suppressing [3 H]TG secretion. Trihydroxylated (cholic acid) and various dihydroxylated (deoxycholic, chenodeoxycholic and ursodeoxycholic acids) bile acids had similar capacities in this respect, suggesting that their common sterol-3 α -OH structure is required for the suppressive effect. Neither non-bile acid organic anions, e.g. bilirubin ditaurate and dibromosulphthalein, nor dianionic bile acid metabolites, e.g. sulphated tauro lithocholic acid and lithocholate-3-*O*-glucuronide, showed any effect on [3 H]TG secretion. These results indicate that bile acids might play a physiological role in regulating VLDL production by the liver, especially in the postprandial state when their enterohepatic circulation is stimulated.

INTRODUCTION

From a number of clinical studies it seems that bile acids have a role in the regulation of triglyceride (TG) metabolism. Treatment of patients with bile acid sequestrants (see, for example, [1]) and interruption of the enterohepatic circulation by ileal resection (see, for example, [2]) are often associated with raised plasma TG levels. Conversely, administration of cholic acid or chenodeoxycholic acid (CDC), which increases bile acid pool sizes and decreases hepatic bile acid synthesis, results in decreased levels of plasma TG in patients with type II or IV hyperlipoproteinaemia [3]. Similar phenomena have been observed in animal experiments. An increase in plasma TG has been reported in the cholestyramine-fed rat [4]. In rhesus monkeys, partial diversion of enterohepatic circulation led to an increase in plasma TG that could be prevented by intraduodenal replacement of taurocholic acid (TC) [5]. The increase in plasma TG level after bile acid withdrawal could be caused by increased very-low-density lipoprotein (VLDL)-TG production, decreased TG metabolism in plasma or altered VLDL composition. Recent studies from our laboratory with a primary culture of human hepatocytes demonstrated that TC is able to suppress hepatic VLDL secretion directly [6]. The mechanism behind this action is not yet understood.

It is well established that individual bile acids have different potentials for regulation of hepatic cholesterol synthesis [7] and biliary lipid secretion [8]. However, no information is available about the effects of individual bile acids on hepatic VLDL secretion. The present study was designed to compare and to quantify the effects of naturally occurring and synthetic bile acids on the secretion of VLDL by rat hepatocytes in primary culture.

The specificity of the bile acid effects was evaluated by comparing them with those of non-bile acid organic anions, which, *in vivo*, are actively secreted into bile.

MATERIALS AND METHODS

Materials

All bile acids, as sodium salts, and rat albumin were obtained from Calbiochem (La Jolla, CA, U.S.A.). Williams' E medium, glutamine, penicillin-streptomycin, fetal calf serum (FCS) and collagenase were purchased from Life Technologies Ltd., Paisley, Scotland, U.K. Insulin and dexamethasone came from Novo Nordisk Pharma B.V. (Amsterdam, The Netherlands). Hydrated colloidal silica (Cab-O-Sil) (fumed silica, particle size 0.011 μ m) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [2 - 3 H]Glycerol (1 Ci/mmol), L-[4,5- 3 H]leucine (61 Ci/mmol) and 14 C-labelled bile acids were obtained from Amersham International (Amersham, Bucks., U.K.). Cell culture plates and flasks were supplied by Costar (Cambridge, MA, U.S.A.). TLC plates were obtained from E. Merck (Darmstadt, Germany). Triglycerides GPO-PAP Kit was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Goat antiplasma to rat albumin, peroxidase-conjugated rabbit IgG fraction against rat albumin, and purified rat albumin were supplied by Organon Teknika and Cappel (Turnhout, Belgium). Molecular mass markers were obtained from BDH Laboratory Supplies (Poole, Dorset, U.K.). All other chemicals and solvents were high-purity commercial materials.

Rat hepatocytes were isolated by the method of Berry and Friend [9] from male Wistar rats of body weight 180–220 g. Rats were maintained in a light- and temperature-controlled room

Abbreviations used: CDC, chenodeoxycholic acid; DBSP, dibromosulphthalein; FCS, fetal calf serum; LOG, lithocholic acid-3-*O*-glucuronide; TC, taurocholic acid; TG, triglyceride; UDC, ursodeoxycholic acid; VLDL, very-low-density lipoprotein.

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and fed with standard lab chow and water *ad lib.* until killed. The viability of hepatocytes after isolation was above 90 % as examined by Trypan Blue exclusion (final concentration 0.2 %). Cells were plated in 35 mm six-well plastic dishes at a density of 1.5×10^6 viable cells per well in 2 ml of culture medium. These cell culture dishes were precoated with collagen by adding 1 ml of 0.2 % collagen R to each well and drying.

The preculture medium consisted of Williams' E medium supplemented with 10 % (v/v) FCS, 2 mM glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, 4 m-units/ml insulin and 0.02 µg/ml dexamethasone as described by Princen et al. [10]. After cells had attached to the plates, usually after 4 h, medium was renewed. Cells were incubated at 37 °C in an atmosphere of air/CO₂ (19:1).

