Lipoprotein cholesterol uptake mediates up-regulation of bile-acid synthesis by increasing cholesterol 7α-hydroxylase but not sterol 27-hydroxylase gene expression in cultured rat hepatocytes

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Lipoproteins may supply substrate for the formation of bile acids, and the amount of hepatic cholesterol can regulate bile-acid synthesis and increase cholesterol 7α-hydroxylase expression. However, the effect of lipoprotein cholesterol on sterol 27-hydroxylase expression and the role of different lipoproteins in regulating both enzymes are not well established. We studied the effect of different rabbit lipoproteins on cholesterol 7α-hydroxylase and sterol 27-hydroxylase in cultured rat hepatocytes. β-Migrating very-low-density lipoprotein (βVLDL) and intermediate-density lipoprotein (IDL) caused a significant increase in the intracellular cholesteryl ester content of cells (2.3- and 2-fold, respectively) at a concentration of 200 μg of cholesterol/ml, whereas high-density lipoprotein (HDL, 50% v/v), containing no apolipoprotein E (apo E), showed no effect after a 24-h incubation. βVLDL and IDL increased bile-acid synthesis (1.9- and 1.6-fold, respectively) by up-regulation of cholesterol 7α-hydroxylase activity (1.7- and 1.5-fold, respectively). Dose- and time-dependent changes in cholesterol 7α-hydroxylase mRNA levels and gene expression underlie the increase in enzyme activity. Incubation of cells with HDL showed no effect. Sterol 27-hydroxylase gene expression was not affected by any of the lipoproteins added. Transient-expression experiments in hepatocytes, transfected with a promoter-reporter construct containing the proximal 348 nucleotides of the rat cholesterol 7α-hydroxylase promoter, showed an enhanced gene transcription (2-fold) with βVLDL, indicating that a sequence important for a cholesterol-induced transcriptional response is located in this part of the cholesterol 7α-hydroxylase gene. The extent of stimulation of cholesterol 7α-hydroxylase is associated with the apo E content of the lipoprotein particle, which is important in the uptake of lipoprotein cholesterol. We conclude that physiological concentrations of cholesterol in apo E-containing lipoproteins increase bile-acid synthesis by stimulating cholesterol 7α-hydroxylase gene transcription, whereas HDL has no effect and sterol 27-hydroxylase is not affected.

Key words: apolipoprotein E, high-density lipoprotein, intermediate-density lipoprotein, β-migrating very-low-density lipoprotein.

INTRODUCTION

Most species respond to an increase in the dietary load of cholesterol by suppressing endogenous cholesterol synthesis and by utilizing the hepatic capacity to store large quantities of cholesterol in its esterified form. Several groups have demonstrated that feeding a high-cholesterol diet also results in up-regulation of bile-acid synthesis (for review, see [1]), illustrating the importance of this metabolic pathway for maintaining cholesterol homoeostasis. Bile-acid synthesis and biliary secretion of cholesterol into the bile is the major way of eliminating cholesterol from the body [2,3].

The primary route to bile acids is initiated by 7α-hydroxylation of cholesterol by cholesterol 7α-hydroxylase, a microsomal cytochrome P450-dependent enzyme [4–6]. However, there are alternative pathways to bile-acid synthesis operational [1,7,8]. One of these pathways is initiated by 27-hydroxylation of cholesterol by sterol 27-hydroxylase, a cytochrome P450-dependent enzyme, located in the mitochondrial inner membrane [9–13]. This alternative pathway can contribute substantially to bile-acid synthesis in cultured human and rat hepatocytes [12,13] and in humans [7], rats [14] and rabbits [15] *in vivo*. Both enzymes are regulated co-ordinately by a number of mediators [13,16–19].

A regulatory role has also been ascribed to cholesterol, although the mechanism of regulation is controversial. A mode of regulating bile-acid biosynthesis by cholesterol has been suggested that involves the saturation of the enzyme cholesterol 7α-hydroxylase [20–22]. However, Einarsson et al. [23,24] did not find such a relationship. Several groups have shown that feeding rats a cholesterol-rich diet led to increased cholesterol 7α-hydroxylase activity, mRNA levels and transcriptional activity [25–30]. It remains unclear, however, whether cholesterol exerts its effect directly on cholesterol 7α-hydroxylase or whether other factors may also contribute. Constant infusion of mevalonate, a cholesterol precursor, in control and bile-fistulated rats stimulated cholesterol 7α-hydroxylase gene expression, suggesting a direct involvement of cholesterol [31,32]. An indirect mechanism was also proposed, in which stimulation of cholesterol 7α-hydroxylase by dietary cholesterol is ascribed to malabsorption of bile acids in the intestine, resulting in a reduced potential for bile acid-induced feedback or to the involvement of an intestinal factor [28,33–35].

Furthermore, the effect of lipoprotein cholesterol on sterol 27-hydroxylase expression and the role of different lipoproteins in regulating both enzymes are not well established. In the current study, we have re-assessed the role of lipoprotein cholesterol as

Abbreviations used: LPDS, lipoprotein-deficient serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; apo E, apolipoprotein E; WE medium, Williams E medium; βVLDL, β-migrating very-low-density lipoprotein; IDL, intermediate-density lipoprotein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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a regulator of bile-acid biosynthesis [21,36–39] in cultured rat hepatocytes, which has the advantage of allowing us to discriminate between direct and indirect events. The effects of different lipoproteins as a source of exogenous cholesterol were assessed on the regulation of the two key enzymes of this metabolic route. Our results show that cholesterol or a metabolite thereof supplied by cholesterol- and apolipoprotein E (apo E)-rich lipoproteins stimulates bile-acid synthesis by directly stimulating cholesterol 7α-hydroxylase gene expression, whereas sterol 27-hydroxylase remains unaffected.

EXPERIMENTAL

Materials and animals

Materials used for the isolation and culturing of rat hepatocytes, and for assaying cholesterol 7α-hydroxylase, were obtained from sources described previously [19,40,41]. [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]dUTP (400 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. Cholesterol kit (catalogue no. 125512) and triglyceride kit (catalogue no. 701904) were provided by Boehringer Mannheim, Mannheim, Germany. The phospholipid kit (catalogue no. 990-54009) was provided by Wako Chemicals, Neuss, Germany. CI-1011 was provided kindly by Dr. Krause, Parke Davis Pharmaceutical Research Division, Ann Arbor, MI, U.S.A.

