REGULATION OF CHOLESTEROL 7α-HYDROXYLASE AND STEROL 27-HYDROXYLASE IN CULTURED RAT HEPATOCYTES

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STELLINGEN

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In het openbaar te verdedigen op dinsdag 22 november 1994
des namiddags te klokke 16.15 uur door

Jaap Twisk

1. De galzuur-gemedieerde onderdrukking van de galzuursynthese, in gekweekte primaire hepatocyten van zowel de big als de rat, toont aan dat een vermeende rol van een gal-flow afhankelijke darm-factor in dit opzicht kan worden uitgesloten.

   Kwekkeboom et al. Hepatology 1990; 12: 1209-1215
   Dit proefschrift.

2. In tegenstelling tot de algemene opvatting vormt sterol 27-hydroxylase wel degelijk een regulatoir punt in de galzuursynthese route. Het enzym verdient derhalve meer aandacht.

   Dit proefschrift.

3. De gedachte dat hydrophobiciteit van galzuren bepalend is voor de mate van galzuur-gemediëerde remming van galzuursynthese, is te beperkt.

   Heuman et al. J. Lipid Res. 1989; 30: 1161-1171.
   Stravitz et al. J. Biol. Chem. 1993; 268: 13987-13993.
   Kwekkeboom et al. Hepatology 1990; 12: 1209-1215.
   Dit proefschrift.

4. Bij het *in vivo* onderzoek naar de onderlinge beïnvloeding van de cholesterol- en de galzuursynthese-routes in de lever, is het onjuist om daarbij gebruik te maken van totale lever homogenaten, daar beide routes ruimtelijk gescheiden zijn.

   Li et al. J. Lipid Res. 1988; 29: 781-786.
   Ugele et al. Biochem. J. 1991; 276: 73-77.
   Dit proefschrift.

5. Uit het onderzoek van Björkhem et al., waarin wordt aangetoond dat een grote hoeveelheid cholesterol in de voeding werkt als een galzuur-binder en daardoor de galzuursynthese stimuleert, mag niet worden geconcludeerd dat cholesterol zelf geen direct regulatoire capaciteit bezit.

   Björkhem et al. Biochim. Biophys. Acta. 1990; 1085: 329-335.

6. *De novo* gesynthetiseerd cholesterol is onder normale omstandigheden geen voorkeurssubstraat voor galzuursynthese.

   Scheibner et al. Hepatology 1993; 17: 1095-1102.
   Dit proefschrift.

7. De normale vascularisatie tijdens de embryonale ontwikkeling van t-PA/u-PA knock-out muizen (Carmeliet et al.), in tegenstelling tot gestoorde angiogenese in het adulte stadium, toont aan dat embryonale bloedvatvorming, en de angiogenese die optreedt in het volwassen individu, als verschillende processen moeten worden gezien.

   Carmeliet et al. Nature 1994; 368: 419-424.

8. Voor bestudering van het effect van bepaalde voedingscomponenten op de ontwikkeling van atherosclerose, verdient het gebruik van de humane APOE*3-Leiden transgene muis de voorkeur boven het gebruik van de *Apoe* knock-out muis.

9. Het toenemend aantal publicaties over het ontbreken van een effect van antioxydanten op de ontwikkeling van atherosclerose in proefdiermodellen, zegt eerder wat over het gebruikte model dan over de werking van het antioxydant.

10. Wie blind is voor andermans resultaten zal altijd een primeur kunnen presenteren.

11. De snelheid waarmee de VN te hulp schieten is mede afhankelijk van het aantal wereldse energiebronnen dat gevaar loopt.

12. Plutonium dient te worden beschouwd als een splijtstof tussen volkeren.

13. Die genen die afwijken, kosten geld.

Regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in cultured rat hepatocytes

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit Leiden,
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hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op dinsdag 22 november 1994
te klokke 16.15 uur

door

Jaap Twisk

geboren te Harlingen in 1965

Promotiecommissie:

Promotor:	Prof.dr. P. Brakman
Co-promotor:	Dr. J.M.G. Princen (Gaubius Laboratorium TNO-PG, Leiden)
Referent:	Prof.dr. R. Gebhardt (Universiteit Tübingen, Duitsland)
Overige leden:	Prof.dr. W.J.H.M. Möller
	Prof.dr. H.M.J. Krans
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Cover: Lobular distribution of cholesterol 7α-hydroxylase.

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Those who find ugly meanings in beautiful things are corrupt without being charming. This is a fault.

Those who find beautiful meanings in beautiful things are the cultivated. For these there is hope.

.....

Oscar Wilde

Aan mijn ouders

CONTENTS

CHAPTER 1
General Introduction

INTRODUCTION

Scientific effort has been devoted to designing new ways to lower the risk of atherosclerosis, main cause of death in Western society. Important risk factors inherent to development of the desease are high serum levels of cholesterol and/or triglycerides (1-4). Allthough potentially pathological, these compounds form a necessity for the proper execution and maintenance of various bodily functions and structures. Cholesterol is used as a building-block for cell-membranes, and is a precursor molecule for the synthesis of steroid hormones, bile acids, and vitamins. Triglycerides (TG) are an essential energy source for muscle- and other tissue, and excess thereof may be stored in adipose tissue.

The liver plays an essential role within cholesterol homeostasis. It is the major site of receptor-mediated uptake of circulating cholesterol, which is transported to and from peripheral tissue via blood-borne lipoprotein particles (Fig. 1). Lipoproteins consist largely of a polar outer shell of phospholipids, free cholesterol, and apolipoproteins, encompassing a core of TG and cholesteryl esters (CE). The apolipoproteins are involved in receptor-mediated recognition and uptake of lipoproteins, and their apo-identity and association with specific lipoproteins determines in part their physiological fate, and targeting to various cell-types (for a review: ref. 5). They may also act as a co-factor, e.g in the esterification of cholesterol by the lecithin:cholesterol acyltransferase (LCAT). Lipoprotein-derived cholesterol originates in part from dietary intake, with subsequent intestinal absorption and transport to the liver via chylomicrons (CM). During circulation in blood, these particles become lypolised, resulting in denser, relatively TG-poor and cholesterol-rich chylomicron remnant particles (CR), which are rapidly cleared by the liver. Alternatively, cholesterol may be distributed from the liver to peripheral tissue, via very-low-density-lipoprotein (VLDL)-secretion, and so enter the circulation. In this case, the cholesterol-entity may originate either from a preformed pool of lipoprotein-cholesterol, entering the liver via receptor-mediated uptake, or from endogenous synthesis from acetate precursors. Approximately 70% of total endogenous cholesterol is liver-synthesized. Finally, cholesterol may enter the circulation by efflux from peripheral tissue. Specifically high-density-lipoprotein (HDL) has been implicated as a cholesterol-acceptor, and to play an important role in reverse cholesterol transport (see for reviews: 6-10). Upon lipolysis of TG, VLDL may acquire cholesterol, and undergo a density shift towards a cholesterol-rich, and relatively TG-poor low-density-lipoprotein (LDL)-particle. Specifically LDL-cholesterol is considered a major contributor to a strong correlation between serum cholesterol and incidence of cardiovascular disease. Prolonged half-life of circulating LDL also promotes oxidative processes acting on the particle, which accumulates in arterial macrophages (11,12). Uptake of modified cholesterol by macrophages is mediated via scavenger receptors, which, unlike the LDL-receptor, are not regulated by cellular cholesterol content (13). Consequently, cholesterol accumulates within the arterial

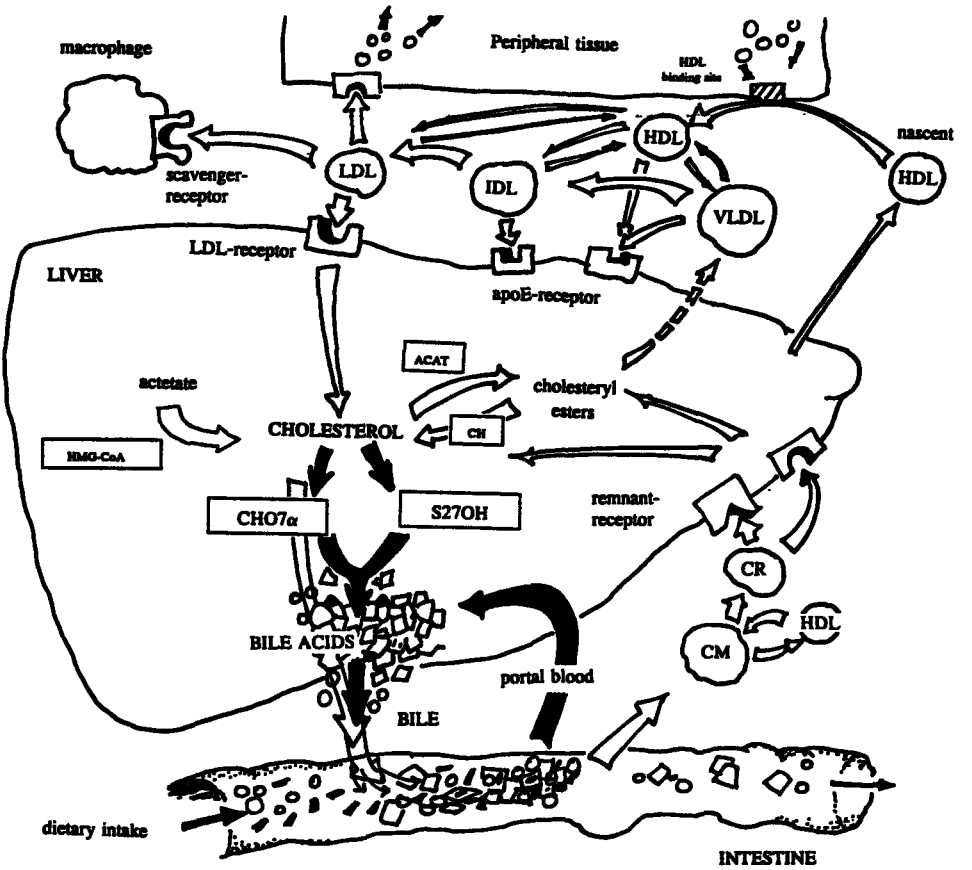


Figure 1. Schematic representation of cholesterol fluxes to and from the liver. Important enzymes involved in homeostatic maintenance of liver cholesterol have been depicted (boxed): HMG-CoA, HMG-CoA reductase; CHO7α, cholesterol 7α-hydroxylase; S27OH, sterol 27-hydroxylase; ACAT, acyl-CoA: cholesterol acyltransferase; CH, cholesteryl ester hydrolase. Cholesterol (○) fluxes are represented by open arrows, and formation and flux of bile acids (◇) by closed arrows. (▯) are dietary fats. CM, chylomicron; CR, chylomicron remnant; HDL, high-density lipoprotein; VLDL, very-low-density lipopprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein. Exchange of proteins and cholesterol between HDL and other lipoproteins has been indicated; enzymes involved (CETP and LCAT) have been ommitted for reasons of clarity. Gradual lipolysis (density shift from VLDL→IDL→LDL, or CM→CR) via TG disposition and storage in adipose tissue, has not been included in the diagram. Intermediate space surrounding the liver represents blood circulation.

lining, resulting in foam cell formation and vessel-occlusion, as one of the first symptoms of onset of atherosclerosis. Basic therapy is therefore concentrated on minimizing arterial concentration of these potentially atherosclerotic particles.

In contrast, high levels of HDL are considered beneficial, in that they promote efflux of cholesterol, and direction of peripheral cholesterol storage pools to the liver and degradational processes localized within this organ (14).

The only quantitive way to excrete cholesterol is by bile-flow-dependent direct excretion of cholesterol as such, or by conversion of cholesterol into bile acids (15-17). A low bile acid synthetic capacity was found to be an independent risk factor for incidence of coronary heart disease (CHD), and subnormal levels of bile acid synthesis could be correlated to progression of atherosclerosis and coronary mortality in patients heterozygous for Familial Hypercholesterolemia (FH) (18). Additionally, an increase in serum levels of LDL occurs parallel with ageing, as can be observed in man (19). A decrease in bile acid synthetic capacity, inversely correlated with age, has also been reported, suggesting the importance of bile acid synthetic capacity within cholesterol homeostasis (20). Bile acid synthesis, and modes to regulate this synthetic pathway, are therefore considered an important tool in cholesterol-lowering therapy. Hypercholesterolemic patients have been successfully treated by enhancement of bile acid synthesis via interruption of the enterohepatic circulation (EHC), either via ileal bypass surgery or by administration of bile acid-binding resins cholestyramine or colestid. Successful lowering of serum cholesterol levels in this way has demonstrated the validity of such therapy in a number of clinical trials and studies (21-24).