Experimental protocol

Because initial experiments had shown that the cell's ability to concentrate bile acids declined rapidly during cell culture, experiments were conducted in cells with an overnight (about 14 h) preculture. BSA–oleic acid complexes (1 mM:0.25 mM) were prepared on the day of an experiment as described previously [11]. Experimental media were warmed to 37 °C before use. The viability of cells was monitored by the leakage of lactate dehydrogenase (EC 1.1.1.27) (routinely less than 7 %) from cells.

As insulin is known to inhibit VLDL secretion [12], FCS-hormone-containing medium was removed from the wells and cells were washed twice with FCS-hormone-free medium and incubated in this medium for 4 h to eliminate the effects of insulin. Then the medium was changed to experimental media (1 ml per well): BSA–oleic acid complex-containing, FCS-hormone-free medium with or without (control) addition of bile acids, or non-bile acid organic anions at various concentrations. Cells were incubated for 1–24 h. To determine the glycerolipid synthesis and secretion rates at indicated times, 10 µl of a solution of [³H]glycerol in water (4.4 µCi, 25 µM) was added to each well 3 h before termination of the experiments. All incubations were performed in triplicate and terminated by placing the culture plates on ice and collection of the media. Media were centrifuged at 10000 g for 2 min to remove suspended cells. Cells were washed twice with PBS and harvested by using a rubber 'policeman' in 2 ml of PBS. Samples were frozen at –20 °C before analysis.

Assessment of cellular lipid content

Cells were thawed and resuspended by passing five times through needles (0.45 mm × 25 mm). The cellular lipids were extracted with chloroform/methanol (1:2, v/v) [13]. TG, cholesterol, cholesteryl ester and phospholipid were separated by TLC with hexane/diethyl ether/acetic acid (80:20:1, by vol.) as developing solvent. After staining with iodine, the spots containing the lipids of interest were scraped into vials and the incorporation of [³H]glycerol into individual lipids was assayed for radioactivity by scintillation counting. Cellular TG mass was assayed by using Triglycerides GPO-PAP Kit in accordance with the manufacturer's instructions, with glycerol as standard. Cellular cholesterol and cholesteryl ester masses were determined by the methods developed by Gamble et al. [14]. Phospholipids were determined by measuring phosphorus content after treatment with perchloric acid [15].

Assessment of the secretion of [³H]TG and TG mass

[³H]TG in medium has been used extensively to estimate VLDL-[³H]TG secretion by primary cultured rat hepatocytes (see, for

example, [16,17]) because more than 95 % of [³H]TG secreted into the medium of cultured rat hepatocytes was recovered in the VLDL fraction ($d < 1.006$ g/ml). In the present studies we analysed [³H]TG secretion as a reflection of hepatocytic VLDL formation. Before medium lipids were extracted, 30 µl of 2 mM TG solution in chloroform was added to each sample as a carrier. TG was separated from other lipids by TLC in a similar procedure to that for the cellular lipids. Silica gel containing TG was scraped into vials and assayed for radioactivity by scintillation counting. In some experiments, indicated in the Results section, the TG mass in the medium was also determined. In this case, lipids were extracted from the medium exposed to cells cultured in a 75 cm² flask. TG mass was determined by a method similar to that for cellular TG mass as described above.

Assessment of mass of VLDL-associated apolipoprotein and lipids

To further investigate the components of VLDL secreted into the medium of specific experiments, VLDL particles were freshly isolated from the $d < 1.006$ g/ml fraction of 12 ml of conditioned medium exposed to hepatocytes for 24 h, by centrifuging for 24 h at 280000 g in a Beckman SW41 rotor. Floating VLDL particles were absorbed on Cab-O-Sil, and VLDL-associated lipids and apolipoproteins were extracted by the methods described by Vance et al. [18]. Briefly, lipids were extracted with chloroform/methanol (2:1, v/v) and apolipoproteins were extracted with buffer containing 2 % (w/v) SDS, 0.05 M Tris, pH 9.0, 6 M urea, 0.1 % EDTA, 0.1 % dithiothreitol, 0.13 % ϵ -aminohexanoic acid and 0.05 % glutathione at 95 °C for 10 min. After extraction, VLDL-associated lipids were quantified by methods identical with those used for cellular lipid as described above. Apolipoproteins were separated by electrophoresis in 3–15 % (w/v) gradient polyacrylamide gels containing 0.1 % SDS. The gels were subsequently stained with Coomassie Blue. Quantification of the bands was performed with an UltroScan XL Enhanced Laser Densitometer.

Electron microscopy

VLDL particles were isolated from the same conditioned medium by ultracentrifugation as mentioned above. The $d > 1.006$ g/ml fractions were fixed for electron microscopy within 1 h. The particles were allowed to adhere to hydrophilic carbon films and immersed in 2 % (w/v) potassium phosphotungstate, pH 7.4, as a negative stain [19]. Electron micrographs were obtained with a Philips EM208 transmission electron microscope. The size of VLDL particles was determined with Quantimet 520+ software (Leica, Cambridge, U.K.); at least 500 particles were evaluated for each condition.