Male Wistar rats weighing 250–350 g were used throughout and were maintained on standard chow and water *ad libitum*. In the experiments described, rats were fed a diet supplemented with 2% cholestyramine (Questran, Bristol Myers B. V. Weesp, The Netherlands) 2 days before the isolation of hepatocytes. For the preparation of hepatocytes, the animals were killed between 9 and 10 a.m. Institutional guidelines for animal care were observed in all experiments.

Rat hepatocyte isolation and culture

Hepatocytes were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor and cultured as described previously [19,40,41]. After a 4-h attachment period, the cell medium was refreshed with 1.0 ml [6-well plates for intracellular lipids (1 well) and transfection experiment (3 wells)] or 2.5 ml [60-mm diameter dishes for bile-acid mass production (2 dishes), enzyme activity (5 dishes), mRNA (1 dish) and nuclear run-off assay (7 dishes)] of Williams E medium (WE medium) supplemented with 10% (v/v) fetal calf serum, and cells were incubated for a further 14 h. Lipoproteins in medium containing 10% (v/v) lipoprotein-deficient serum (LPDS) were added to the cells after this period, between hours 18 and 42 of culture age, unless otherwise stated. Cells were harvested at the same time after a 42-h culture period for measurement of intracellular lipids, cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, mRNA levels and the determination of transcriptional activity.

Isolation of LPDS; lipoprotein isolation and characterization

LPDS was isolated from fetal calf serum (Boehringer Mannheim) by ultracentrifugation at 4 °C for 48 h after a density adjustment with solid KBr. The LPDS fraction ($d > 1.21$) was dialysed at 4 °C against 10 mM sodium phosphate/0.15 M NaCl (pH 7.4) for 24 h, and subsequently against WE medium for an additional 24 h, and sterilized by filtering through a 0.22-μm membrane.

For the isolation of lipoproteins, blood was obtained from rabbits fed a diet supplemented with 1% (w/w) cholesterol for 7 days. Rabbit lipoproteins were chosen based on the fact that only one rabbit (without being killed) is needed to obtain sufficient amounts of lipoproteins in contrast to the large number of rats needed for isolation of rat lipoproteins. In addition, sufficient amounts of β-migrating very-low-density lipoprotein (βVLDL)-like particles in rat can only be obtained by feeding cholate-containing diets rich in fat and cholesterol, having the disadvantage of contamination of lipoproteins with cholate even after dialysis. Lipoproteins were isolated by ultracentrifugation (202000 $g_{av.}$) in a SW40 rotor (18 h, 4 °C) on a NaCl density gradient: 4 ml of serum [d (ρ), 1.21 with KBr]/2.6 ml of NaCl (ρ 1.063)/8.6 ml of H_2O. Gradients were fractionated and cholesterol and triglyceride levels in lipoprotein fractions were determined enzymically, using commercial kits. The different lipoprotein fractions were pooled on the basis of the determination of density (density-measuring cell DMA 602M, Mettler/Paar, Graz, Austria) and cholesterol profile (see Figure 1, top panel). The βVLDL fraction ($d < 1.006$) contained most of the lipoprotein-associated serum cholesterol (12.3 mg/mg of protein), was rich in phospholipids (2.16 mg/mg of protein) and contained relatively low amounts of triglycerides (0.22 mg/mg of protein) as a consequence of displacement by cholesterol, giving

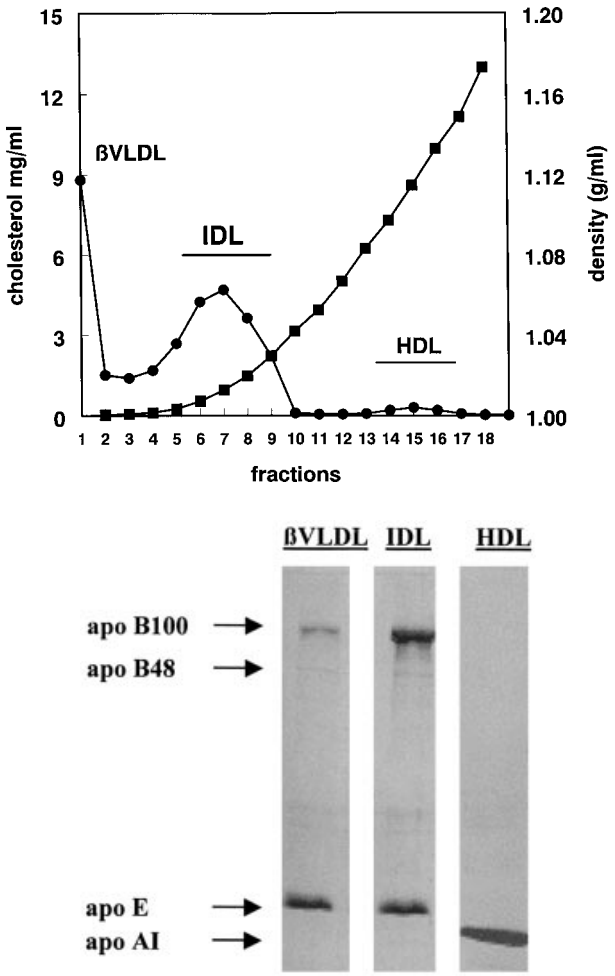

Figure 1 Lipoprotein profile

Top panel: serum from rabbits fed a 1% (w/w) cholesterol diet for 1 week was fractionated by ultracentrifugation on a NaCl density gradient (■). The cholesterol content (●) of each individual fraction was measured enzymically. Fraction 1 represents βVLDL, fractions 5–9 IDL and fractions 13–17 HDL. Bottom panel: lipoprotein fractions were further analysed by SDS/PAGE with 4–20% gradient gels.

this particle a β-migrating mobility. Intermediate-density lipoprotein (IDL, $d = 1.004$–1.030) contained less cholesterol (3.79 mg/mg of protein), phospholipid (1.11 mg/mg of protein) and triglycerides (0.03 mg/mg of protein). The high-density lipoprotein (HDL)-fraction ($d = 1.081$–1.146) contained low amounts of cholesterol (0.57 mg/mg of protein) and phospholipids (0.42 mg/mg of protein), and hardly any triglycerides (0.011 mg/mg of protein). Lipoprotein fractions were further analysed by SDS/PAGE (Figure 1, bottom panel) with 4–20 % gradient gels. βVLDL predominantly contained apo E, with minor amounts of apo B100 and apo B48. IDL contained apo E and more apo B100 and apo B48 than βVLDL. There was no contamination with HDL, since apo AI was absent. HDL was rich in apo AI and contained no apo E. The ratio of apo E to apo B was determined by scanning the SDS/polyacrylamide gels with a Hewlett Packard Scanjet 4c and quantifying the bands by image analysis using the program Tina version 2.09. The various fractions were stabilized by the addition of 10 % (v/v) heat-inactivated LPDS (to prevent aggregation and oxidation), dialysed for 24 h against sodium phosphate buffer, for another 24 h against WE medium and then filtered through a 0.45-μm membrane. The fractions were stored at 4 °C and used within 3 days.