Although stimulation of bile acid synthesis has yielded positive results, there are disadvantages to the use of bile acid binders such as cholestyramine, being the limited effect (lowering of serum cholesterol by 20-30%), and a poor patient-acceptability. Side-effects may be various gastrointestinal complaints (flatulence, fullness, epigastrive pain, bloating) and nausea (24-26). Research into the mechanisms involved in regulation of bile acid synthesis may reveal new strategies for development of more efficient regulators of cholesterol metabolism. A thorough study into the molecular mechanisms underlying regulation mainly by endogenous effectors, was therefore undertaken, utilizing a system of monolayer cultures of isolated rat hepatocytes as a model. Different pathways to the synthesis of bile acids, known to date, and how they relate to other processes involved in cholesterol homeostasis, are discussed below.

LIVER MORPHOLOGY IN RELATION TO FORMATION OF BILE

Primary bile acids (or bile salts), being cholic- and chenodeoxycholic acid in most animals, are synthesized exclusively in the liver, and are subsequently secreted together with cholesterol, phospholipids, and electrolytes, into bile. In man and pig, bile is stored in the gallbladder, and is postprandially discharged into the duodenum. In some other species, such as rats, absence of a gallbladder results in a more continuous secretion. Thus, these animals exhibit a much higher synthesis and secretion rate of bile acids, as compared to humans (15). Biological functions of bile acids are many-fold. In the liver, bile acids serve to stimulate bile flow, and the subsequent facilitated excretion of hepatic cholesterol, along with elimination of a number of different endogenous and xenobiotic lipophilic compounds (17,27,28). Bile acids are also involved in regulation of cholesterol synthetic- and bile acid synthetic pathways (see below), and may play a role in the regulation of hepatic lipoprotein receptors, consequently influencing the rate of hepatic uptake of lipoprotein cholesterol (29).

Following synthesis, bile acids are actively secreted from the parenchymal cells into the common bile duct (27,30). In most mammals, the majority of bile acids is then stored in the gallbladder, to await release upon hormonal stimulation in the duodenum (17). After intestinal excretion, bile acids act as detergents, forming mixed micelles with various dietary factors, i.e. fats and cholesterol, enhancing their solubilization and uptake by enterocytes (31). Within the intestinal tract bile acids undergo various biotransformations, exerted by a diverse bacterial flora, resulting in the synthesis of *secondary* bile acids, being deoxycholic acid and lithocholic acid, from cholic acid and chenodeoxycholic acid respectively. Other transformations include bacterial deconjugation. The bile acid pool is largely conserved, as a result of efficient resorption of approximately 95% of bile acids in the terminal ileum, followed by return to the liver via enterohepatic circulation (EHC) (17). Solubilization of dietary cholesterol, and a decreased need for bile acid synthesis due to efficient recycling, will increase the overall cholesterol pool and thereby suppress its supply pathways. Several mechanisms, including passive diffusion and active transport, may be involved in the reabsorption process, relative contributions of which may be determined by the hydrophobic:hydrophilic balance of the bile acid pool. Conversion in the intestinal tract may therefore play an additional role in regulation of bile acid synthesis (see below). Additionally, bile acids may be involved in feedback regulation of ileal bile acid transport, and so regulate the composition and size of the bile acid pool (32).

Liver morphology. The liver is composed of numerous acini (33), with at their corners portal canals consisting of small branches of the portal vein, delivering blood rich in intestinally-derived nutrients and hormones from the mesenteric bed, and the hepatic arteries, providing oxygen (Fig. 2i). The efferent bile ductules are the third component of the portal triad, forming the center of the basic structural and functional unit of microcirculation within the

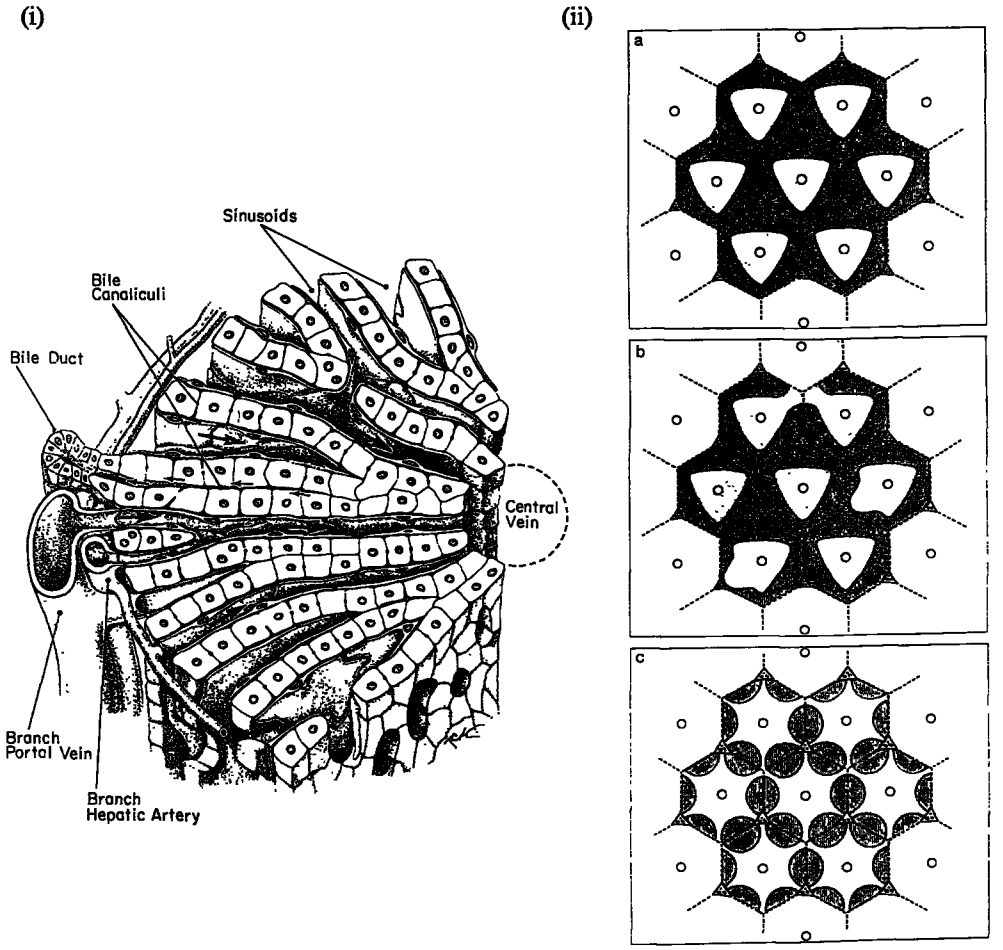


Figure 2. (i) Diagram representing the radial architecture of parenchymal cells, sinusoids and bile canaliculi, situated around the central vein. Large arrows indicate the portocentral flow of sinusoidal blood, originating from afferent portal veins (carrying nutrients from the intestine) and hepatic arteries (providing oxygen); small arrows represent centrifugal flow of bile towards the bile ducts. Reprinted from Bloom et al (1975) A textbook of Histology. (ii) Schematic drawing of Fig. 2i., expressing different views on hepatic architecture. Circles refer to central veins, and triangles to portal triads. Shaded areas represent the periportal zone, which may form a continuum between adjacent hepatic units, as in the metabolic lobular concept (a,b), or be discrete within an acinar concept (c). Conversely, the acinar concept allows limited coninuity of the pericentral zone (open areas), being discrete within a lobular architecture. In (b), the pericentral area may touch the tract boundaries of the larger portal triads, disconnecting the periportal continuum in that case. Based on hepatic expression patterns of several liver-specific enzymes, the situation as depicted in (a,b: lobular concept) is considered to appropriately reflect distribution under normal conditions. In contrast, under certain pathological conditions the acinar concept may provide a better representation (c). Originally reprinted from Lamers et al (36).

liver, within the acinar concept as described by Rappaport (33). However, the pattern of decoloration observed after antegrade and retrograde perfusion of the liver with digitonin, does not fit with a pattern of acinar zones (34,35). Exact definition the liver unit was recently reviewed and reevaluated by Lamers et al. (36), to be more of a lobular than acinar type (Fig. 2ii). Based on spatial distribution patterns of certain liver-specific enzymes, a continuous network is orientated around the terminal vessels, also known as the periportal (PP) zone, while the pericentral (PC) domain consists of a discrete concentric area around each central vein (36,37). The lobular concept is considered to reflect the normal liver-architectural situation, while the acinar model may be more appropriate under certain pathological conditions.

Within a lobule, the majority of cells (both in number and in volume) are parenchymal (also denoted as hepatocytes), arranged in plates situated in a radial fashion, towards the terminal veins (central vein). The parenchymal plates are interconnected to form a three-dimensional network. On either side, blood flows through sinusoids, consisting of fenestrated endothelium, and supplying substrates to the underlying hepatocytes in a portocentral direction. Numerous compounds are transported intracellularly, from basolateral (or sinusoidal) membranes to the apical (or canalicular) membranes, followed by their excretion into the bile canicular lumen. This lumen is formed by the membranes of adjacent hepatocytes, and is sealed off from the sinusoidal compartment by tight junctions. Centrifugal flow of bile transports these compounds to the hepatic bile ducts.

Other liver-cell types are the Kupffer cells, located within the sinusoids, which belong to the mononuclear phagocytotic system (MPS), and liver endothelial cells. The latter may also participate in the hepatic uptake of LDL, followed by HDL-mediated delivery of cholesterol to the site of bile acid synthesis, the parenchymal cells (38). Fat-storing or Ito cells are found in the spaces between sinusoids and parenchymal plates (perisinusoidal space).

Apart from cholesterol synthetic- and metabolic processes, the liver plays an important role in homeostatic regulation of intermediary metabolism of carbohydrates, amino acids, and lipids. It appears that not all hepatocytes contribute equally to this task. In contrast, uptake, storage, interconversion and release of various compounds shows a large degree of heterogeneity along the portocentral axis, even up to a point that only a few cells are involved in a given function (39-41). It has been postulated that the heterogeneous lobular distribution of certain enzymes is a major determinant for proper execution and regulation of a number of liver-specific functions (42). Opposite metabolic pathways like gluconeogenesis and glycolysis are carried out simultaneously in every hepatocyte, but the ratio of both synthetic- and catabolic routes differs in cells from the central and portal zones (43,44). Such heterogeneity of opposite pathways limits futile cycling of metabolites. Importantly, the distribution may be dynamic under different physiological and pathological conditions, in the sense that the liver may adapt to certain requirements by changes in

distribution patterns. Liver cell heterogeneity thus forms the basis for an efficient regulation mechanism, and adaptation to different metabolic states.

The importance of specific liver morphology, and subdivision of liver lobuli into different zones, with respect to supply of oxygen and nutrients via the blood circulation, has been described in the concept of "metabolic zonation" (42). Specific lobular structure may have important implications with respect to portocentral concentrations of various compounds. Thus, bile acid concentrations differ 6-fold from the periportal to pericentral extremes (45,46). Hepatocytes exhibit a general over-capacity to take up, and excrete bile acids into the bile caniculus, irrespective of their zonal origin (45,46), allthough there may be some differences in uptake system used (47). The unequal rates of uptake and biliary excretion of bile acids observed, being highest in the portal area, appear to be directly resultant from portocentral flow of bile acids, and a gradual removal of substances during sinusoidal passage. Increasing the bile acid load results in recruitment of more hepatocytes towards the central area. Conversely, reversing afferent bloodflow shows efficient uptake of bile acids by the pericentral hepatocytes (46). The high uptake rates for bile acids in the periportal area appears to be responsible for the preferential sensitivity of the periportal zone towards lithogenic bile acids (48).

The significance of liver cell heterogeneity for regulation of bile acid synthesis, and overall cholesterol homeostasis, will be discussed separately below.

PATHWAYS TO BILE ACID SYNTHESIS

Primary bile acids are synthesized from cholesterol via a sequence of reactions involving more than 14 different enzymes. In humans, the rat, and hamster, the major primary bile acids formed are cholic acid and chenodeoxycholic acid (49), but there are many species related differences. Thus in rat, a major bile component is β-muricholic acid, a primary bile acid synthesized from chenodeoxycholic acid upon second pass through the liver.