Assessment of albumin secretion

The amount of albumin secreted by rat hepatocytes into the medium was estimated by sandwich ELISA. Microplates were precoated with monoclonal anti-rat-albumin antibody in PBS by incubating overnight at 4 °C. The buffer containing the unbound antibodies was drained from the plate and the wells were washed four times with PBS containing 0.05 % Tween-20. The unbound sites on the wells were blocked by incubation of 200 µl of a block solution [2 % (w/v) milk powder in PBS] in each well for 2 h at room temperature. The wells were then washed as described above. Rat albumin standard was diluted with PBS buffer (buffer A) containing 0.05 % Tween-20 and 0.1 % milk powder in concentrations ranging from 0 to 5 ng per 100 µl. An aliquot (100 µl) of these rat albumin standards was added to each well in duplicate. Samples of media were diluted (1:20 to 1:200) with

buffer A and added to the wells in duplicate, so that the resulting albumin concentrations fell within the standard curve. The plate was incubated for 1 h at room temperature. The well was then washed as described above. Buffer A (200 μ l) containing polyclonal anti-rat-albumin antibody conjugated with horseradish peroxidase was added to each well and incubated for 1 h, and the wells were washed again. Colour development was started by adding 100 μ l of substrate solution prepared freshly [3,3',5,5'-tetramethylbenzidine (0.2 mg/ml) and H_2O_2 (0.3 mg/ml) in 0.1 M Na_2HPO_4 and citric acid buffer, pH 4.3]. The reaction was stopped by adding 100 μ l of 1 M H_2SO_4 , after which the absorbance was determined at 450 nm with a Titertek Multiscan MCC/340 plate reader.

Miscellaneous methods

Protein was measured by the method of Lowry [20] with BSA as standard. Lactate dehydrogenase activity in media and cells was determined as reported previously [11]. [^3H]Leucine incorporation into the trichloroacetic acid-precipitable proteins was investigated to determine total protein synthesis. Hepatocytes were cultured for 3 h in the experimental medium supplied with [^3H]leucine (2.5 $\mu\text{Ci}/\text{ml}$ per well). Proteins contained in cell and medium were purified by repeating precipitation with trichloroacetic acid and resuspension. ^3H -Labelled proteins were determined by scintillation counting. Cellular total bilirubin was assessed by the method of Novros et al. [21]. Cellular dibromosulphthalein (DBSP) was determined by diluting cell suspension with NaOH (final concentration 0.1 M) and measuring spectrophotometrically at 580 nm.

Calculations and statistical analysis

Data were normalized to the amount of cellular protein. Statistical differences were assessed using a two-way analysis of variance (for multi-group comparisons) or a paired Student's *t*-test (for comparing two groups). Values of $P < 0.05$ (two-tailed) were considered significant. Regression analysis was performed by a least-squares method.

RESULTS

The effects of cell culture time on TC uptake and TG secretion by rat hepatocytes

When investigating the effects of bile acids on hepatic VLDL secretion, factors that might influence the processes of bile acid

Figure 1 Intracellular accumulation of TC in rat hepatocytes as a function of extracellular TC concentration

Hepatocytes were incubated with the indicated extracellular concentration of unlabelled TC with [^{14}C]TC (0.1 $\mu\text{Ci}/\text{ml}$) for 3 h. Intracellular accumulation of TC was calculated from intracellular [^{14}C]TC content, which was determined by scintillation counting. Points represent means $\pm$ S.D. for at least three independent experiments, each experiment performed in triplicate.

uptake and VLDL secretion by hepatocytes have to be considered. It has been reported for rat hepatocytes that the uptake capacity for TC declines during cell culture [22]. Therefore our initial studies addressed the influence of preculture time on TC uptake and [^3H]TG secretion (Table 1). The results show that the TC uptake and basal [^3H]TG secretion (control) by hepatocytes precultured for 6 or 24 h were similar. In both situations TC was able to suppress [^3H]TG secretion by approx. 50 % in comparison with parallel control incubations. After 48 h of preincubation, intracellular accumulation of TC declined by 81 % from the value for 6 h of preincubation. In parallel with this phenomenon, inhibition of secretion of [^3H]TG declined. In addition, [^3H]TG secretion (48 h control) decreased by 37 % from the value for 6 h of preincubation. The reason(s) for this decline has not yet been established. On the basis of these results, subsequent experiments were routinely performed with cells precultured overnight (approx. 14 h).