Measurement of the mass of intracellular lipids

After a 24-h incubation period, with or without lipoproteins, cells were washed three times with cold PBS (pH 7.4) and harvested by scraping. Scraped cells were homogenized, samples were taken for measuring the protein content and lipids were extracted from the cell suspension, after the addition of cholesterol acetate (2 μg per sample) as an internal standard. The neutral lipids were separated by HPTLC on silica-gel-60-precoated plates as described [19,42]. Quantification of the amounts was done by scanning the plates with a Hewlett Packard Scanjet 4c and image analysis using the program Tina version 2.09.

Quantification of mass production of bile acids

Mass production of bile acids by rat hepatocytes was measured by GLC. Rat hepatocytes were cultured for 24 h (from 26–50 h of culture age) after a preincubation period of 8 h (from 18–26 h of culture age) in WE medium containing 10 % LPDS in both periods in the absence (control) or presence of different lipoproteins as described previously [19].

Assay of cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activity

Enzyme activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase were measured by HPLC according to [43] with minor modifications measuring mass conversion of cholesterol into 7α-hydroxy- and 27-hydroxy-cholesterol. In short, 4 mg of protein of cell homogenates was incubated in 1 ml of buffer containing 0.1 M potassium phosphate buffer (pH 7.2), 50 mM NaF, 5 mM dithiothreitol, 1 mM EDTA, 20 % (w/v) glycerol and 0.015 % (w/w) CHAPS. Then, 20 μl of 1 mg of cholesterol in 45 % (w/v) hydroxypropyl-β-cyclodextrin was added and the mixture was incubated with agitation for 10 min at 37 °C. A regenerating system (200 μl) was added containing 10 mM sodium isocitrate, 10 mM $MgCl_2$, 1 mM NADPH and 0.15 units of isocitrate-dehydrogenase at 37 °C. After 20 min, 60 μl of a stop solution containing 20 % (w/v) sodium cholate and 1 μg 20α-hydroxycholesterol, which served as a recovery standard, were added. Steroid products were oxidized at 37 °C for 45 min after the addition of 100 μl of buffer containing 0.1 % cholesteroloxidase (w/v; Calbiochem, catalogue no. 228250), 10 mM potassium phosphate (pH 7.4), 1 mM dithiothreitol and 20 % (w/w) glycerine. The reaction was stopped by 2 ml of ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleum ether. The ether layer was evaporated under a stream of nitrogen. Residues resuspended in a mixture of 60 % acetonitrile, 30 % methanol and 10 % (v/v) chloroform were analysed by using C_{18} reverse-phase HPLC using a Tosohaas TSK gel-ODS 80TM column equilibrated with 70 % acetonitrile/30 % methanol at a flow rate of 0.8 ml/min. The amount of product formed was determined by monitoring the absorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, Cambridge, U.K.).

RNA isolation, blotting and hybridization procedures

Isolation of total RNA and subsequent electrophoresis, Northern-blotting and hybridization techniques were performed as described previously [19]. The following DNA fragments were used as probes in hybridization experiments: a 1.6-kb PCR-synthesized fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region [41], a 1.6-kb *Hin*dIII/*Xba*I fragment of rat sterol 27-hydroxylase cDNA, a 1.2-kb *Pst*I fragment of hamster β-actin cDNA and a 1.2-kb *Pst*I fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were used [19]. The actin or GAPDH mRNA was used as an internal standard to correct for differences in the amount of total RNA applied on to the gel or filter. mRNA levels were quantified using a PhosphorImager BAS-reader (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and Tina version 2.09.

Nuclear run-off studies

Nuclear run-off studies were conducted essentially as described in [41]. For hybridization, target DNA, being 5 μg of plasmid material containing cDNA sequences of rat cholesterol 7α-hydroxylase, rat sterol 27-hydroxylase, hamster actin, rat GAPDH (see above) and the empty vector pUC19, were slot-blotted on to strips of Hybond-N^+ filter (Amersham), and crosslinked with 0.4 M NaOH for 30 min. The filters were preincubated for 30 min at 65 °C in a sodium phosphate buffer, and hybridized with the labelled RNA for 36 h. Labelled RNA generated by incorporation of [^{32}P]dUTP had been incorporated into nascent RNA, using isolated nuclei from cells that had been cultured with or without βVLDL for different lengths of time between 24 and 48 h of culture. After hybridization, the various filters were washed once for 5 min and twice for 30 min in 2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/1 % SDS at 65 °C, and exposed to a PhosphorImager plate for 2–5 days. Quantification of relative amounts of transcribed mRNA was performed using a PhosphorImager BAS-reader (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and Tina version 2.09.

Transfection experiments and chloramphenicol acetyltransferase (CAT) assays

At 22 h after isolation, cells were subjected to transfection using plasmid −348R*cat*, subsequently cultured in WE medium containing 10 % LPDS with or without βVLDL (400 μg of cholesterol/ml) for 48 h, and CAT assays were performed as described previously [44]. −348R*cat* contains the proximal 348 nucleotides of the rat cholesterol 7α-hydroxylase promoter fused to the bacterial *CAT* gene, used as a reporter. The amounts of acetylated product as represented by autoradiography were quantified by

PhosphorImager analysis. Data were corrected for protein content and transfection efficiency.

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 ± S.E.M. Spearman's correlation coefficients were calculated to determine the association between apo E/apo B ratio and the induction of cholesterol 7α-hydroxylase mRNA or cholesteryl ester content in the cell.