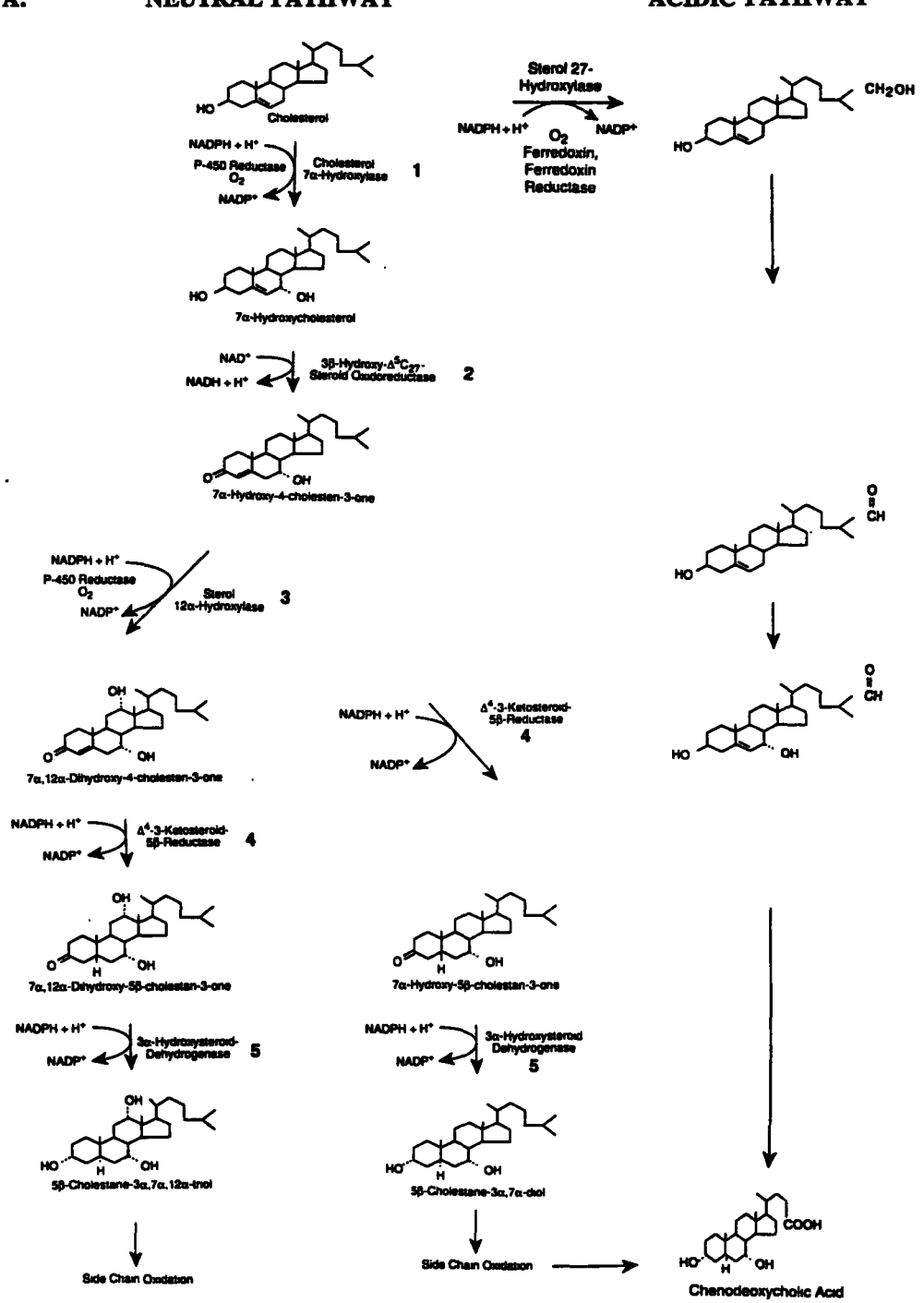
Sequential steps leading to bile acid biosynthesis can be out-lined in two broad categories: (i) those that modify the steroid nucleus, and (ii) those that involve oxidation and subsequent shortening of the side-chain. The order in which these reactions take place is not entirely fixed, resulting in two major pathways towards formation of bile acids (Fig. 3). Additionally, some of the enzymes exhibit substrate promiscuity, and are located in various different organelles within the heaptocytes, involving complex intracellular trafficking and the translocation of cholesterol and various intermediates to bile acids.

The neutral route

The main route to bile acids is considered to be initiated by 7α-hydroxylation of cholesterol (reaction 1), catalyzed by the enzyme cholesterol 7α-hydroxylase, which is located in the smooth endoplasmatic reticulum, and considered the rate-limiting and main point of regulation within the synthetic pathway (see below). In the second step, catalyzed by another microsomal enzyme, 3β-hydroxy-C_{27}-steroid oxidoreductase (reaction 2), 7α-hydroxy-4-cholesten-3-one is formed, which may undergo one of two possible biotransformations. If the intermediate is acted upon by sterol 12α-hydroxylase (reaction 3), also a microsomal cytochrome P-450 enzyme, this will result in the formation of cholic acid. Thus the enzyme forms the branching point within the biosynthetic route, and may be important in determination of the cholic:chenodeoxycholic acid ratio within the bile acid pool, at least in the hamster (50).

Alternatively, 7α-hydroxy-4-cholesten-3-one can serve as a substrate for a soluble Δ^4-3-oxosteroid 5β-reductase (reaction 4), indicating transport of the intermediate to the cytosol, which will result in the formation of chenodeoxycholic acid. This enzyme also catalyzes the conversion of the product of 12α-hydroxylation, 7α,12α-dihydroxy-4-cholesten-3-one. All subsequent enzymatic transformations are shared by the two intermediates (reactions 5-14). A soluble 3α-hydroxysteroid dehydrogenase (reaction 5) catalyzes both oxidation and reduction of a number of different substrates (51). In addition, this enzyme may serve to shuttle bile acids from the sinusoidal to apical membranes of the rat hepatocyte (52). Products of 3α-hydroxysteroid dehydrogenase undergo side-chain degradation. The first step is catalyzed by a mitochondrial cytochrome P-450 sterol 27-hydroxylase (reaction 6), selectively introducing a hydroxyl group at C-27 of bile alcohols 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol (53). Substrate for this enzyme, however, is not limited to the bile alcohols described; the enzyme will also oxidize other sterols, including cholesterol, and vitamin D_3 (54-58). Hence this enzyme may also be involved in other pathways to bile acid synthesis, as described below. Subsequent formation of the carboxylic derivative of the product of reaction 6, may be accomplished by the same enzyme, yielding 3α,7α,12α-trihydroxy-5β-cholestanoic acid. Alternatively, formation of the latter may be a result of a concerted action of a cytoplasmatic NAD-linked alcohol dehydrogenase (reaction 7), followed by a NAD-requiring aldehyde dehydrogenase (reaction 8) (59,60). Relative contributions of these 3 enzymes to the formation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid *in vivo* is unknown. Reaction 9 is catalyzed by a microsomal coenzyme A ligase, utilizing ATP and coenzyme A, to form a thioesterified 5β-cholestanoic acid.

The final biotransformations of the side-chain take place mainly in the peroxisome (61), following a pathway very similar to β-oxidation of fatty acids in this organelle (62). The CoA-ester is acted upon by an oxidase and bifunctional dehydrogenase/hydratase, yielding a C_{24}-derivative (reactions 10,11). 3α,7α12α,24-tetrahydroxy-5β-cholestanoyl-CoA formation


A.
NEUTRAL PATHWAY
ACIDIC PATHWAY
Cholesterol
NADPH + H+
P-450 Reductase
O2
NADP+
Cholesterol 7α-Hydroxylase
1
7α-Hydroxycholesterol
NAD+
NADH + H+
3β-Hydroxy-Δ5C27-Steroid Oxidoreductase
2
7α-Hydroxy-4-cholesten-3-one
Sterol 27-Hydroxylase
Ferredoxin, Ferredoxin Reductase
CH2OH
HO
O=CH
NADPH + H+
P-450 Reductase
O2
NADP+
Sterol 12α-Hydroxylase
3
7α,12α-Dihydroxy-4-cholesten-3-one
NADPH + H+
NADP+
Δ4-3-Ketosteroid-5β-Reductase
4
7α,12α-Dihydroxy-5β-cholestan-3-one
7α-Hydroxy-5β-cholestan-3-one
NADPH + H+
NADP+
3α-Hydroxysteroid Dehydrogenase
5
5β-Cholestane-3α,7α,12α-triol
5β-Cholestane-3α,7α-diol
Side Chain Oxidation
COOH
Chenodeoxycholic Acid

B. Side-chain degradation

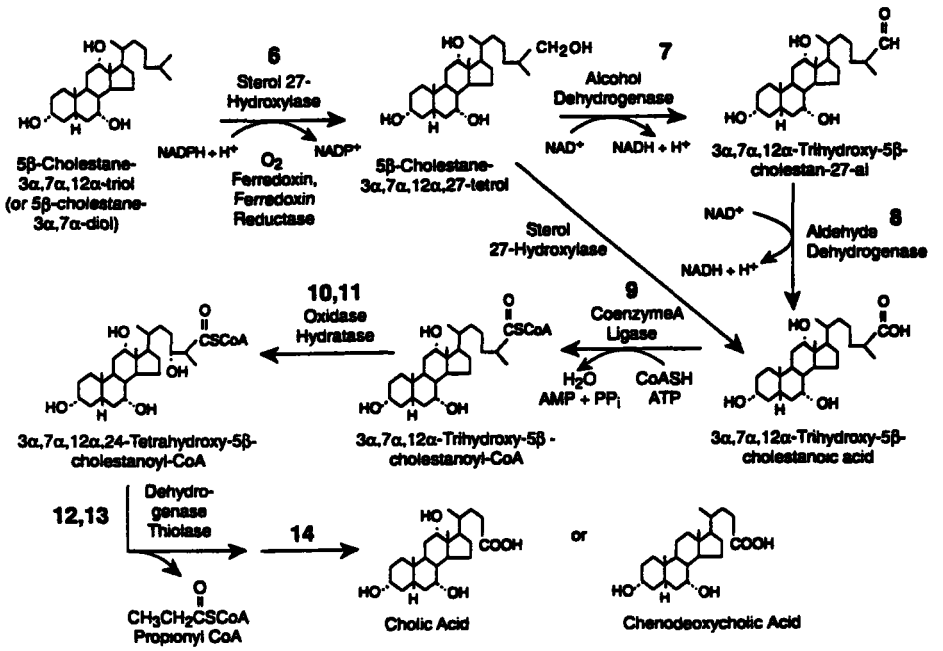
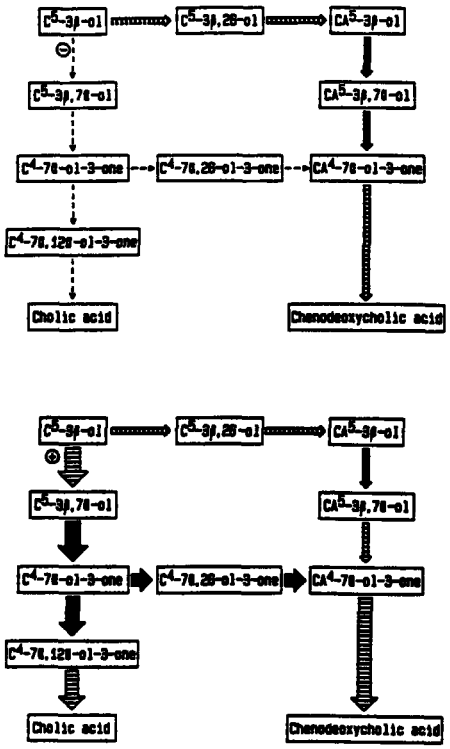


Figure 3. Pathways to formation of bile acids. (A) Neutral or '7α-hydroxylase' pathway (reactions 1 to 5), as opposed to the acidic or '27-hydroxylase' pathway, which has been simplified for reasons of clarity. Enzymes depicted can show promiscuity with respect to substrates, and may feature in both synthetic routes. Hence, enzymes starring in side-chain degradation of bile acid intermediates (B), also function in the acidic pathway. (B) Side-chain degradation of products generated via the neutral pathway (reactions 6 to 14). Adapted from Russell & Setchell (162).