Additional experiments were conducted with hepatocytes incubated with TC for 3 h to determine the relationship between extracellular TC concentration and intracellular TC content. The results (Figure 1) show that the accumulation of TC reached a plateau at an extracellular TC concentration of 100 μM , at a

Table 1 Effects of preculture time on cellular [^3H]TG content, [^3H]TG secretion and TC uptake by hepatocytes incubated in the absence or presence of 100 μM TC

Rat hepatocytes were precultured for various periods as indicated, before incubation experiments were performed. For [^3H]TG secretion experiments, hepatocytes were incubated in the absence (control) or presence of 100 μM TC for 3 h, and [^3H]glycerol (4.4 $\mu\text{Ci}/\text{ml}$, 25 μM final concentration) was added to the media to monitor [^3H]TG secretion. Lipids in the medium were extracted and purified by TLC. [^3H]TG was determined by scintillation counting. For TC uptake experiments, cells were incubated in medium with 100 μM [^{14}C]TC (0.1 $\mu\text{Ci}/100 \mu\text{M}$) for 3 h. Intracellular accumulation of TC was calculated from intracellular [^{14}C]TC content, which was determined by scintillation counting. Data represent the range of two independent determinations for TC uptake, and the means $\pm$ S.D. for three determinations for [^3H]TG secretion, conducted in a representative experiment. Statistical significance: *, [^3H]TG secretion is significantly different from parallel control incubations ($P < 0.01$); †, [^3H]TG secretion is significantly different from that in 6 or 24 h of incubation ($P < 0.05$).

Time (h)	$10^{-4} \times$ Cellular [^3H]TG (d.p.m./mg)		$10^{-4} \times$ Medium [^3H]TG (d.p.m./mg)		Cellular TC (nmol/mg)
	Control	TC	Control	TC	
6	111.2 $\pm$ 25.1	134.9 $\pm$ 12.7	2.1 $\pm$ 0.1	1.2 $\pm$ 0.1*	15.5–17.6
24	151.6 $\pm$ 26.2	163.1 $\pm$ 17.4	1.9 $\pm$ 0.1	0.8 $\pm$ 0.1*	16.5–18.4
48	130.9 $\pm$ 17.3	149.6 $\pm$ 25.2	1.3 $\pm$ 0.1†	1.2 $\pm$ 0.1	3.0–3.4

Table 2 Effects of TC on hepatocellular content and secretion of [³H]TG and TG mass

Rat hepatocytes were incubated with the indicated concentrations of TC and [³H]glycerol (4.4 μ Ci/ml; 25 μ M final concentration) for 3 h. Lipids in the medium were extracted and purified by TLC. [³H]TG was determined by scintillation counting. The mass of TG was measured by using Triglycerides GPO-PAP Kit in accordance with the manufacturer's instructions. Values are shown as a percentage of the control. Data represent means $\pm$ S.D. for at least three independent experiments with triplicate incubations. The significant differences of the values from those of control are indicated with * ($P < 0.05$) and ** ($P < 0.01$). ND, not determined.

TC (μ M)	Medium [³ H]TG (% of control)	Medium TG mass (% of control)	Cell [³ H]TG (% of control)	Cell TG mass (% of control)
10	85.7 $\pm$ 18.5	ND	100.0 $\pm$ 5.1	97.2 $\pm$ 19.8
25	88.7 $\pm$ 4.0	ND	127.0 $\pm$ 5.0*	109.3 $\pm$ 10.4
50	70.1 $\pm$ 8.1*	69.2 $\pm$ 3.1**	136.2 $\pm$ 8.1*	97.4 $\pm$ 11.2
100	34.2 $\pm$ 10.1*	52.1 $\pm$ 7.1**	138.9 $\pm$ 27.6	110.8 $\pm$ 17.8
200	28.4 $\pm$ 8.2**	41.0 $\pm$ 4.3**	123.2 $\pm$ 28.5	98.7 $\pm$ 8.4

Figure 2 Relationship between intracellular TC content and changes in [³H]TG secretion

Rat hepatocytes were incubated as described in the caption to Table 2. Each symbol represents the mean $\pm$ S.D. for at least three independent experiments with triplicate incubations. The regression line is described by $y = -4.76x + 101.66$ ($r = 0.985$, $P < 0.01$).

level of about 15 nmol per mg of cell protein. This value is similar to that reported by Kwekkeboom et al. [22].

The effects of TC on VLDL-associated TG secretion

The relationship between extracellular bile acid concentration and synthesis and secretion of [³H]TG was studied with 3 h incubations. The results are shown in Table 2. Cells incubated with TC showed a nonlinear, dose-dependent decrease in secretion of both TG mass and [³H]TG. The cell-associated [³H]TG concentration in the presence of TC at concentrations exceeding 50 μ M was approx. 20% higher than in the control. As our initial experiments (Table 1) indicated that TC affects VLDL secretion only after uptake by the cells, a regression analysis between intracellular [³H]TC concentrations and [³H]TG secretion was conducted (Figure 2). The results show that there is a strong negative relationship between [³H]TG secretion and intracellular TC content ($y = -4.76x + 101.66$, $r = 0.985$,

Table 3 Time course of the effects of TC on cellular content and secretion of [³H]TG and TG mass

Rat hepatocytes were incubated with 100 μ M TC for various periods as indicated. [³H]Glycerol (4.4 μ Ci/ml; 25 μ M final concentration) was added 3 h before termination of the experiments (except in the 1 h incubation experiment). Values are shown as a percentage of the parallel control. Data represent means $\pm$ S.D. for at least three independent experiments with triplicate incubations. The significant differences of the values from those of parallel controls are indicated with * ($P < 0.05$) or ** ($P < 0.01$). ND, not determined.