RESULTS

Effect of different lipoproteins on intracellular lipid levels

To address the effects of an exogenous cholesterol source on the bile-acid-synthetic capacity of a liver cell, rat hepatocytes were incubated with rabbit lipoproteins of varying buoyant density, and cellular lipid content was determined as a measure of their internalization (Table 1). Incubation with βVLDL or IDL (both 200 μg of cholesterol/ml) for 24 h resulted in 2.3- and 2-fold increases in intracellular cholesteryl ester content, respectively. HDL (50%, v/v, equal to approx. 60 μg of cholesterol/ml) did not change the cholesteryl ester content. Under the various incubation conditions, intracellular free cholesterol and triglycerides levels were not significantly affected. The increase in cholesteryl ester content by βVLDL was prevented by the simultaneous addition of the acyl-CoA:cholesterol acyltransferase inhibitor CI-1011. A similar result was obtained when IDL was added together with CI-1011 (results not shown).

Effect of different lipoproteins on bile-acid mass production

Bile-acid mass production was measured over a 24-h incubation period after a preincubation of 8 h with the various lipoproteins, as described in the Experimental section. Incubation of hepatocytes with βVLDL or IDL resulted in 1.9- and 1.7-fold increases in bile-acid mass production, respectively (Table 1). The major bile acids formed were cholic acid and β-muricholic acid, in a ratio of approx. 20:80. This ratio did not change upon incubation with the various lipoproteins.

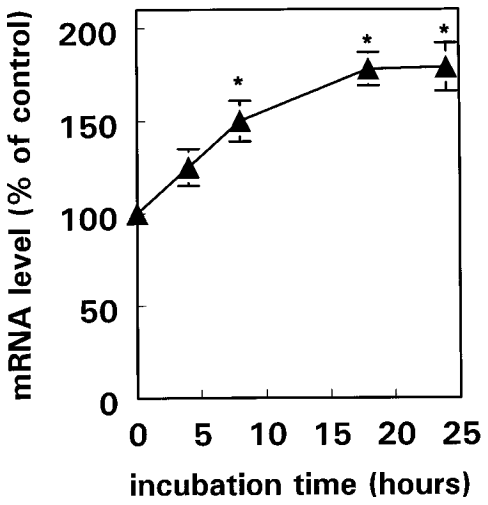

Figure 2 Time course of stimulation of cholesterol 7α-hydroxylase mRNA by βVLDL

Rat hepatocytes were incubated with βVLDL (200 μg of cholesterol/ml) for different lengths of time, between 18 and 42 h of culture, and were harvested simultaneously at 42 h of culture time. Total RNA was isolated and cholesterol 7α-hydroxylase mRNA levels were determined relative to GAPDH mRNA expression, as described in the Experimental section. Data are expressed as a percentage of mRNA levels in control cells and are means ± S.E.M. of independent experiments with hepatocytes from three rats. A significant difference ($P < 0.05$) between control and treated cells is indicated by an asterisk.

Effect of different lipoproteins on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and mRNA levels

To assess the level at which exogenous cholesterol, supplied by its physiological carriers, enhances bile-acid mass production, enzyme activities and mRNA levels of cholesterol 7α-hydroxylase and sterol 27-hydroxylase were determined. Rat hepatocytes were cultured for 24 h in the presence of βVLDL, IDL or HDL, since cholesterol 7α-hydroxylase mRNA levels were increased maximally after a 18–24-h incubation period (Figure 2). Addition of βVLDL or IDL stimulated cholesterol 7α-hydroxylase activity 1.7- and 1.5-fold, respectively. The increase of cholesterol 7α-hydroxylase activity was similar to the induction of its mRNA, being 1.8-fold with βVLDL and 1.7-fold with IDL (Table 2). The

Table 1 Effect of different lipoproteins on intracellular lipid levels and bile-acid mass production

For measurement of intracellular lipids, hepatocytes were incubated for 24 h, from 18 to 42 h of culture, in the presence of lipoproteins [βVLDL and IDL (200 μg of cholesterol/ml), or 50% (v/v) HDL (approx. 60 μg of cholesterol/ml)]. Additional incubations with βVLDL contained 3 μM of the acyl-CoA:cholesterol acyltransferase inhibitor CI-1011. Lipoproteins used were isolated from rabbits fed a 1% cholesterol-enriched diet as described in the Experimental section. Cells were harvested and the cellular contents of cholesteryl ester (CE), free cholesterol (FC), triglycerides (TG) were determined as described. Values shown are expressed as mean absolute amounts (μg/mg of cellular protein) ± S.E.M. For measurement of bile-acid synthesis, rat hepatocytes were cultured for 24 h (26–50 h of culture) after an 8-h preincubation period (18–26 h of culture), in both periods in the absence (control medium containing 10% LPDS) or in the presence of the different lipoproteins. Bile-acid synthesis is expressed as a percentage of the synthesis in control incubations and values shown are means ± S.E.M of μg/mg of cellular protein per 24 h. The absolute bile-acid-synthesis rate in controls was 3.68 ± 0.86 μg of bile acids/mg of cellular protein per 24 h. All values shown were obtained from independent experiments with hepatocytes from 3–7 rats. A significant difference between control and treated cells is indicated by asterisks; $^{*}P < 0.05$, $^{**}P < 0.005$ and $^{***}P < 0.0005$.

	Intracellular lipid levels			
Cholesterol source	CE	FC	TG	Bile-acid synthesis (% of control)
Control	10.6 ± 1.5	6.1 ± 1.2	10.5 ± 2.2	100
βVLDL	24.8 ± 1.6***	6.5 ± 0.4	10.3 ± 0.2	191 ± 14*
IDL	21.2 ± 1.5**	6.4 ± 0.2	9.9 ± 0.6	164 ± 17*
HDL	11.9 ± 0.6	6.1 ± 0.2	11.7 ± 2.0	101 ± 20
βVLDL + CI-1011	11.2 ± 0.6	7.1 ± 0.4	11.2 ± 0.8	Not determined

Table 2 Effect of different lipoproteins on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities and mRNA levels

Rat hepatocytes were incubated for 24 h, from 18 to 42 h of culture, without (10% LPDS) or with βVLDL, IDL (both 200 μg of cholesterol/ml) or 50% (v/v) HDL (approx. 60 μg of cholesterol/ml). Cells were harvested after 24 h of incubation to measure cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activities and mRNA levels. Values shown are expressed as percentages of enzyme activity or mRNA levels in control cells and are means ± S.E.M. of independent experiments with hepatocytes from 3–6 rats. The absolute values for enzyme activity in controls cultured in 10% LPDS only were 352 ± 38 and 79 ± 18 pmol/mg of cellular protein per h, respectively, for cholesterol 7α-hydroxylase and sterol 27-hydroxylase. A significance difference between control and treated cells is indicated by an asterisk; $^{*}P < 0.05$ and $^{**}P < 0.005$.