C. Contribution of neutral and acidic pathways to bile acid synthesis

Bile acid synthesis in humans, as proposed by Axelsson & Sjövall (66), and relative contributions under different physiological conditions. Level of cholesterol 7α-hydroxylase activity is indicated by a '+'(e.g. reduced intestinal reabsorption of bile acids) or '-' (liver disease, e.g. alcoholic liver cirrhosis), determining the flux of cholesterol towards the neutral or acidic pathway, respectively.

goes through a Δ^{24} intermediate (63), followed by hydration. A 24-oxo intermediate is subsequently formed, and thiolytic cleavage results in the loss of a propionyl-CoA, to form a C_{24}-bile acid-CoA (reactions 12,13).

The final steps in bile acid biosynthesis are somewhat unclear (reaction 14). The thiolase product may exit the peroxisome as a CoA derivative, or as an unesterified cholic or chenodeoxycholic acid. Reconjugation may occur outside the peroxisome, as a microsomal bile acid CoA synthase has been reported (64). This derivative is substrate for a bile acid-CoA:amino acid N-acyltransferase, utilizing either glycine or taurine to form the corresponding N-acyl bile acids. 98% of bile acids are excreted in the conjugated form, lowering their pK and solubility at physiological intestinal pH.

Enrichment of the latter enzyme, which has equal affinity for both glycine and taurine as substrates, within a peroxisomal fraction (65), suggests that all final steps of the bile acid synthetic pathway take place in the peroxisomes. Nevertheless, the subcellular localization of enzymes of the bile acid biosynthetic route, within a number of different organelles, demonstrates that bile acid intermediates undergo complex intracellular transport prior to

becoming primary bile acids. Thus, within the route described, intermediates travel from ER→cytoplasm→mitochondria→cytoplasm→ER→peroxisome→cytoplasm to bile. How cellular trafficking is mediated is, at present, largely unknown, allthough the soluble 3α-hydroxy steroid dehydrogenase has been assigned such a function (52).

Alternative pathway

Based on the occurrance of bile acid intermediates in human serum, which can not be explained by the sequence of events depicted in the above, an alternative route to bile acids has been proposed. In this case, side-chain degradation precedes biotransformation of the steroid nucleus, yielding acidic C_{24}-intermediates (Fig. 3) (66). This pathway has therefore been termed 'acidic' pathway, in contrast to the 'classical', or 'neutral' pathway described, initiated by 7α-hydroxylation of cholesterol. As stated, the mitochondrial sterol 27-hydroxylase exhibits activity towards several C_{27}-sterol substrates, including cholesterol (56,67). The enzyme has been referred to as sterol 26-hydroxylase in past literature; however, on the basis of stereochemistry of the reaction, it should be termed a sterol 27-hydroxylase (16).It has been demonstrated that the resulting 27-hydroxycholesterol is further metabolized to 3β-hydroxy-5-cholenoic acid in rat liver peroxisomes (68), and to 7α-hydroxy intermediates by human liver microsomes and mitochondria (69,70), ultimately leading to preferential formation of chenodeoxycholic acid in both rat and man (71-74). It appears that the microsomal 12α-hydroxylase is specific to side-chain length; once side-chain degradation has been performed, the enzym can no longer utilize the resulting products, yielding only chenodeoxycholic acid (16).

There is accumulating evidence for an important contribution of both neutral and acidic routes, to overall bile acid synthesis both in humans (66), and in cultured human and rat hepatocytes (75,76). The bile acid intermediates 27-hydroxycholesterol and 3β-hydroxy-5-cholenoic acid are present in relative high concentrations in human blood (66,77,78). It was recently described that baboons showing a low response to an atherosclerotic diet, i.e. supplemented with 2% cholesterol, have a markedly higher sterol 27-hydroxylase activity than do high-responders on a similar diet (79), indicating the importance of the enzyme for cholesterol maintenance. It has been postulated that the alternative route contributes substantially to bile acid synthesis in normal subjects, but that this pathway may even become the major one under certain pathological conditions (66). In cultured rat and human hepatocytes, the immunosuppressive drug cyclosporin A was shown to selectively inhibit sterol 27-hydroxylase activity towards cholesterol, resulting in approximately 50% reduction in total bile acid synthesis. Interestingly, only chenodeoxycholic acid synthesis was markedly suppressed, demonstrative of the selective production of the particular bile acid via this pathway (75,76). Patients suffering from a defect in sterol 27-hydroxylase (CTX), similarly exhibit decreased levels of chenodeoxycholic acid synthesis (80).

CYTOCHROME P-450 ENZYMES INVOLVED IN BILE ACID BIOSYNTHESIS - LINKS TO REGULATORY MECHANISMS

Members of the large cytochrome P-450 superfamily have been shown to be subject to regulation bynumerous endogenous and exogenous compounds (81). It is therefore not surprising that also this subgroup of bile acid biosynthetic enzymes, has been implicated to play an important role in the overall regulation of the bile acid synthetic pathways.

Cholesterol 7α-hydroxylase

The first and rate-limiting step in the neutral pathway to bile acids involves modification of the ringstructure of cholesterol by introduction of an hydroxyl group at position C-7, in the axial (α) configuration. This reaction is catalyzed by cholesterol 7α-hydroxylase (EC 1.14.13.17), member of the large cytochrome P-450 superfamily, and a mixed function monooxygenase (81). Generally, the latter enzymes catalyze oxidation reactions, introducing one atom of molecular oxygen into the substrate, and reducing the other to water (68). Cholesterol 7α-hydroxylase utilizes NADPH as an electron donor, and a protein cofactor, cytochrome P-450 reductase, for transfer of the electrons to the cytochrome P-450 entity (82). The enzyme is located in the smooth endoplasmatic reticulum, and is one of several cytochrome P-450 enzymes involved in primary bile acid synthesis. It is highly specific to 7α-hydroxylation of cholesterol and cholestanol (83). The enzyme is present in trace quantities in the liver, and was purified in 1985 by Andersson et al. (84), followed by subsequent adaptation of the procedure described to obtain sufficient amounts for protein sequencing, antibody production, and cloning (85-93). The amino acid sequence deduced from these studies revealed a unique P-450 protein of 503 (rat) or 504 (human) amino acids, belonging to a novel gene family (VII or *CYP7*), with a predicted molecular weight between 56-57 KD. Further analysis revealed that cholesterol 7α-hydroxylase bears most similarity to lauric acid ω-hydroxylase, mitochondrial side-chain cleavage P-450 and sterol 27-hydroxylase (90). A cluster of conserved amino acids between cholesterol 7α-hydroxylase and other cholesterol-metabolizing cytochrome P-450-enzymes suggest that these residues are related to recognition of cholesterol substrate. Based on Southern-blot analysis (92,93), it was postulated that only a single copy of the gene is present in rat chromosomes (chromosome 8q11-q12) (94). Expression of the enzyme is largely limited to the liver, allthough minor amounts of activity were detected in lung, kidney, and heart tissue (87). Expression of cholesterol 7α-hydroxylase mRNA, however, has only been found in liver (89). Northern-blotting revealed the presence of multiple mRNAs originating from the single gene copy, both *in vivo* and in cultured rat hepatocytes, with reported lengths of 1.8, 2.1, 3.6 and 4.0 kb (89,95), allthough reports vary. Variable lengths are the result of multiple poly-adenylation sites present in the 3'-untranslated region (UTR), generating mRNAs with

variable polyA-tails. Furthermore, the 3'-UTR is rich in AU-sequence, containing AUUUA motifs (29 times in rat), and 5'-AAU-3' or 5'-UAA-3' trinucleotides (96), which have been linked to rapid degradation of mRNA (97,98). In accordance, the mRNA half-life for this enzyme has been estimated to be 4 hours (95), in line with reported short half-life of 2-3 hours for the enzyme (99,100).

The gene encoding cholesterol 7α-hydroxylase has been isolated from rat (92,93), human (94,101), hamster (102), and mouse (103) genomic DNA. The rat gene spans 11 kilobases, containing six exons and five introns, of which individual locations share no homology with any other P-450 genes (93). The 5'-flanking region of the human gene has been characterized (101,104), harboring a number of basic and liver-specific sequences involved in recognition by transcriptional factors. The first 400 nucleotides appeared to contain the fundamental elements required for cell-specific expression, e.g. a TATA box at -30, a modified CAAT box at position -92, and three potential hepatocyte nuclear factor 3 (HNF-3) recognition sites at nucleotides -316, -288, and -255, implicating the latter as essential for cell-specific enhancement of cholesterol 7α-hydroxylase promoter activity (Fig. 4) (104). Homology between promoter sequences of human and rat, specifically within the region of -432 and -220 which apppears to confer cell specificity of human CYP7, is limited to approximately 50%, and variance in the regulatory elements present within the promoter regions of different species, suggests that regulation at the DNA level may show species related differences. Demonstrating the latter, the 5'-flanking region of the rat cholesterol 7α-hydroxylase promoter harbors recognition sequences for a liver factor B1(LFB-1, 5'-GTTATT), and a basic transcription element (BTE, 5'-AGTAGGAGG) (92,105). In addition, a number of sequences related to physiological regulatory processes (i.e. regulation by bile acids, hormones, cholesterol, diurnal rhythm) have been identified (105-107), which will be discussed below.

Mitochondrial sterol 27-hydroxylase

The mitochondrial cytochrome P-450 sterol 27-hydroxylase (EC 1.14.13.15) is a mixed-function monooxygenase, exhibiting specificity for various substrates. Thus, it not only utilizes products of the 3α-hydroxysteroid dehydrogenase reaction, being di- and tri-hydroxy bile acid intermediates, but is also specific towards cholesterol, resulting in oxidation and shortening of the side-chain to yield 27-hydroxycholesterol. The enzyme resides in the inner mitochondrial membrane (60), and utilizes molecular oxygen, NADPH, and two protein cofactors, ferredoxin and ferredoxin reductase, to introduce an hydroxyl group at the C27 position (53).

cDNAs encoding the rabbit, rat, and human sterol 27-hydroxylase have been isolated, and have been shown to encode an enzyme of 530 amino acids (108-111). The cDNAs all encode a cleavable mitochondrial signal-sequence, and a relatively short 3'-UTR, within which

A.

```
-401 GTTCATTTAA ATAATTTTCC CCGAAGTACA TTATGGGCAG CCAGTGTTGT GATGGGAAGC

                                                    E
                                                 *********
     TTCTGCCTGT TTTGCTTTGC GTCGTGCTCC ACACCTTTGA CAGATGTGCT CATCTGTTTA

                                                                  D
                                                              **********
-281 CTTCTTTTTC TACACACAGA GCACAGCATT AGCTGCTGTC CCGGCTTTGG ATGTTATGTC

                                          C
     *                                 * **********             ^^^^^^
     AGCACATGAG GGACAGACCT TCAGCTTATC GAGTATTGCA GCTCTCTGTT TGTTCTGGAG

                                         B
                                      ******* *
-161 CCTCTTCTGA GACTATGGAC TTAGTTCAAG GCCGGGTAAT GCTATTTTTT TCTTCTTTTT

        ooooooo oo          .........                 xxxx xx
     TCTAGTAGGA GGACAAATAG TGTTTGCTTT GGTCACTCAA GTTCAAGTTA TTGGATCATG

                                                    *
- 41 GTCCTGTGCA CATATAAAGT CTAGTCAGAC CCACTGTTTC GGgacagccttgctttgctag

     gcaaagagtctccccttg gaaattttcc tgcttttgca aa +61
```

B.