Time (h)	Medium [³ H]TG (% of control)	Medium TG mass (% of control)	Cell [³ H]TG (% of control)	Cell TG mass (% of control)
1	47.9 $\pm$ 15.9*	ND	128.2 $\pm$ 22.4	107.1 $\pm$ 2.9
3	34.2 $\pm$ 10.1**	52.1 $\pm$ 7.1**	136.1 $\pm$ 30.9	110.1 $\pm$ 17.8
8	34.4 $\pm$ 5.9**	53.8 $\pm$ 3.8**	155.1 $\pm$ 13.1*	137.7 $\pm$ 7.6*
24	35.5 $\pm$ 4.4**	49.8 $\pm$ 2.6**	150.2 $\pm$ 13.7*	124.2 $\pm$ 6.5*

Table 4 Lipid composition of VLDL particles isolated from media of cells incubated with or without taurocholic acid

Cells were cultured for 24 h in FCS-hormone-free medium supplemented with 1 mM organic anions and in the presence or absence (control) of 100 μ M TC. VLDLs were isolated from these media ($d < 1.006$) by ultracentrifugation (at 280 000 g in a SW 41 rotor for 24 h). VLDL-lipids were extracted with chloroform/methanol and assayed as described in the Materials and methods section. Data represent the means $\pm$ S.D. for three independent VLDL isolations, and are given in units of nmol/mg per 24 h; results in parentheses are percentages of total lipids. No statistical significance was obtained when the ratio of lipids was compared between the two groups ($P < 0.10$). Abbreviations used: CH, cholesterol; CHE, cholesteryl ester; PL, phospholipid.

	TG	CH	CHE	PL
Control	18.8 $\pm$ 3.2 (73.7 $\pm$ 4.8)	2.8 $\pm$ 0.8 (10.7 $\pm$ 0.9)	1.6 $\pm$ 0.9 (6.1 $\pm$ 2.4)	2.5 $\pm$ 0.9 (9.5 $\pm$ 1.8)
100 μ M TC	9.4 $\pm$ 0.5 (68.3 $\pm$ 4.9)	1.9 $\pm$ 0.4 (13.5 $\pm$ 2.2)	1.2 $\pm$ 0.3 (8.5 $\pm$ 2.0)	1.3 $\pm$ 0.2 (9.8 $\pm$ 1.0)

$P < 0.01$); 50% inhibition is reached at an intracellular TC concentration of approx. 10.8 nmol/mg.

The relationship between cellular TC accumulation and secretion of TG was further evaluated during a time-course experiment in which cells were incubated with 100 μ M TC for 1–24 h with parallel controls. Table 3 shows that suppression of the secretion of both TG mass and [³H]TG by TC was maximal at about 3 h of incubation. The suppression of TG secretion was maintained at this level for at least 24 h. Hepatocytes treated with TC accumulated TG mass and [³H]TG progressively.

The effects of TC on the size and composition of VLDL

Transmission electron microscopy was performed on VLDL particles ($d < 1.006$ g/ml) isolated from media exposed to hepatocytes for 24 h. The VLDL particles isolated from cell culture media were similar in size to those isolated from fasted rat plasma. The size distribution of the VLDL particles isolated from the medium of cells exposed to 100 μ M TC was comparable to that of control incubations (39.3 $\pm$ 14.3 nm compared with 41.3 $\pm$ 14.9 nm, mean diameter $\pm$ S.D.). No significant differences in relative lipid composition were observed between the two groups, whereas a 50% decrease in total amount of VLDL-associated lipids was seen (Table 4) with a simultaneous 45% decrease in both apoB-100 and apoB-48 content in the TC group (results not shown).

Figure 3 The effects of TC on hepatocytic secretion of albumin

The experiments were the same as those described in Table 2. The amount of albumin secreted by rat hepatocytes into the medium was estimated by sandwich ELISA as described in the Materials and methods section. Points represent means $\pm$ S.D. for three independent experiments, each experiment performed in triplicate. Statistical significance: *, $P < 0.05$ compared with no supplement of TC.

The effect of bile acids on albumin and [^3H]protein secretion

To investigate the specificity of the effect of bile acids on protein secretion by rat hepatocytes, both albumin and [^3H]leucine-labelled proteins secreted into the medium were measured. In contrast with the suppression of apoB secretion by approx. 45 %, albumin secretion was maximally suppressed by 23 % in the cells incubated with TC (Figure 3). At an extracellular concentration of 50 μM , maximal suppression of albumin secretion was reached. Other taurine-conjugated bile acid species also suppressed albumin secretion to a similar extent (results not shown). The incorporation of [^3H]leucine into cellular protein and secretion of labelled protein by rat hepatocytes were unaltered, as summarized in Table 5.