	Cholesterol 7α-hydroxylase		Sterol 27-hydroxylase	
Cholesterol source	Activity	mRNA	Activity	mRNA
Control	100	100	100	100
βVLDL	170 ± 21*	179 ± 13**	91 ± 18	97 ± 5
IDL	151 ± 10*	165 ± 25*	102 ± 13	96 ± 8
HDL	61 ± 16	107 ± 14	82 ± 3	97 ± 4

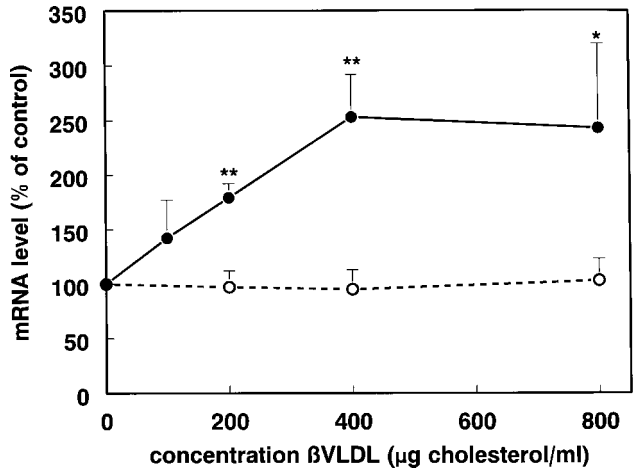

Figure 3 Dose-dependency of stimulation of cholesterol 7α-hydroxylase mRNA by βVLDL

Rat hepatocytes were incubated for 24 h, from 18 to 42 h of culture, with various amounts of βVLDL (100–800 μg of cholesterol/ml). Total RNA was isolated and cholesterol 7α-hydroxylase (●) and sterol 27-hydroxylase (○) mRNA amounts were determined, relative to GAPDH mRNA expression, as described in the Experimental section. Data are expressed as percentages of mRNA levels in control cells and are means ± S.E.M. of independent experiments with hepatocytes from 3–6 rats. Significant differences between control and treated cells are indicated by asterisks ($^{*}P < 0.05$ and $^{**}P < 0.005$).

induction of cholesterol 7α-hydroxylase mRNA levels was time- (Figure 2) and dose-dependent (Figure 3). Upon incubation of hepatocytes with βVLDL, a maximal induction of cholesterol 7α-hydroxylase mRNA was observed with an equivalent of 400 μg of cholesterol/ml. HDL did not increase cholesterol 7α-hydroxylase activity or mRNA levels. In contrast, sterol 27-hydroxylase activity was not affected by any of the lipoprotein fractions tested, indicating that the two enzymes diverge with respect to regulation by substrate cholesterol.

As indicated above, intracellular free cholesterol levels remained unchanged. Small changes in free cholesterol are difficult to detect due to the high amount of free cholesterol in the cell membranes, which may overshadow subtle changes in free

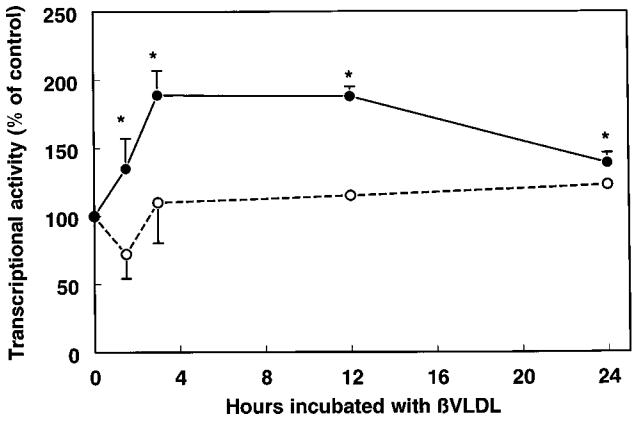

Figure 4 Time course of cholesterol 7α-hydroxylase and sterol 27-hydroxylase transcriptional activity in response to βVLDL

Rat hepatocytes were incubated with βVLDL (200 μg of cholesterol/ml) for different lengths of time between 18 and 42 h of culture, and hepatocytes were harvested simultaneously after 42 h of culture for the preparation of nuclei. Transcriptional activities of the different genes were determined by nuclear run-off assays, as described in the Experimental section, and values are presented relative to transcriptional activity of the β-actin gene, used as an internal standard. Non-specific hybridization was checked using the empty vector pUC19. Relative transcriptional activities of cholesterol 7α-hydroxylase (●) and sterol 27-hydroxylase (○) are expressed as percentages of control values and are means ± S.E.M. of independent experiments with hepatocytes from 3–4 rats. Significant differences between control and treated cells are indicated by asterisks ($^{*}P < 0.05$).

cholesterol, and the rapid esterification of free cholesterol. To postulate a role for the regulatory pool of cholesterol in the regulation of cholesterol 7α-hydroxylase, we measured mRNA levels of the low-density-lipoprotein (LDL) receptor and 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase as sensitive measures to detect subtle changes in this pool. The latter mRNA levels decreased significantly ($P < 0.05$) upon incubation with βVLDL, by $-33 \pm 11\%$ and $-49 \pm 7\%$, respectively ($n = 4$). These data point to the involvement of cholesterol or a metabolite as a regulatory factor. mRNA levels of β-actin and GAPDH, used as internal standards, were not affected by the lipoproteins. Simultaneous addition of both βVLDL (200 μg of cholesterol/ml) and CI-1011 (3 μM), to further increase the amount of regulatory cholesterol, resulted in even higher cholesterol 7α-hydroxylase mRNA levels as compared with incubations with βVLDL alone (286 ± 21 versus 179 ± 13%, $n = 5$, $P < 0.05$), indicating further that cholesterol or a metabolite is the regulatory compound. Even under these conditions with an enhanced intracellular regulatory free cholesterol content, sterol 27-hydroxylase remained unaffected.