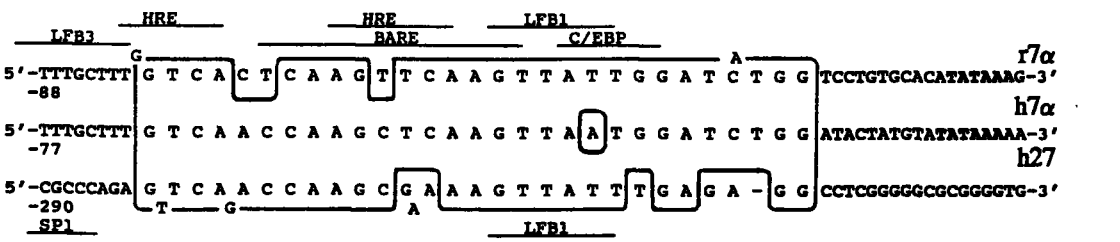


Figure 4. (A) Nucleotide sequence of the 5'-flanking region of the rat cholesterol 7α-hydroxylase gene. The transcriptional initiation site is indicated by a single asterix (+1). The TATA box is printed in boldface. Lowercase lettering indicates the beginning of the leader region. The -49 to -79 region, as indicated by Hoekman et al (105), contains several putative regulatory elements: a possible bile-acid-responsive-element (BARE; 5'-TCAAGTTCAAGT-3'), consisting of a direct repeat, is underlined, as well as a CCAAT-Box (5'-ATTGG-3', C/EBPα binding site); (xxxxx), LFB1 binding site; 3 putative HREs are overlined, the first two of which form an imperfect repeat of a consensus type II steroid/thyroid HRE (5'-AG(G/T)TCA-3'). Other sequences include a TGT3 element (5'-TGTTTGCTTT-3' (....); binding site for LFB3), and just downstream a basic transcription element (BTE; 5'-AGTAGGAGG-3' (oooo)). Further downstream are several DBP-binding sites (B-E, (*****)), as reported in ref. 106 and 174), and a human glucocorticoid responsive element 5 (HGRE5; 5'-TGTTCT-3' (^^^^)) (105). (B) A distinct promoter region of the rat and human cholesterol 7α-hydroxylase shows homology with a region within the human sterol 27-hydroxylase promoter (sequence from ref. 122).

sequences related to instability of the cholesterol 7α-hydroxylase mRNA are virtually absent. Sterol 27-hydroxylase activity and mRNA is present in many tissues (108,109,112), suggesting that the activity of the enzyme is not restricted to bile acid synthesis. mRNA expression for the enzyme in different tissues could be correlated to cholesterol synthetic capacity (109). Combined with the knowledge that oxysterols are known to be potent regulators of cholesterol synthetic enzyme HMG-CoA reductase, and expression of the LDL receptor gene (113,114), it has been suggested that sterol 27-hydroxylase plays a role in regulation of endogenous cholesterol synthesis and lipoprotein uptake in extrahepatic tissue (109,115). Uncontrolled endogenous cholesterol synthesis, and abnormal cholesterol metabolism resulting from a molecular defect in the sterol 27-hydroxylase (111,112,116-119), was recognized to underly the build-up of abnormal levels of cholesterol and C27 bile acid derivatives in tissues and serum, and occurrence of multiple xanthomas and neurological disorders in patients suffering from cerebrotendinous xanthomatosis (CTX) (80,120). Recently, high levels of sterol 27-hydroxylase have been described by Reiss et al. (121), to be present in vascular endothelium, providing a mechanism for preventing the accumulation of intracellular cholesterol in the vascular wall, by down-regulation of LDL-receptor expression and HMG-CoA reductase. In addition, presence of the enzyme can be taken as the initiation of extrahepatic bile acid synthesis. Subsequent excretion of oxysterols in the blood circulation is followed by rapid clearance by the liver and fueling into bile acid synthesis.

Multiple mRNAs have been detected for the rat sterol 27-hydroxylase, of both 2.4 and 2.1 kb. The liver only exhibits expression of the single 2.4 kb mRNA, but both may be present in the ovaries (108), when rats are stimulated with gonadotropin.

The human sterol 27-hydroxylase gene has been isolated, and was shown to span a length of 18.6 kb, consisting of nine exons and eight introns. The promoter region apppeared GC-rich, and contained putative recognition sites for transcription factor SP1 and LFB-1 (122).

Virtually nothing is known about regulation of this enzyme. It has been suggested that sterol 27-hydroxylase is of minor importance for the regulation of bile acid synthesis or composition of bile acids formed (123), allthough cholic acid and starvation were reported to reduce activity by 30-60% (124).

Sterol 12α-hydroxylase

The sterol 12α-hydroxylase has been purified from rat liver, and shown to be a microsomal cytochrome P-450 enzyme with a molecular weight of 56 KD (125). A cDNA for this enzyme has, however, not been reported. Being the branching point within bile acid synthesis, this enzyme - as well as sterol 27-hydroxylase- may be important for the determination of the cholic:chenodeoxycholic ratio, and hence the relative hydrophobicity of the bile acid pool, since chenodeoxycholic acid is readily converted to hydrophilic β-

muricholic acid in rats, both *in vivo* and *in vitro*. A strong correlation between 12α-hydroxylase and bile acid composition has been found in hamsters (50), allthough such a correlation is not evident in humans (126).

Concerning regulatory processes affecting this enzyme, limited data is available. Biliary diversion increases specific activity (127), and the enzyme is suppressed by chenodeoxycholic acid treatment in humans (128), and by thyroxine (129,130). Suppression of activity of 12α-hydroxylase is also seen in rats with streptozotocin-induced diabetes, with a subsequent normalization by insulin treatment (131,132).

Other potentially important enzymes

- *3α-hydroxysteroid dehydrogenase*. As stated, this particular dehydrogenase is not only responsible for the metabolism of certain bile acid intermediates, but it shows high affinity for certain bile acids, which are subsequently translocated from basolateral to canalicular membranes, without being metabolized by the enzyme (50). Specificity with regard to affinity for different bile acids, and traversement through of the cytoplasmatic space, may be important in bile acid mediated feedback control (see below).
- *lithocholic acid 6α/6β-hydroxylase*. In some species, secondary bile acids may undergo further metabolism, following intestinal reabsorption and transport back to the liver via portal blood. In rat and hamster, the bacterial transformation product of chenodeoxycholic acid, lithocholic acid, may be hydroxylated upon return to the liver by a 6α- or 6β-hydroxylase, yielding hyodeoxycholic and murideoxycholic acids, respectively. These metabolic events may serve to prevent damage to the liver by hydrophobic bile acids. The lithocholic acid 6β-hydroxylase has been shown to be stimulated by cholic acid feeding of hamsters under 6 weeks of age (133). Similarly, expression of mRNA and transcriptional activity of this enzyme has been shown to be localized periportally, where blood rich in nutrients (and bile acids) enters the liver (134). Thus, the enzyme may contribute to bile acid synthesis-mediated excretion of compounds, and regulation of bile acid synthesis, by acting on the overall hydrophobic:hydrophilic balance of the bile acid pool to which hepatocytes are exposed.

REGULATORY PROCESSES AFFECTING BILE ACID BIOSYNTHESIS

Hepatic hydroxylases involved in regulation of bile acid biosynthesis

Most of our knowledge regarding regulation of bile acid synthesis has been gathered in the rat. Due to the absence of a gall bladder, and the presence of an unusually long small intestine, these animals exhibit a high capacity to form bile acids (15). These anatomical and biochemical differences have to be kept in mind, when extrapolating results obtained in these animals to other species. Nevertheless, most animals share the same rate-limiting steps in bile

acid biosynthesis, and many forms of regulation of the pathway, known to date, have been recognized over a wide range of different species.

Bile acid-induced biofeedback

According to current concepts, the most important way in which bile acid synthesis is regulated, is by the flux of bile acids returning to the liver via enterohepatic circulation (EHC). This hypothesis was initially based on experiments of Eriksson (135), Thompson and Vars (136), and others (127,137,138), showing a several-fold stimulation of bile acid synthesis in rats, after complete biliary diversion (CBD), or by administration of anion-exchange copolymers that bind bile acids in the small intestine and thereby prevent their uptake and feedback regulation (139). Further evidence was provided by administration of bile acids to CBD-rats, either intraduodenally or intravenously, leading to normalization of bile acid synthetic capacity (137,140). Shefer et al. (141) subsequently showed that administration of taurocholate inhibited the conversion of radio-labeled acetate, mevalonate, and cholesterol, but not of 7α-hydroxycholesterol, indicating that the enzyme responsible for the first and rate-limiting step in the conversion of cholesterol to bile acids, cholesterol 7α-hydroxylase, was the regulatory point. In addition, biliary drainage or cholestyramine-treatment were found to stimulate cholesterol 7α-hydroxylase activity 5 to 8-fold, while enzymes involved in subsequent steps of bile acid formation were affected to a lesser extent, or not at all (127,142).

Further resolution as to the level of regulation, and the molecular mechanism involved, had to await the isolation of cDNA and antibody probes for cholesterol 7α-hydroxylase. It has now been established that bile acid feeding reduces, and cholesterol and cholestyramine-treatment increases cholesterol 7α-hydroxylase *in vivo* at the level of activity, protein mass, mRNA, and rate of gene transcription (87,89,90,143).

While regulation studies have focussed mainly on cholesterol 7α-hydroxylase as major point of regulation by bile acids, and other factors, it was recently shown in a system of cultured rat hepatocytes that the sterol 27-hydroxylase is subject to similar forms of regulation. The enzyme was shown to be suppressed by various bile acids, parallel to an effect on cholesterol 7α-hydroxylase, at the level of activity, mRNA, and gene transcription (144). Results led to suggest that co-ordinate suppression of both enzymes may efficiently lead to homeostatic regulation of bile acid synthesis.

Mechanistic aspects: There was still concern, however, whether bile acids must be regarded as the actual regulators of cholesterol 7α-hydroxylase, or if effects were exerted indirectly. Failure to detect a direct effect of bile acids on bile acid synthesis in rat hepatocytes in suspension (145), or in monolayer cultures of rat (146,147) and rabbit (148), even in concentrations greatly exceeding those found in portal blood of rats, seriously challenged the

concept of feedback regulation. It was suggested that an intact EHC be a prerequisite for bile acid-induced feedback regulation, resulting in the speculation that possible intestinal factors are the real regulators of cholesterol 7α-hydroxylase (149). These factors would be dependent on bile acid flux for their absorption from the intestine (150). However, Kwekkeboom et al. (151,152), and later Twisk et al. (95) demonstrated that addition of physiological concentrations of bile acids to the culture medium of pig and rat hepatocytes, respectively, resulted in a strong decrease of bile acid synthesis and cholesterol 7α-hydroxylase activity, mRNA, and gene transcription, indicative of a direct effect of these compounds on the hepatocyte.

Another suggestion was based upon the observation of similar induction of both HMG-CoA reductase and cholesterol 7α-hydroxylase as a result of biliary diversion, or, alternatively, co-ordinate suppression of the two enzymes after bile acid feeding (153,154, and others). The possibility that cholesterol 7α-hydroxylase might be regulated by the flux of newly synthesized cholesterol in this way, was addressed by Pandak (155). Experiments, in which taurocholate infusion was combined with constant substrate supply, in the form of mevalonate, showed sustained inhibition of cholesterol 7α-hydroxylase, indicating this not to be a likely option.

Regulation at the level of cholesterol 7α-hydroxylase gene transcription could, however, only partially explain the strong suppression of cholesterol 7α-hydroxylase activity and mRNA levels by bile acids, as observed in cultured rat hepatocytes (95,105), suggesting that bile acids exert an effect both at the transcriptional- and post-transcriptional level. In line with this assumption, analysis of the primary and secondary structure of the 3'-non-coding region of the cholesterol 7α-hydroxylase mRNA has revealed the presence of multiple AU-rich sequences (96), shown to be involved in destabilization of various mRNAs (97,98). A perhaps direct or indirect action of bile acids towards these destabilizing sequences, however, has not been demonstrated. Interestingly, these sequences are virtually absent within the 3'-non-coding region of sterol 27-hydroxylase mRNA (108,109), suggesting that destabilization of mRNA does not play a major role in the regulation of sterol 27-hydroxylase mRNA. In agreement with this thought, a much longer half-life (13 hours) for this particular messenger was calculated from incubation studies using taurocholate (144), whereas similar experiments revealed a half-life for cholesterol 7α-hydroxylase mRNA of only 4 hours (95).

Analysis of the cholesterol 7α-hydroxylase promoter has resulted in the identification of several sequences, possibly involved in transcriptional expression of the cholesterol 7α-hydroxylase gene (92,104,105,107). In addition, using functional analysis in transient expression assays with promoter-reporter constructs, a putative bile acid responsive element (BARE) has been characterized (105) within a region of -79 to -49 nucleotides preceding the transcriptional initiation site (Fig. 4A). A direct repeat TCAAGTTCAAGT is located within this region, which has recently been shown to interact with a 57,000 dalton protein, possibly

acting as a bile acid responsive protein (BARP). It is envisioned that this protein may be involved in mediating an interaction between a putative bile acid:bile acid-receptor complex, and the responsive DNA-element (107). Low specificity was shown, however, with respect to binding of the direct repeat to factors from various rat liver nuclear extracts, be they from control, bile acid-fed, or cholestyramine-treated rats (107). Other putative regulatory sequences within this region are imperfect repeats of hormone receptor elements (HREs, 5-AG(T)TCA), together forming a region possibly responsive to certain steroids and/or thyroid hormones (TRE) (105,107). The superficial resemblance of bile acids to steroids could provoke an interaction with these HREs via general hormone receptors (see below).