The effects of individual bile acids on VLDL secretion

Subsequent studies were performed with various bile acid species as well as with some non-bile acid organic anions to determine whether a specific structural fraction or charge of bile acids is responsible for their inhibitory effect on VLDL secretion. The results show that trihydroxylated (i.e. TC) and dihydroxylated [i.e. ursodeoxycholic acid (UDC), deoxycholic acid and CDC]

Table 6 Effects of differently structured bile acids and non-bile acid organic anions on hepatocytic secretion of [^3H]TG

Hepatocytes were incubated in medium with or without (control) addition of the indicated bile acids or organic anions. [^3H]Glycerol was added to the medium (4.4 $\mu\text{Ci}/\text{ml}$, 25 μM final concentration) to quantify lipid secretion. Lipids in the medium were extracted and purified by TLC. [^3H]TG was determined by scintillation counting. Data represent the means $\pm$ S.D. for at least three independent experiments with triplicate incubations. The absolute values of [^3H]TG secretion by control cells were varied between 13471 and 29611 d.p.m. per mg of cell proteins in different cell preparations. Significant differences from control are marked with * ($P < 0.05$) or ** ($P < 0.01$). Abbreviations used: C, cholic acid; GC, glycocholic acid; TCDC, taurochenodeoxycholic acid; DC, deoxycholic acid; TDC, taurodeoxycholic acid; GUDC, glyco-ursodeoxycholic acid; TUDC, tauroursodeoxycholic acid; DHC, dehydrocholic acid; TDHC, taurodehydrocholic acid; STLC, sulphated tauroolithocholic acid; BDT, bilirubin ditaurate; DBSP, dibromosulphthalein.

Compound	Medium [^3H]TG (% of control)	Compound	Medium [^3H]TG (% of control)
C	67.8 $\pm$ 12.7*	GUDC	43.0 $\pm$ 2.3**
GC	38.8 $\pm$ 5.7**	TUDC	53.7 $\pm$ 5.9**
TC	25.4 $\pm$ 6.1**	DHC	94.8 $\pm$ 4.3
CDC	66.2 $\pm$ 4.2**	TDHC	53.4 $\pm$ 20.1*
TCDC	30.8 $\pm$ 13.1*	LOG	90.5 $\pm$ 10.6
DC	58.9 $\pm$ 14.8*	STLC	76.5 $\pm$ 9.7
TDC	30.7 $\pm$ 6.5**	BDT	91.2 $\pm$ 4.5
UDC	72.5 $\pm$ 3.6**	DBSP	93.9 $\pm$ 3.0

have a stronger potency than the sulphated and glucuronidated compounds [sulphated tauroolithocholic acid and lithocholic acid-3-*O*-glucuronide (LOG)] in suppression of [^3H]TG secretion (Table 6). Conjugated bile acids show a greater ability to suppress [^3H]TG secretion than their unconjugated counterparts. This may reflect a lower uptake efficiency of the unconjugated species, as they are rapidly conjugated to taurine after their uptake [23]. No attempts were made to quantify bile acid conjugation in these studies. In addition it should be stressed that rat liver rapidly hydroxylates dihydroxy bile acids to muricholic acids [24]. As the inhibitory potency of all compounds was similar, no attempts were made to identify these metabolites, which all share the 3- α -OH structure. As equilibrium intracellular bile acid concentrations varied widely between various bile acid species, a clear inverse relationship between intracellular bile acid content and [^3H]TG production ($y = -4.72x + 87.84$, $r = 0.823$, $P < 0.01$) could be demonstrated (Figure 4). According to this equation, 50 % suppression of [^3H]TG secretion corresponds to 8.02 nmol/mg intracellular bile acid content, which is similar to

Table 5 Protein synthesis and secretion by rat hepatocytes incubated with bile acids

Rat hepatocytes were incubated for 3 h in FCS-hormone-free medium supplemented with 1 mM oleic acid in the presence or absence of the indicated bile acids. ^3H -labelled protein synthesis and secretion were estimated by determining [^3H]leucine (2.5 $\mu\text{Ci}/\text{ml}$) incorporation into trichloroacetic acid-precipitable cellular and medium proteins respectively, which were quantified by scintillation counting. Data are expressed as means $\pm$ S.D. for at least three independent experiments, with each experiment performed in triplicate. No statistically significant differences were observed between control and bile acid-treated cells. Abbreviation used: TCDC, taurochenodeoxycholic acid.

	Protein in cells		Protein in medium	
	(d.p.m./mg)	(% of control)	(d.p.m./mg)	(% of control)
Control	50 357 $\pm$ 13 630	100.0	16 445 $\pm$ 1849	100.0
100 μM TC	46 580 $\pm$ 692	92.5	17 450 $\pm$ 1721	106.1
200 μM TC	49 702 $\pm$ 7554	98.7	16 369 $\pm$ 2467	99.5
100 μM TCDC	57 407 $\pm$ 6043	114.0	18 747 $\pm$ 1973	114.0
100 μM LOG	42 300 $\pm$ 6849	84.0	17 103 $\pm$ 5098	104.0

Figure 4 Relationship between intracellular bile acid (solid symbols) or organic anion (open symbols) content and change in $[^3\text{H}]\text{TG}$ secretion

Rat hepatocytes were incubated as described in Table 6. Data shown are from three independent experiments. Each symbol represents the mean of triplicate incubations per experiment. The regression line for bile acids (solid line) is described by $y = -4.72x + 87.84$ and the correlation between intracellular bile acid content and the suppression of $[^3\text{H}]\text{TG}$ secretion is highly significant ($r = 0.823$, $P < 0.01$). The correlation between cellular organic anion content and $[^3\text{H}]\text{TG}$ secretion (broken line) is not statistically significant ($P > 0.10$).