Effect of βVLDL on the transcriptional activity of the cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes

To further examine the molecular level at which the effect of lipoproteins is exerted, the transcriptional activity of the cholesterol 7α-hydroxylase gene was determined by means of nuclear run-off assays. Hepatocytes were incubated with βVLDL for up to 24 h, and harvested for the isolation of nuclei. Cholesterol 7α-hydroxylase transcriptional activity was induced rapidly upon incubation of cells with βVLDL (200 μg of cholesterol/ml): gene expression was significantly higher (1.4-fold) after 1.5 h of incubation, and stimulated maximally upon 3–12 h of βVLDL treatment (1.9-fold; Figure 4).

The transcriptional activity of the sterol 27-hydroxylase gene was not affected by βVLDL, in accordance with the absence of

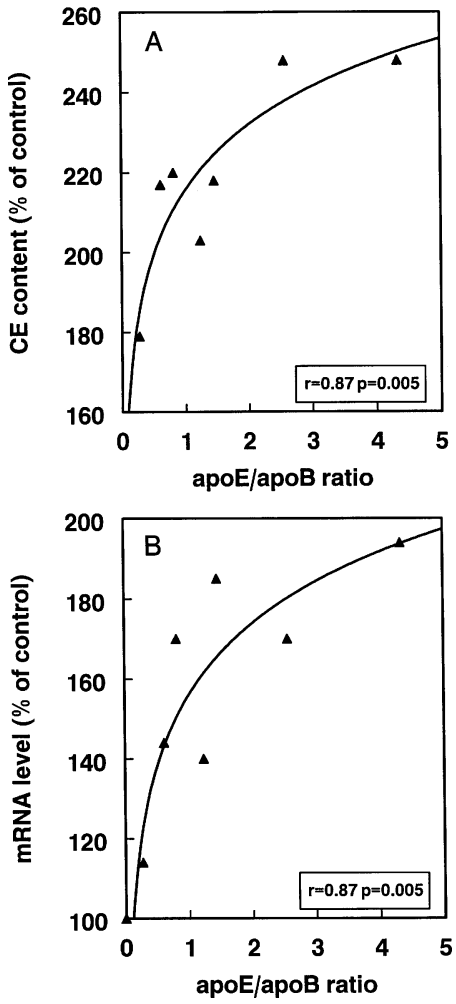

Figure 5 Relationship between cellular cholesteryl ester contents or cholesterol 7α-hydroxylase mRNA levels and apo E/apo B ratios of lipoproteins

Rat hepatocytes were incubated with different βVLDL and IDL fractions (all at 200 μg of cholesterol/ml) from 26 to 42 h of culture. Cells were harvested at 42 h to measure the intracellular cholesteryl ester (CE) contents and mRNA levels. The ratios of apo E/apo B were determined as described in the Experimental section. Spearman's correlation coefficients (*r*) were calculated to determine the association.

effects on sterol 27-hydroxylase activity and mRNA expression. The transcriptional activities of the β-actin and GAPDH genes, used as internal standards, showed no changes upon the addition of βVLDL.

Effect of βVLDL on cholesterol 7α-hydroxylase promoter activity in cells

Hoekman et al. [44], and others [45,46], have described a region in the proximal promoter of the rat cholesterol 7α-hydroxylase gene, harbouring major transcription-regulating elements, responsive to a variety of physiological signals. To assess whether this particular region of the cholesterol 7α-hydroxylase promoter is also responsive to lipoprotein cholesterol, transient-expression studies were performed. The −348R*cat* construct, consisting of the first 348 base pairs of the rat cholesterol 7α-hydroxylase promoter fused to the CAT-reporter gene [44], was used in these experiments. Promoter activity of the construct was significantly ($P < 0.05$) stimulated in the presence of βVLDL (400 μg of cholesterol/ml) as compared with control incubations (195 ± 7 versus 100 ± 10 %). The data are expressed as a percentages of control and are means ± S.E.M. of independent experiments with hepatocytes from four rats. The data are in accordance with the approx. 2-fold stimulation of the transcriptional activity of the cholesterol 7α-hydroxylase gene as determined by nuclear run-off assays.

Relationship between cellular cholesteryl ester content or cholesterol 7α-hydroxylase mRNA levels and apo E/apo B ratio of lipoproteins

The apo E content of a particle determines in part the efficiency of uptake by a liver cell [47]. Hence, apo E-rich lipoproteins are cleared more rapidly than those that rely solely on apo B-mediated uptake by the LDL receptor. We therefore determined whether there was a relationship between the apo E content of a particle and its capacity to elevate intracellular cholesteryl ester levels. Subsequently, we also determined whether apo E content correlated with ability to induce cholesterol 7α-hydroxylase mRNA levels.

Cells were incubated with different βVLDL and IDL subfractions, with different apo E/apo B ratios (fractions 1, 2–4, 5–7, and 8–9; Figure 1, top panel). Equal amounts of cholesterol were added in each case. There was a positive correlation between the apo E/apo B ratio of the particle, and the extent of increase in intracellular cholesteryl ester content (Figure 5A), indicating a more efficient uptake of apo E-rich lipoproteins. As a consequence of the greater elevation in intracellular cholesteryl ester content, the higher apo E/apo B ratio also resulted in elevated cholesterol 7α-hydroxylase mRNA levels (Figure 5B).

DISCUSSION

This study shows that, in rat hepatocytes, exogenous cholesterol supply in the form of lipoproteins stimulates the rate-limiting enzyme in the neutral pathway to the formation of bile acids, cholesterol 7α-hydroxylase. βVLDL and IDL, both cholesterol-rich lipoproteins, induced cholesterol 7α-hydroxylase at the level of gene transcription, whereas the expression of sterol 27-hydroxylase was not affected. Elevation of the initial level of bile-acid synthesis by feeding the rats with chow supplemented with 2 % cholestyramine prior to isolation of the hepatocytes [13,19,41] was not found to be obligatory to observe up-regulation of cholesterol 7α-hydroxylase by lipoprotein cholesterol.