The sterol 27-hydroxylase promoter has not been studied as extensively as that of cholesterol 7α-hydroxylase, for the simple reason that the enzyme was not considered to be involved in regulation of bile acid synthesis (123). Promoter sequences of the human sterol 27-hydroxylase gene have been published (122), however, and a region between -254 and -280, very homologous to part of the rat cholesterol 7α-hydroxylase promoter, has been described (Fig. 4B) (144). This very region harbors two putative HRE-sequences, in part overlapping a consensus sequence for LFB-1, indicating a similar topology of several binding sequences, which have been conserved between the two different enzymes. This may explain the co-ordinate transcriptional regulation of both enzymes by similar bile acids (144).

Bile acid structure: With regard to a functional structure of a bile acid in terms of potency to inhibit bile acid synthesis and cholesterol 7α-hydroxylase, it has been postulated that repressional activity is directly correlated to hydrophobicity of a given bile acid (153,156). After feeding different bile acids to rats with an intact EHC, it was observed that relatively hydrophilic bile acids (ursocholate, ursodeoxycholate, hyocholate, hyodeoxycholate) did not affect cholesterol 7α-hydroxylase activity. However, more hydrophobic bile acids (cholate, chenodeoxycholate, deoxycholate) inhibited cholesterol 7α-hydroxylase and HMG-CoA-reductase activities, in order of increasing hydrophobicity (153). Other views exist, reporting taurocholate not active, whereas taurodeoxycholate and taurolithocholate were strong inhibitors of bile acid synthesis. It was suggested that primary bile acids first have to be converted intestinally to become regulatory (157,158). This view was substantiated by the observation that direct intravenous infusion of taurocholate did not result in enhanced feed-back regulation, in contrast to intraduodenal administration of the compound (159). Alternatively, specifically monohydroxy bile acids, derived either from intestinal or hepatic sources, were reported to effectively down-regulate bile acid synthesis in the rabbit, indicating hydroxylation status *per se* to be important for inhibitory potency (158). It was also reported the 7β-hydroxy epimers of both cholic acid and chenodeoxycholic acid had little or no ability to regulate expression of the enzyme (154,160,161), leading to suggest that differences in regulatory potency be related to a decreased ability of 7β-epimers to promote

cholesterol uptake in the small intestine (162). However, the knowledge that bile acids act directly on the hepatocyte, does not point to such a mechanism (95,151,152). Furthermore, cholesterol has been reported to have stimulatory effects on cholesterol 7α-hydroxylase (see below), excluding such a possibility.

Studies in primary hepatocytes of both pig and rat, have indicated an important role for hydrophobic bile acids in eliciting biofeedback. However, within the limited group of bile acids used, considerable differences in hydrophobicity index (HI_x) could not always be correlated to the extent of inhibition (95,152). A large study was conducted with monolayer cultures of primary rat hepatocytes, incubated with a variety of bile acids differing highly in HI_x, and number, position, and orientation (α/β) of the hydroxylgroups present on the steroid backbone. From this study, indirect effects of bile acids related to intestinal events (conversion, processes related to cholesterol uptake, intestinal or lymph-factor) could be excluded. Only a weak correlation between potency of down-regulation of cholesterol 7α-hydroxylase, and the hydrophobicity index was found ($R = 0.61$). It was postulated that the hydroxyl groups present on the steroid backbone may form a hydrophilic microenviroment within an otherwise hydrophobic molecule, provided that the different hydroxyl groups are located in the proper position and orientation (163). Creation of such an environment could be responsible for the direct or indirect selective binding to a putative BARP or BARE described, eliciting an event at the transcriptional level.

Hormonal regulation in relation to diurnal rhythm

Several hormones have been implicated to play an important role in regulation of bile acid synthesis, both *in vitro* and *in vivo* (ref. 82 for a review).

(i) In rats, daily injections of thyroxine led to a 2 to 3-fold increase in the activity of cholesterol 7α-hydroxylase (164,165). Thyroid hormones also increase the chenodeoxycholic acid to cholic acid ratio, indicating an effect on 12α-hydroxylase (129,130), and possibly on sterol 27-hydroxylase (129). Hypophysectomized rats administered thyroid hormones showed a rapid increase in levels of cholesterol 7α-hydroxylase activity and mRNA expression (166). Possible interaction via the TRE described (Fig. 4A) was suggested.

(ii) Specific involvement of glucocorticoids in up-regulation of cholesterol 7α-hydroxylase *in vivo* was apparent after adrenalectomy (167). It was subsequently shown that dexamethasone, and other members of the class of glucocorticoids, stimulated cholesterol 7α-hydroxylase activity, while this is not the case for sex steroid hormones, or pregnenolone or the mineralocorticoid aldosterone (168). Hylemon et al. (169) subsequently demonstrated that addition of both T_4 and dexamethasone to hepatocytes in culture led to strong up-regulation of cholesterol 7α-hydroxylase mRNA, and gene transcription, allthough in the absence of these hormones, cholesterol 7α-hydroxylase was almost undetectable. Evidence seems to point to mRNA stability as a regulatory point of action by dexamethasone (170), and a

general up-regulation of transcriptional activity of multiple genes, while a more direct role in transcriptional activation of the cholesterol 7α-hydroxylase gene has been shown for thyroid hormone (105).

Diurnal rhythm: In previous reports, the regulatory action of glucocorticoids has been connected to a diurnal rhythm, exhibited by both cholesterol 7α-hydroxylase and HMG-CoA reductase. These enzymes show a peak in protein, enzyme activity, and mRNA at midnight, parallel to the nocturnal feeding-behaviour of most rodents (82,87,96). Serum concentrations of glucocorticoids precede cholesterol 7α-hydroxylase activity levels somewhat (171). Additionally, complete loss of diurnal rhythm in cholesterol 7α-hydroxylase was demonstrated upon bilateral adrenalectomy, or by suppressing the secretion of adrenocorticotrophine (ACTH) (167,172). Recent studies by Lavery & Schibler (106), however, have shown that daily fluctuations are the result of a parallel expression of liver-enriched rat albumin D-element binding protein (DBP) (173), member of the basic leucine zipper (bZIP) family. Sequences responsive to DBP have been shown to be present in the promoter region of cholesterol 7α-hydroxylase (106,174), and DBP may co-operate with C/EBP to transcriptionally activate the cholesterol 7α-hydroxylase gene (174). DBP expression activated cholesterol 7α-hydroxylase promoter activity in cotransfection experiments. Interestingly, addition of dexamethasone in these experiments resulted in reduced cholesterol 7α-hydroxylase promoter activity, suggesting that glucocorticoids are not the mediators of diurnal rhythm (106). Recently, LDL-receptor expression has also been shown to fluctuate in a diurnal manner (175). Treatment of rats with dexamethasone increased expression at both lowest and highest point in diurnal rhythm, but the rhythm *per se* persisted, again indicating that dexamethasone may not be involved in circadian rhythm of specific gene expression.

(iii) Removal of the pituitary has profound effects on the activity of cholesterol 7α-hydroxylase. Administration of growth factor to children with growth hormone deficiency resulted in an increase of the CDCA pool, presumably as a result of modulated cholesterol 7α-hydroxylase activity (176).

(iv) Hormones of the pancreas, insulin and glucagon, also have an impact on bile acid synthesis, and key-enzymes thereof. A role for insulin in regulation of bile acid synthesis has been suggested, based on abnormal bile acid synthetic capacity of subjects suffering from diabetes mellitus (177). Treatment with insulin resulted in subsequent normalization of these levels (177-179). Insulin was shown to affect both cholesterol 7α-hydroxylase and sterol 27-hydroxylase in cultured rat hepatocytes, by suppression of transcriptional activity of the respective genes (180). Transient expression experiments in cultured rat hepatocytes have revealed responsiveness to insulin within the proximal part of the cholesterol 7α-hydroxylase promoter (170).

Glucagon has been shown to suppress bile acid synthesis in freshly isolated rat liver cells in suspension (181), mediated via down-regulation of intracellular cAMP. Conversely, addition of the cAMP analogue Bt_2cAMP led to increased bile acid synthesis, specifically of chenodeoxycholic acid formation (182,183). In contrast, addition of either glucagon or cAMP to isolated rat hepatocytes in monolayer culture, led to down-regulation of cholesterol 7α-hydroxylase mRNA, indicating an opposite role for cAMP (171). No evidence was found for a role of other compounds involved in cellular signal-transduction, with respect to regulation of cholesterol 7α-hydroxylase (169).

Relative levels of almost all hormones discribed in the above, insulin, glucagon, cAMP, hydrocortison, and insulin, have been implicated to regulate the amplitude of diurnal variation in HMG-CoA reductase activity (184). It can not be excluded that similar concerted actions of these compounds regulate diurnal rhythm of cholestrol 7α-hydroxylase, allthough opposite effects of insulin on cholesterol 7α-hydroxylase and HMG-CoA reductase suggest differences in relative contributions to regulation of both enzymes (180).

Regulation by endogenous and exogenous cholesterol

A regulatory role within bile acid biosynthesis has also been ascribed to cholesterol. As a mode of regulation, different from transcriptional events described in the above, it was suggested that stimulation of cholesterol 7α-hydroxylase activity, by cholesterol-feeding or incubation of isolated hepatocytes with cholesterol-rich lipoproteins, might involve the level of substrate saturation of the enzyme (185-187). Einarsson and coworkers (188,189) showed this not to be the case: while certain feeding conditions caused large fluctuations in levels of cholesterol 7α-hydroxylase activity in the rat *in vivo*, these could not be correlated to hepatic cholesterol levels, nor to respective saturation indices of cholesterol 7α-hydroxylase under the various physiological conditions applied. At best, stimulation of the enzyme by cholestyramine treatment led to lowering of the saturation index, indicating a limitation for substrate under these specific conditions (189).

In vivo studies have focussed on the role of cholesterol by manipulating the flux of cholesterol within the liver. Inhibition of endogenous cholesterol synthesis by administration of HMG-CoA reductase inhibitors, e.g. lovastatin, to rats equipped with a short- or long-term bile fistula, has been shown to lead to a rapid down-regulation of cholesterol 7α-hydroxylase activity, mRNA, and gene transcription (190,191). This suppression could be prevented by simultaneous infusion with mevalonate, indicating a need for, and regulatory role of, *de novo* cholesterol. In addition, these experiments led to the conclusion that apparently circulating lipoprotein-derived cholesterol could not function as such (190). These experiments were not quite conclusive as to the regulatory role of cholesterol, as administration of mevalonate alone failed to stimulate cholesterol 7α-hydroxylase, both in stimulated- and control rats (155,191), while inhibiting transcriptional activity of cholesterol 7α-hydroxylase (155).

Similarly, long-term administration of lovastatin to control rats did not affect cholesterol 7α-hydroxylase (192). In contrast, a stimulatory effect of mevalonate was reported by Jones (191), using control rats, and intraperitoneal infusion of mevalonate to these animals was shown to stimulate cholesterol 7α-hydroxylase activity and mRNA 2.5-fold and 3 to 6-fold, respectively (91). At least some discrepancies may have arisen from differences in substrate-saturation of the cholesterol 7α-hydroxylase enzyme, inherent to the models used (189).

Another approach has been to supplement diet with high amounts of cholesterol (2%), which undisputably stimulates cholesterol 7α-hydroxylase (89,90,143,193) at the level of gene transcription (143). Delivery of dietary cholesterol to the liver does not seem necessary, however, as lymph-fistulated rats still exhibited stimulation in response to cholesterol-enriched diet in the face of a blocked transport of chylomicrons (149,194). This phenomenon raised questions as to the underlying mechanism of cholesterol-induced stimulation of cholesterol 7α-hydroxylase. A decreased half-life for cholic acid, in addition to an increase in faecal excretion of bile acids, led to the supposition that a high cholesterol load caused malabsorption of bile acids in the intestine. Within this concept, bile acids remained the main regulators of cholesterol 7α-hydroxylase (193). In agreement with this hypothesis, intraveneously administered Intralipid, enriched in cholesterol, failed to stimulate bile acid synthesis (193,195).

Although an attractive view, a number of observations are not in line with the the latter. A recent publication by Duane (196) reported a decreased half-life for cholic acid in humans, in reponse to a high cholesterol-diet (1071 mg/day). Conversely, the half-life of chenodeoxycholic acid was not affected, indicating that some bile acids may escape intestinal entrapping, possibly resulting in a shift in composition of the bile acid pool. Furthermore, simultaneous administration of a diet enriched in both cholesterol and bile acids to rats, led to up-regulation of cholesterol 7α-hydroxylase in these animals, and a concomitant down-regulation of HMG-CoA reductase activity, suggesting that dietary cholesterol does reach the liver, eliciting multiple effects (see below) (197).