Table 7 Effects of various bile acids and DBSP on cellular lipid content

Hepatocytes were incubated in the medium with the indicated compounds at $100 \mu\text{M}$ or without them (control) for 3 h. Cellular lipids were extracted and quantified as described in the Materials and methods section. Data represent means $\pm$ S.D. for three independent experiments with triplicate incubations, and are in nmol per mg of cell protein. There were no statistically significant differences between the parameters across these groups. Abbreviations used: CH, cholesterol; CHE, cholesteryl ester; PL, phospholipid.

Compound	TG	CH	CHE	PL
Control	146 $\pm$ 14	27 $\pm$ 3	9 $\pm$ 2	199 $\pm$ 17
TC	160 $\pm$ 18	26 $\pm$ 3	10 $\pm$ 5	180 $\pm$ 12
TCDC	150 $\pm$ 13	24 $\pm$ 3	13 $\pm$ 4	224 $\pm$ 24
TDH	138 $\pm$ 10	25 $\pm$ 4	13 $\pm$ 2	172 $\pm$ 18
DBSP	138 $\pm$ 14	28 $\pm$ 4	10 $\pm$ 3	195 $\pm$ 22

that obtained from the incubation with various extracellular TC concentrations (Figure 2). Non-bile acid organic anions (e.g. bilirubin ditaurate and DBSP) did not affect VLDL secretion, in spite of the fact that the intracellular content of DBSP was as high as 7.5 ± 0.7 nmol per mg of cell protein. This indicates that the inhibition of VLDL secretion represents a specific bile acid effect.

As availability of VLDL-lipid constituents may be limiting for VLDL production, cellular lipid content was determined in a number of experiments. Table 7 shows that there were no significant differences in intracellular contents of TG, cholesterol, cholesteryl ester and phospholipid between incubations with TC, taurochenodeoxycholic acid (TCDC), TDH and DBSP, compared with control incubations. In addition, no decreases in the cellular amounts of $[^3\text{H}]\text{phospholipid}$ formed from $[^3\text{H}]\text{glycerol}$ and of $[^{14}\text{C}]\text{cholesterol}$ and $[^{14}\text{C}]\text{cholesteryl ester}$ synthesized from $[^{14}\text{C}]\text{acetate}$ were observed during 3 h incubations with these compounds (results not shown).

DISCUSSION

The role of bile acids in lipid absorption and bile formation is well established, but their potential role in regulation of hepatic VLDL production is still poorly understood. The present study was performed to characterize the effects of bile acids on VLDL secretion by rat hepatocytes in primary culture. Our results show that TC, the predominant bile acid species in humans and rats, suppresses VLDL-associated TG secretion at a concentration as low as $50 \mu\text{M}$. The maximal suppression of VLDL secretion by TC was observed at an extracellular concentration of approx. $100 \mu\text{M}$, which is in the range of physiological portal bile acid concentrations. Portal blood bile acid concentrations as high as $175 \mu\text{M}$ has been reported in the postprandial condition in rats [25]. Only scant information is available on the postprandial portal bile acid concentrations in humans. In fasting patients undergoing cholecystectomy, the concentration of bile acids in portal venous blood was $22.2 \pm 5.1 \mu\text{M}$ [26]. In the postprandial state, peripheral blood bile acid concentrations may increase 2–5-fold [27,28]. Assuming that a similar increase occurs in portal blood, a concentration of $100 \mu\text{M}$ will be reached under this condition. The present results lead us to hypothesize that the enterohepatic circulation of bile acids plays a physiological role in the regulation of plasma lipoprotein level by inhibiting VLDL production.

TC did not alter the size and composition of medium VLDL particles (Table 4). This observation implies that the observed decrease in VLDL-associated $[^3\text{H}]\text{TG}$ and TG mass is a result of a decrease in the number of VLDL particles secreted, suggesting that bile acids interfere either with the number of VLDL particles assembled intracellularly or with the efficiency of VLDL secretion.

The degree of the suppressive effect is related to the intracellular bile acid concentration, as appears from the a strong negative correlation between intracellular bile acid content and $[^3\text{H}]\text{TG}$ secretion (Figures 2 and 4) and from the fact that TC does not exert inhibitory effects in cells that have largely lost the capacity for bile acid uptake (Table 1). The greater inhibitory potency of conjugated (with taurine or glycine) bile acids in comparison with their unconjugated counterparts might be explained at least partly by a more efficient carrier-mediated hepatic uptake of the former. Several studies have demonstrated bile acid accumulation in endoplasmic reticulum and Golgi (see, for example, [29]) where VLDL is known to be assembled. This allows us to hypothesize that the effect of bile acids is mediated via interference of intracellular bile acids with VLDL assembly, its transport routing towards secretion, or its degradation. The concept that the VLDL secretion pathway is influenced by bile acids is corroborated by the observation that bile acids decrease albumin secretion, a finding recently also reported in cultured human hepatocytes [30]. It has been suggested that albumin is secreted along a pathway similar to that of VLDL, and both apoB and albumin have high-affinity bile acid binding sites, as demonstrated *in vitro* [31,32]. The difference between albumin secretion (-23%) and total protein secretion (no change) under the influence of TC can be explained by the fact that the amount of albumin accounts for only one-third of the total secreted protein in cultured rat hepatocytes [33] and that the decrease may be too small to be detected in total protein secretion. Alternatively, it may be that the decrease in albumin secretion is compensated for by an increase of other secretory proteins, possibly stimulated by bile acids [34].