We demonstrated that lipoprotein cholesterol has a direct stimulatory effect on cholesterol 7α-hydroxylase gene expression, in addition to having a merely stimulatory effect on bile-acid synthesis by supplying cholesterol [21,36–39], and that not all lipoprotein fractions contribute to the stimulation. βVLDL and IDL were active inducers, whereas HDL did not have an effect. The effect was accompanied by the accumulation of cholesteryl esters in the hepatocytes, as also observed *in vivo* [29], indicating that uptake of the lipoprotein cholesterol is important for regulation. This contention is supported by the strong association between the apo E content of the lipoprotein particle, expressed as the apo E/apo B ratio, and the extent of stimulation of cholesterol 7α-hydroxylase. The role of apo E in receptor-mediated uptake of lipoproteins is well known [47]. The HDL fraction did not have an effect on cholesterol 7α-hydroxylase expression, due to the fact that this fraction did not contain apo E and contained significantly less cholesterol than either βVLDL or IDL (approx. 60 μg/ml), which is too little to induce chol-

esterol 7α-hydroxylase expression. The HDL particle also did not deliver cholesterol to the cells, as is reflected by the absence of an effect on intracellular lipid levels and bile-acid synthesis, indicating that there may be insufficient supply of regulatory cholesterol to enhance cholesterol 7α-hydroxylase expression. This is in line with previous studies, which have shown that apo E-rich HDL is indeed able to stimulate bile-acid synthesis in cultured rat hepatocytes [36–38] and that this increase is proportional to the apo E content of the particle [38]. In contrast, Whiting et al. reported an increased bile-acid synthesis after incubation of cultured rabbit hepatocytes with apo E-free HDL [39].

Our results are in line with studies *in vivo*, showing that feeding a cholesterol-rich diet led to an increased cholesterol 7α-hydroxylase gene expression [25–29]. Continuous intravenous infusion of mevalonate, as a source of cholesterol, in control and bile-fistulated rats stimulated cholesterol 7α-hydroxylase enzyme activity, mRNA levels and transcriptional activity [31,32]. The latter studies *in vivo*, in which the intestinal route of cholesterol administration was avoided, and our results, favour a direct stimulatory mechanism over an indirect mechanism via intestinal malabsorption of different bile acids, as proposed by others [28,33–35]. Moreover, Duane [48] showed marked differences in the absorption of different bile acids after cholesterol feeding in rats, indicating that some potent regulators of bile-acid synthesis (chenodeoxycholic acid) escape intestinal entrapment and others (cholic acid) do not. This would lead to a scenario where the ratio of cholesterol to bile acids determines the transcriptional activity of the cholesterol 7α-hydroxylase gene, which seems more likely.

Previous reports [13,16–19,49] have shown co-ordinate regulation of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase by the same mediators. This study and those of others *in vivo* [14,15] and *in vitro* [49] demonstrate that both enzymes diverge, however, with respect to regulation by exogenous and endogenous cholesterol, respectively. We showed that strongly increasing the regulatory pool of cholesterol by incubations with lipoproteins and the acyl-CoA:cholesterol acyltransferase inhibitor CI-1011 did not affect sterol 27-hydroxylase. In contrast, an increasing effect on sterol 27-hydroxylase was found *in vivo* in other species fed a cholesterol-rich diet [15,50] with no concomitant induction [50], or even a decline [15], in cholesterol 7α-hydroxylase expression. This indicates that species differences exist for both the expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase by cholesterol.

Nuclear run-off studies showed increased transcription of cholesterol 7α-hydroxylase by cholesterol-rich lipoproteins, suggesting the presence of a sterol-responsive element within its promoter. These motifs may differ from those in the promoter region of the LDL-receptor and HMG-CoA reductase genes, as cholesterol or metabolite(s) suppress the expression of these genes [51]. During the preparation of this work a paper appeared [52], which suggests that it is not cholesterol but its oxidized metabolites that are responsible for stimulating cholesterol 7α-hydroxylase expression. In mice lacking the nuclear oxysterol receptor LXRα, no induction of cholesterol 7α-hydroxylase was found after they were fed a cholesterol-rich diet. In our study, a cholesterol-responsive element was localized within the proximal 348 nucleotides of the cholesterol 7α-hydroxylase promoter, which harbours a large amount of different responsive elements to various physiological signals [18,44–46,53]. The exact location and nature of this sterol-responsive site has yet to be identified. Transcriptional regulation of cholesterol 7α-hydroxylase by exogenous cholesterol has also been reported in H2.35 and HepG2 cells, but regulatory elements were found far more distally [30].

In conclusion, we have shown that circulating cholesterol-laden lipoproteins are not only substrates for bile-acid synthesis, but that cholesterol from these lipoproteins or a metabolite thereof is directly regulatory at the level of cholesterol 7α-hydroxylase gene transcription.