It was also recently described that specific lipoproteins stimulate cholesterol 7α-hydroxylase (198). Differential effects of various lipoproteins have also been observed to be exerted on cholesterol synthetic capacity (146), and expression of the LDL-receptor (199,200). Thus it was apparent that only βVLDL was capable of stimulating expression of cholesterol 7α-hydroxylase activity, mRNA and gene transcription (198), in addition to supplying substrate *per se*, while LDL or HDL showed no such regulatory capacity. In this light, administation of cholesterol-enriched Intralipid (193) may not result in stimulation of cholesterol 7α-hydroxylase due to lack of certain additional lipoprotein entities, e.g. certain apolipoproteins, or specific TG and/or cholesterol content.

Cholesterol-responsiveness is elicited via as yet unidentified sequences within the cholesterol 7α-hydroxylase promoter (170,201). Such positive sterol regulatory elements

(SREs) may differ from those identified within promoter regions of HMG-CoA reductase, and the LDL-receptor (114), as cholesterol exhibits suppressive effects on promoter activity of these genes, in contrast to a positive effect on cholesterol 7α-hydroxylase. An elegant view to regulation of cholesterol 7α-hydroxylase at the DNA-level has been expressed by Russell & Setchell (162), envisioning positive and negative effects, elicited by both cholesterol and bile acids, respectively, via SREs (or HREs) and BAREs (see Fig. 4A). These effects ultimately titrate expression of the cholesterol 7α-hydroxylase gene. In this respect, simultaneous administration of cholesterol and bile acids (197), and resulting stimulated expression of cholesterol 7α-hydroxylase, suggests that positive SREs may outweigh negative regulation via BAREs under these circumstances.

Liver heterogeneity of bile acid synthesis- a possible link to substrate utilization, and regulatory cross-talk between cholesterol synthetic and bile acid synthetic pathways

A close link between cholesterol synthesis and bile acid synthesis has been observed under various physiological conditions, and activities of key-enzymes in the respective pathways, HMG-CoA reductase and cholesterol 7α-hydroxylase, often act in parallel in response to different physiological conditions (153,202-205). The close relationship has led to suggest regulation of both enzymes by similar factors. Alternatively, cholesterol 7α-hydroxylase has been suggested to be primarily regulated by the cholesterol synthesis pathway (204). An opposite view is that high bile acid synthetic capacity may be responsible for the efficient routing of cholesterol away from the site of negative regulation by the sterol, resulting in stimulated expression of the HMG-CoA reductase gene.

Several views exist as to the identity of the substrate pool for bile acid synthesis, and possible preference of cholesterol 7α-hydroxylase for either preformed- or *de novo* cholesterol. Both associative and direct evidence for the latter has been provided by several groups (206,207). In addition, bile fistulation of rats, and subsequent up-regulation of bile acid synthesis and cholesterol 7α-hydroxylase, appeared dependent on *de novo* cholesterol synthesis (190,191,205), indicating a preference of the enzyme for this particular cholesterol pool. Furthermore, several reports have indicated that different cholesterol pools may be substrate for certain bile acids. Thus it was indicated that *de novo* cholesterol forms substrate for the synthesis of cholic acid, while preformed cholesterol is specifically routed to chenodeoxycholic acid (208,209).

Several clues regarding utilization of cholesterol pools for bile acid synthesis may be found within the knowledge that cholesterol synthesis, bile acid formation, and a number of enzymes related to these processes, are not evenly distributed within the liver acinus (134,209-211). Thus it has been shown that cholesterol synthetic enzymes, HMG-CoA reductase and HMG-CoA synthase, are localized exclusively in the portal zone of the liver acinus (210,211). In contrast, bile acid synthesis, and key-enzymes involved therein,

cholesterol 7α-hydroxylase and sterol 27-hydroxylase, are pericentrally localized (134,209). Under normal conditions, therefore, synthetic and catabolic routes of cholesterol are separated, limiting linkage of both synthetic and catabolic routes to a few cells within the acinus, and suggesting the utilization of preformed cholesterol for bile acid synthesis under these circumstances. Conversely, *de novo* cholesterol may be routed to an ACAT-accessible pool (Fig. 1), followed by VLDL synthesis and excretion thereof, or to subsequent bile-flow-dependent biliary excretion of cholesterol, rather than forming precursor for bile acid synthesis. In agreement with our data (134,209), it has been shown by Scheibner et al. (212) that under normal conditions, the major part of bile acids is formed from exogenous cholesterol. Additionally, efficient excretion of particularly *de novo* cholesterol may result from the high concentration of bile acids in the portal area, acting as dissolvents of microsomal cholesterol as they are translocated from basolateral to canalicular membranes in these hepatocytes (213).

Distribution patterns of bile acid synthetic enzymes may change, however, upon dietary manipulation, i.e. administration of bile acid sequestrants such as colestid or cholestyramine (134,209), whereas expression of cholesterol synthetic enzymes remains portal under these circumstances (211). Administration of these resins results in reduced portal blood concentrations (214), and a reduced bile acid gradient and biofeedback (209), leading to enhanced bile acid synthesis and extension thereof to the portal area. The possibility of a direct link between cholesterol synthetic- and bile acid synthetic processes is thus provided (134,209). In line with this view, prolonged bile fistulation of rats has been shown to lead to enhanced utilization of *de novo* cholesterol for bile acid synthetic purposes (207,212,215). Taken together, utilization of certain cholesterol pools as substrate for bile acid synthesis seems to be dictated by relative acinar distribution of cholesterol synthetic- and bile acid synthetic enzymes, rather than the inherent preference of cholesterol 7α-hydroxylase for a particular cholesterol pool.

Another aspect of heterogeneity is related to the molecular mechanism of bile acid induced biofeedback. As reported, specific liver morphology results in the presence of a bile acid gradient over the acinus. Thus, bile acid concentrations differ 6-fold between the periportal and pericentral extremes (45,46), showing an inverse relationship with bile acid synthetic capacity (209). Hepatocytes exhibit a general over-capacity to take up and to excrete bile acids into the bile caniculus, irrespective of their zonal origin, resulting in low bile acid concentrations in the pericentral area (45,46). It is here that major bile acid synthesis is localized, and specific expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, mRNA, and transcriptional activity, has been demonstrated in this part of the acinus (134).

Administration of bile acid sequestrants lowers the acinar bile acid gradient, resulting in increased expression of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase over a

large part of the liver acinus. The latter is in line with concentration dependent down-regulation of bile acid synthetic enzymes, as demonstrated in cultured rat hepatocytes (95,144).

Thus the liver can efficiently regulate formation of bile acids, in response to a particular demand, by displacing expression of key-enzymes to a larger, or smaller area within the liver acinus, thereby either linking bile acid synthesis to cholesterol synthesis for sufficient supply of substrate, or uncoupling the two routes by creating a spacial barrier between them (Fig. 5).

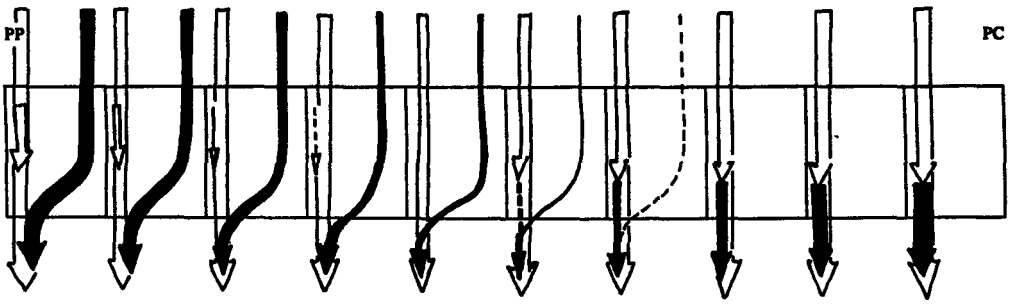
Liver morphology may play a major role in the heterogeneous distribution of various ligands involved in transcriptional regulation, probably not limited to regulation of bile acid synthesis alone (41). For example, the general transcriptional factor C/EBP shows heterogeneous expression, being abundant particularly in the pericentral area after induction with glucose or dexamethasone (216). Many hormones are taken up from the blood during passage through the sinusoids, thereby forming acinar gradients as in the case of bile acids. Portocentral differences have been measured for known effectors of bile acid synthetic enzymes, e.g. insulin, glucagon, and glucocorticoids (217-219). Relative contributions of these hormones may vary considerably with dietary status (218). Heterogeneous acinar distribution of bile acid synthesis may thus result from concerted action of these compounds, superimposed on a bile acid-induced effect.

OUTLINE OF THE THESIS

Our first aim was to achieve a good understanding of the various endogenous mediators involved in regulatory mechanisms of this biosynthetic pathway, specifically with respect to important key-enzymes that could act as a target for regulation. Concerning the latter, previous work had focussed on cholesterol 7α-hydroxylase as rate-limiting enzyme and major regulatory point in bile acid biosynthesis, but growing evidence for an important alternative route to bile acids, raised the question whether other enzymes might be involved in similar regulatory processes. Sterol 27-hydroxylase formed a possible candidate, responsible for catalysis of the first and rate-limiting step in the alternative pathway to formation of bile acids.

As a model system for most of our studies, we made use of the cultured rat hepatocyte, exhibiting characteristics very similar to the hepatocyte *in vivo*. The hepatocyte exhibits an over-capacity to take up and excrete bile acids from the sinusoidal compartment into the bile-caniculus, as well as being the site of primary bile acid synthesis. In previous studies, these hall-marks for the proper functioning of hepatocytes have been demonstrated, provided the proper culture conditions were applied (220-224). Thus, both primary rat and pig hepatocytes

A. Control liver



B. Cholestyramine-treated

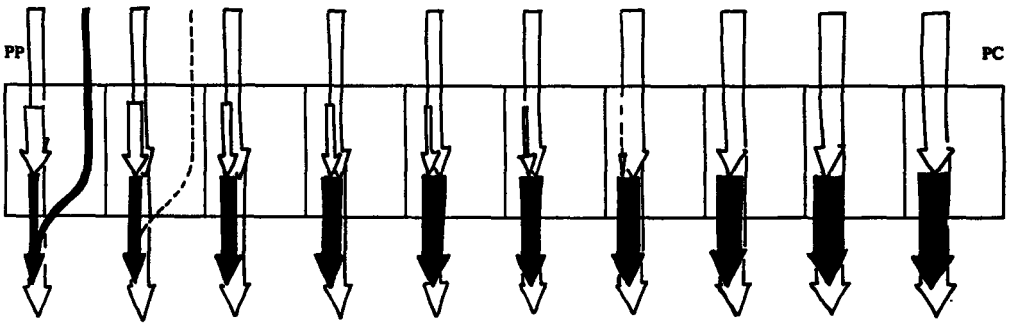


Figure 5. Hypothetical scheme of the relationship between lobular bile acid concentration and cholesterol synthetic and bile acid synthetic pathways within the liver lobulus. Open arrows indicate endogenous cholesterol synthesis, or uptake of exogenous cholesterol. Exiting of cholesterol from the cell has been indicated by a single arrow, which implies both biliary excretion as well as VLDL synthesis. Black arrows indicate uptake of bile acids from sinusoidal blood, or hepatic synthesis (from endogenous- or exogenous cholesterol). (A) Liver under control conditions; *de novo* cholesterol synthesis and bile acid synthesis are not linked. Bile acids are synthesized from preformed cholesterol, in the pericentral (PC) zone. (B) Diversion of bile acids from the EHC by cholestyramine-treatment; extension of bile acid synthesis up to the periportal (PP) zone because of reduced feedback within the lobulus. Bile acids are mainly formed from *de novo* cholesterol in the PP zone, while preformed cholesterol still provides substrate for the pathway in the PC zone. Depicted increase in uptake of preformed cholesterol in the PC zone may not be entirely applicable to rats, which do not exhibit high levels of circulating LDL, and compensate for the increased demand for substrate by stimulated utilization of *de novo* cholesterol.

were shown to efficiently take-up, synthesize, and excrete bile acids over a period of several days of culturing (221,222). In addition, activity and bile acid responsiveness of bile acid synthesis and of cholesterol 7α-hydroxylase was demonstrated in cultured pig hepatocytes (151,152) presenting a valuable tool for regulating studies. The rat hepatocyte was chosen for subsequent studies, after publication of the cDNA-sequence for cholesterol 7α-hydroxylase (86). Based upon the latter sequence, a cDNA probe for detection of cholesterol 7α-hydroxylase mRNA was synthesized via RT-PCR, providing the possibility of studying regulation of this enzyme up to the molecular level.