Our results demonstrate that a number of bile acid species are able to suppress VLDL secretion. This potency did not correlate with the degree of hydrophobicity of the bile acids: UDC and

CDC showed similar suppressive capacities, in spite of their large difference in hydrophobicity. However, it should be realized, as mentioned in the Results section, that CDC is rapidly converted to muricholic acid by rat hepatocytes, thereby giving rise to a very hydrophilic species. In any case, our results demonstrate that all bile acids with a sterol-3 α -OH structure are able to suppress VLDL secretion. DBSP and bilirubin ditaurate, which are non-bile acid organic anions, had no effect on VLDL secretion. Therefore the present results suggest that the common sterol-3 α -OH structure of bile acids and not their anionic character is related to inhibitory effects on VLDL secretion. Bile acids whose sterol-3 α -OH group is modified by sulphate (e.g. sulphated tauroolithocholic acid) or glucuronide (e.g. LOG) showed no inhibitory effect (Table 6), although this phenomenon might also be explained by a lower intracellular accumulation (2.5 nmol/mg; Figure 4) of LOG.

The results of clinical studies demonstrated that CDC decreases plasma TG concentration more markedly than cholic acid [35] and UDC [3,36] do. These results *in vivo* differ from our studies *in vitro* on rat hepatocytes, in which the various bile acids have similar potencies in suppressing VLDL secretion. The differences between results *in vivo* (human) and *in vitro* (rat hepatocytes) might be caused by species-specific factors in bile acid metabolism or by differences in intestinal bile acid absorption *in vivo*. CDC is more readily absorbed than cholic acid and UDC in the upper small intestine [37]. In the postprandial state, the concentration of TCDC in blood increases more rapidly and to a greater extent than that of TC [27,37].

Hepatic secretion of VLDL has been shown to be influenced by the cellular availability of TG, cholesterol, cholesteryl ester and newly synthesized phosphatidylcholine [38–40]. It is possible that bile acids interfere with VLDL production *in vivo* by influencing the cellular availability of lipids for VLDL assembly through interfering with lipid synthesis or by stimulating biliary lipid secretion [8], as biliary cholesterol output has been shown to be reciprocally related to VLDL-CH secretion [41]. However, our results *in vitro* do not support this possibility. First, taurodehydrocholic acid, which has little or no effect on biliary cholesterol and PL secretion *in vivo*, also suppresses VLDL secretion *in vitro*. Secondly, no effects on cellular lipid mass and the amount of newly synthesized lipids from [3 H]glycerol or [14 C]acetate were found concomitantly with suppression of VLDL secretion. Obviously these results do not exclude effects of bile acids on specific pools of cellular lipids and/or shifts between intracellular compartments. Clearly, more studies are needed to clarify this issue.

Recent studies have demonstrated that VLDL secretion can be modulated via intracellular Ca $^{2+}$ [42]. Some bile acids are known to affect cytosolic calcium mobilization [43]. However, TC, which strongly suppresses VLDL secretion, was demonstrated to have little or no effect on intracellular Ca $^{2+}$ mobilization [43]. On other hand, tauroursodeoxycholic acid, which shows less potency in suppression of VLDL secretion, strongly alters intracellular Ca $^{2+}$ distribution [43]. These results indicate that bile acid-induced suppression of VLDL secretion is unlikely to be mediated by cellular Ca $^{2+}$ mobilization.

Theoretically, the inhibitory effect of bile acids on VLDL and albumin secretion could reflect a rather non-specific 'toxic' effect, for example on vesicle-mediated transport. However, the following evidence indicates that the inhibition of VLDL secretion by bile acids, as observed in this study, is not due to their potential cytotoxic effects: (1) the overall parameters of cellular lipid metabolism studied were not affected by bile acids (Tables 2, 3 and 7); (2) the leakage of lactate dehydrogenase from cells and total protein synthesis and secretion were not altered by bile

acids (Table 5); (3) the bile acid concentration needed to suppress VLDL secretion was within the physiological portal blood range, which is far lower than the reported concentration needed to exert a toxic effect in cultured rat hepatocytes [44]; (4) tauroursodeoxycholic acid, a 'non-toxic' bile acid both *in vivo* and *in vitro* [45,46], shows a similar suppressive potency to TCDC, a more toxic bile acid.

In summary, hepatic intracellular bile acid content can influence VLDL secretion by rat hepatocytes, suggesting that bile acids play a role in regulating postprandial lipid metabolism.

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