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REFERENCES

1 Princen, H. M. G., Post, S. M. and Twisk, J. (1997) Curr. Pharmaceut. Design **3**, 59–84
2 Björkhem, I. (1985) in Sterols and Bile Acids (Danielsson, H. and Sjövall, J., eds.), pp. 231–278, Elsevier Scientific, Amsterdam
3 Carey, M. and Duane, W. C. (1994) in The Liver: Biology and Pathology, 3rd edn. (Arias, I., Boyer, J. L., Jakoby, W. B., Schachter, D. and Shafritz, D. A., eds.), pp. 719–768, Raven Press, New York
4 Danielsson, H., Einarsson, K. and Johansson, G. (1967) Eur. J. Biochem. **2**, 44–49
5 Shefer, S., Hauser, S., Bekersky, I. and Mosbach, E. H. (1970) J. Lipid Res. **11**, 404–411
6 Myant, N. B. and Mitropoulos, K. A. (1977) J. Lipid Res. **18**, 135–153
7 Axelson, M. and Sjövall, J. (1990) J. Steroid Biochem. **36**, 631–640
8 Schwarz, M., Lund, E. G. and Russell, D. W. (1998) Curr. Opin. Lipidol. **9**, 113–118
9 Danielsson, H. (1961) Ark. Kemi **17**, 373–379
10 Anderson, K. E., Kok, E. and Javitt, N. B. (1972) J. Clin. Invest. **51**, 112–117
11 Swell, L., Gustafsson, J., Schwartz, C. C., Halloran, L. G., Danielsson, H. and Vlahcevic, Z. R. (1980) J. Lipid Res. **21**, 455–466
12 Princen, H. M. G., Meijer, P., Wolthers, B. G., Vonk, R. J. and Kuipers, F. (1991) Biochem. J. **275**, 501–505
13 Twisk, J., De Wit, E. C. M. and Princen, H. M. G. (1995) Biochem. J. **305**, 505–511
14 Vlahcevic, Z. R., Stravitz, R. T., Heuman, D. M., Hylemon, P. B. and Pandak, W. M. (1997) Gasteroenterology **113**, 1949–1957
15 Xu, G., Salen, G., Shefer, S., Tint, G. S., Kren, B. T., Nguyen, L. B., Steer, C. J., Chen, T. S. and Salen, L. (1997) Gasteroenterology **113**, 1958–1965
16 Twisk, J., Hoekman, M. F., Muller, L. M., Iida, T., Tamaru, T., Ijzerman, A., Mager, W. H. and Princen, H. M. G. (1995) Eur. J. Biochem. **15**, 596–604
17 Vlahcevic, Z. R., Jairathm, S. K., Heuman, D. M. and Stravitz, R. T. (1996) Am. J. Physiol. **270**, G626–G652
18 Twisk, J., Hoekman, M. F. M., Lehmann, E. M., Meijer, P., Mager, W. H. and Princen, H. M. G. (1995) Hepatology **21**, 501–510
19 Post, S. M., de Wit, E. C. M. and Princen, H. M. G. (1997) Arterioscler. Thromb. Vasc. Biol. **17**, 3064–3070
20 Shefer, S., Cheng, F. W., Hauser, S., Batta, A. K. and Salen, G. (1981) J. Lipid Res. **22**, 532–536
21 Davis, R. A., Hyde, P. M., Kuan, J. C. W., Malone-McNeal, M. and Archambault-Schexnayder, J. (1983) J. Biol. Chem. **258**, 3661–3667
22 Straka, M. S., Junker, L. H., Zacarro, L., Zogg, D. L., Dueland, S., Everson, G. T. and Davis, R. A. (1990) J. Biol. Chem. **265**, 7145–7149
23 Einarsson, K., Åkerlund, J.-E. and Björkhem, I. (1987) J. Lipid Res. **28**, 253–256
24 Einarsson, K., Reihnér, E. and Björkhem, I. (1989) J. Lipid Res. **30**, 1477–1481
25 Jelinek, D. F., Andersson, S., Slaughter, C. R. and Russell, D. W. (1990) J. Biol. Chem. **265**, 8190–8197
26 Li, Y. C., Wang, D. P. and Chiang, J. Y. L. (1990) J. Biol. Chem. **265**, 12012–12017
27 Pandak, W. M., Li, Y. C., Chiang, J. Y. L., Studer, E. J., Gurley, E. C., Heuman, D. M., Vlahcevic, Z. R. and Hylemon, P. B. (1991) J. Biol. Chem. **266**, 3416–3421
28 Björkhem, I., Eggertsen, G. and Andersson, U. (1991) Biochim. Biophys. Acta **1085**, 329–335
29 Spady, D. K. and Cuthbert, J. A. (1992) J. Biol. Chem. **267**, 5584–5591
30 Ramirez, M. I., Karaoglu, D., Haro, D., Barillas, C., Bashirzadeh, R. and Gil, G. (1994) Mol. Cell Biol. **14**, 2809–2821
31 Jones, M. P., Pandak, W. M., Heuman, D. M., Chiang, J. Y. L. and Hylemon, P. B. (1993) J. Lipid Res. **34**, 885–892
32 Vlahcevic, Z. R., Pandak, W. M. and Hylemon, P. B. (1993) Hepatology **18**, 660–668
33 Björkhem, I. and Åkerlund, J. E. (1988) J. Lipid Res. **29**, 136–143
34 Stone, B. G., Schreiber, D., Alleman, L. D. and Ho, C. Y. (1987) J. Lipid Res. **28**, 162–172
35 Björkhem, I., Blomstrand, R., Lewenhaupt, A. and Svensson, L. (1978) Biochem. Biophys. Res. Commun. **85**, 532–540
36 Ford, R. P., Botham, K. M., Suckling, K. E. and Boyd, G. S. (1985) Biochim. Biophys. Acta **836**, 185–191
37 Sampson, W. J., Botham, K. M., Jackson, B. and Suckling, K. E. (1988) FEBS Lett. **227**, 179–182

38 Mackinnon, A. M., Drevon, C. A., Sand, T. M. and Davis, R. A. (1987) J. Lipid Res. **28**, 847–855
39 Whiting, M. J., Wishart, R. A., Lewis, G. and Mackinnon, A. M. (1989) Biochim. Biophys. Acta **1005**, 137–142
40 Princen, H. M. G., Huijsmans, C. M. G., Kuipers, F., Vonk, R. J. and Kempen, H. J. M. (1986) J. Clin. Invest. **78**, 1064–1071
41 Twisk, J., Lehmann, E. M. and Princen, H. M. G. (1993) Biochem. J. **290**, 685–691
42 Havekes, L. M., de Wit, E. C. M. and Princen, H. M. G. (1987) Biochem. J. **247**, 739–746
43 Chiang, J. Y. L. (1991) Methods Enzymol. **206**, 483–491
44 Hoekman, M. F. M., Rientjes, J. M. J., Twisk, J., Planta, R. J., Princen, H. M. G. and Mager, W. H. (1993) Gene **130**, 217–223
45 Chiang, J. Y. L. and Stroup, D. (1994) J. Biol. Chem. **269**, 17502–17507
46 Cooper, A. D., Chen, J., Botelho-Yetkinler, M. J., Cao, Y., Taniguchi, T. and Levy-Wilson, B. (1997) J. Biol. Chem. **272**, 3444–3452
47 Mahley, R. W. (1988) Science **240**, 622–630
48 Duane, W. C. (1994) J. Lipid Res. **35**, 501–509
49 Stravitz, R. T., Vlahcevic, Z. R., Russell, T. L., Heizer, M. L., Avadhani, N. G. and Hylemon, PB. (1996) J. Steroid Biochem. **57**, 337–347
50 Hasan, S. Q. and Kushwaka, R. S. (1993) Biochim. Biophys. Acta **1182**, 299–302
51 Goldstein, J. L. and Brown, M. S. (1990) Nature (London) **343**, 425–430
52 Peet, D. J., Turley, S. D., Ma, W., Janowski, B. A., Lobaccaro, J.-M. A., Hammer, R. E. and Mangelsdorf, D. J. (1998) Cell **93**, 693–704
53 Crestani, M., Stroup, D. and Chiang, J. Y. L. (1995) J. Lipid Res. **36**, 2419–2432

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