As presented in chapters 2 and 3, we investigated the molecular mechanism underlying regulation of bile acid synthesis by bile acids, considered the most important way to regulate this pathway, as it was still unclear, despite numerous *in vivo* and *in vitro* studies, whether bile acids themselves were responsible for down-regulation of bile acid synthesis, or if effects were exerted indirectly. As discussed in the above, evidence for bile acid-mediated feedback control was obtained from *in vivo* studies in rats, either fed the bile acid sequestring agent cholestyramine, or administered bile acids, but these have often led to divergent results. Moreover, the absence of an effect of bile acids in a cultured rat hepatocyte system challenged the feed-back hypothesis (145-148). A major break-through was made with the development of a system of cultured hepatocytes that did respond to bile acids (151,152), making further regulation studies feasable. In chapters 2 and 3, we investigated the molecular level at which bile acids exert an effect on key-enzymes within pathways to bile acids. In chapter 3, attention was devoted to an entirely new aspect of regulation of bile acid formation: the co-ordinate suppression of two rate-limiting enzymes, cholesterol 7α-hydroxylase and sterol 27-hydroxylase, involved in separate pathways to bile acids, by similar bile acids.

In chapter 4, a search for possible strucural elements of bile acids involved in determining potency to down-regulate these enzymes, was undertaken. Apart from a mechanistical point of view, such knowledge is important for development of alternatives to bile acid sequestrants, which may involve the synthesis of compounds built to interfere with interactions of bile acids with a possible negative regulator of bile acid synthesis (e.g. bile acid-receptor-antagonists). Structural requirements for such compounds may be derived from the determination of specific bile acid structure(s) involved in feedback regulation.

In chapter 5, a more macromolecular approach was used to investigate how bile acid-induced inhibition of bile acid synthesis may be brought about within the liver *in vivo*, and at which molecular level this is installed. Using isolated periportal and pericentral hepatocytes, distribution patterns for key-enzymes cholesterol 7α-hydroxylase and sterol 27-hydroxylase were assessed, and changes thereof in response to dietary manipulation, i.e. cholestyramine-treatment. Additionally, results were related to cholesterol synthesis within the liver, and how both synthetic and catabolic routes are interregulated to attain cholesterol

homeostasis within this organ.

Chapter 6 deals with the potential of cholesterol to act as a positive regulator of bile acid synthesis, in addition to supplying a source for substrate. The molecular level at which various forms of lipoprotein-derived cholesterol modulate bile acid synthesis is investigated, again using cultured rat hepatocytes as a model system.

A final study was aimed at elucidating the molecular basis for normalization of bile acid biosynthetic capacity in human subjects suffering from diabetes mellitus, after treatment with insulin (chapter 7). Using cultured rat hepatocytes, the effect of insulin on bile acid synthesis and important enzymes involved therein, was assessed.

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CHAPTER 2

DIFFERENTIAL FEEDBACK REGULATION OF CHOLESTEROL 7α-HYDROXYLASE mRNA AND TRANSCRIPTIONAL ACTIVITY BY RAT BILE ACIDS IN PRIMARY MONOLAYER CULTURES OF RAT HEPATOCYTES

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ABSTRACT

We have used primary monolayer cultures of rat hepatocytes to study the effects of physiological concentrations of various bile acids, commonly found in bile of normal rats, on the mechanism of regulation of cholesterol 7α-hydroxylase and bile acid synthesis.

Addition of taurocholic acid, the most predominant bile acid in rat bile, to the culture medium suppressed cholesterol 7α-hydroxylase activity and mRNA time- and dose-dependently. The decrease in enzyme activity paralleled the changes in mRNA. Maximal suppression of cholesterol 7α-hydroxylase mRNA (-91%) and enzyme activity (-89%) was observed after a 16-hour incubation period with 50μM taurocholic acid. The declines of the mRNA and enzyme caused by taurocholic acid were tightly coupled and followed first order kinetics with a half-life of 4 hours. Transcriptional activity, as assessed with nuclear run-on assays, was decreased by 43% at 50 μM taurocholic acid. Mass production of bile acids (chenodeoxycholic and β-muricholic acids) was inhibited to a similar extent as the cholesterol 7α-hydroxylase when different concentrations of taurocholic acid were used, giving maximal inhibition (-81%) at 50 μM of taurocholic acid.

Glycocholic acid and unconjugated cholic acid were equally effective as taurocholic acid in suppressing cholesterol 7α-hydroxylase mRNA. The more hydrophobic bile acids, chenodeoxycholic and deoxycholic acids showed profound suppression of the cholesterol 7α-hydroxylase mRNA by 85% and 75%, respectively, whereas the other trihydroxy-bile acids in rat bile, α- and β-muricholic acid, were not or only marginally active.

We conclude that rat bile acids, in particular the more hydrophobic ones, in concentrations commonly observed in portal blood, exert negative feedback control at the level of cholesterol 7α-hydroxylase mRNA in cultured rat hepatocytes through a direct effect on the hepatocytes, and that down-regulation of transcription is only one of the mechanisms involved in this regulation.

INTRODUCTION

The hepatic conversion of cholesterol into bile acids is a major route for the elimination of cholesterol from the mammalian body (1,2). According to current concepts, the primary pathway of bile acid biosynthesis in rats and humans is initiated by the 7α-hydroxylation of cholesterol. This reaction is catalyzed by cholesterol 7α-hydroxylase, a specific cytochrome P450 isozyme located in the smooth endoplasmic reticulum (3,4). The enzyme has recently been purified to homogeneity and the molecular cloning of its cDNA has been reported by several research groups (5-10). Cholesterol 7α-hydroxylase is regulated in a number of ways, the most important of which is considered to be exerted through the enterohepatic circulation of bile acids (1,2). Interruption of this circulation by biliary diversion or administration of

bile acid sequestrants has been shown to lead to a several-fold increase in bile acid biosynthesis and cholesterol 7α-hydroxylase activity (11-15). Intraduodenal or intravenous infusion of taurocholate returned rates of bile acid synthesis and the enzyme activity in these rats back to normal (13,16,17).

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

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

EXPERIMENTAL PROCEDURES

Materials

Materials used for isolation and culturing of rat hepatocytes, determination of mass production of bile acids, and assaying cholesterol 7α-hydroxylase activity were obtained from sources described previously (30-32). Tauro-, glyco- and unconjugated cholic acid, and deoxycholic acid were obtained from Sigma Chemicals (St Louis, MO, USA), chenodeoxycholic acid was from Serva (Heidelberg, Germany) and lithocholic-, α-

muricholic- and β-muricholic acids were obtained from Steraloids (Wilton, NH, USA). [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (400 Ci/mmol) and [4-^{14}C]-cholesterol (60 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, UK.

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

Rat Hepatocyte Isolation and Culture

Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously (30-32). Viability, as determined by trypan blue exclusion, was higher than 90%. The cells were seeded on 60-mm diameter plastic tissue culture dishes or 6-well cluster plates (Costar, Cambridge, MA, USA) at a density of $1.5*10^5$ cells/cm^2 in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 140 nM insulin, 50 nM dexamethasone, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37°C in a 5% CO_2/95% air atmosphere (30,31). After a 4-hour attachment period, medium was refreshed with 1.0 ml (6-well plates) or 2.5 ml (dishes) of culture medium with hormones as described above. Cells were left to recover for 18 hours before being used for further experiments (32). Bile acids were added to the hepatocytes at various times between 18 and 42 hours of culture age as indicated in the results section. Cells were harvested at the same time after a 42 hour culture period for measurement of cholesterol 7α-hydroxylase activity, mRNA and transcriptional activity.

Quantitation of Mass Production of Bile Acids

Mass production of bile acids by rat hepatocytes was measured by gas liquid chromatography after a preincubation period of 8 hours (from 18-26 hours of culture age), during the following 24-hour culture period from 26 to 50 hours as described before (33), in the presence or absence of bile acids. Rat hepatocytes in monolayer culture synthesize predominantly cholic acid (20-30% of total bile acid synthesis) and β-muricholic acid, together with minor amounts of chenodeoxycholic and α-muricholic acids (70-80%) (33,34). After addition of taurocholic acid as inhibiting agent, synthesis of this bile acid can not be measured. Values reported are, therefore, expressed as a percentage of the production of α- and β-muricholic acids and chenodeoxycholic acid.

Assay of Cholesterol 7α-hydroxylase

Cholesterol 7α-hydroxylase activity in homogenates of hepatocytes cultured for 42 hours was measured as reported previously (31). Protein and cholesterol were assayed according to

Lowry et al (35) and Gamble et al (36).

Cholesterol 7α-hydroxylase probe synthesis and identification

A probe directed against the cholesterol 7α-hydroxylase mRNA was synthesized with the polymerase chain reaction (PCR), using conditions as described in ref 37. cDNA was synthesized by using Moloney Murine Leukemia Virus RNAseH⁻Reverse Transcriptase (Superscript; Bethesda Research Laboratories), according to the manufacturers protocol, with total rat liver RNA as template and 0.1 mM oligo$(dT)_{12\text{-}18}$ as primer. Oligonucleotides were synthesized using a DNA-synthesizer (Applied Biosystems, model 381A); their sequence being:

oligo1 5'-AGCCGCCAAGTGACATCATCCAGTGTTCGCTTCTTCC-3'

oligo2 5'-ATGATGACTATTTCTTTGATTTGGGGAATTGCCGTG-3'

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

PCR was performed in a 100 μl reaction mixture containing 4 μl cDNA mixture, 20 μl of a 5x buffer (250 mM KCl, 100 mM Tris/HCl pH 8.4, 15 mM $MgCl_2$, 0.005% gelatin), and 10 μl of each primer (8 ng/μl). After denaturing at 95°C for 7 min, 10 μl of dNTP's and 1 unit of Taq-polymerase (Cetus Corporation) were added. The reaction mixture was covered with 70 μl of mineral oil to prevent evaporation. The actual PCR was then carried out by denaturing the RNA-cDNA hybrid at 95°C for 1 min, annealing the primers for 30 sec at 58°C, and extending the primers at 70°C for 5 min. This cycle was repeated 30 times using a programmable heat block manufactured by Cetus (Emmeryville, CA). After the final cycle, the temperature of the reaction mixture was kept at 70°C for several minutes to allow reannealing of the amplification products and the mixture was chilled. To ascertain that the PCR-probe was indeed of cholesterol 7α-hydroxylase origin, it was analyzed by restriction enzyme analysis (enzymes from Bethesda Research Laboratories). After cloning the probe via TA-cloning procedures (Invitrogen Corporation), it was further analyzed by sequencing of the first and last 200 basepairs of the probe, using the oligonucleotides 1 and 2 as sequencing primers (TaqTrack™, Promega).

RNA isolation, Blotting and Hybridization Procedures

Total RNA was isolated from cultured rat hepatocytes using the isolation procedure of Chomczynski and Sacchi (38). After washing the RNA pellets with 70% ethanol, they were dissolved in water and concentrations were determined spectrophotometrically assuming that A_{260}= 1 at 40 μg/ml RNA. Equal amounts of total RNA from different incubations were fractionated by electrophoresis on a 0.8% agarose gel containing 1 M formaldehyde, and transferred to Hybond-N filter (Amersham) in accordance with the manufacturers

instructions. For slotblotting of total RNA, samples were diluted to appropriate concentrations in a buffer containing 1 M NaCl, 50 mM sodium phosphate, pH 7.0, and 6% v/v formaldehyde and applied onto a filter using the Minifold II slotblotting apparatus (Schleicher and Schuell) according to Krawczyk and Wu (39).

After both procedures, filters were crosslinked with UV-light for 5 min and then hybridized with different probes at 65°C in a 0.5 M sodium phosphate buffer, pH 7.5, containing 7% w/v SDS and 1 mM EDTA. DNA fragments used as probes were isolated from low-melting agarose (Biorad) (40). Each blot was hybridized with 25 ng of probe, labelled by the random-primer method (Multi-prime, Amersham) to approximately $6*10^8$ cpm/μg DNA. After hybridization, blots were washed twice with 2 x SSC/1% SDS (30 min at 65°C), (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The filters were exposed to Hyperfilm MP (Amersham) together with an intensifying screen (Eastman-Kodak Co.) for 48-120 h at -80°C. For quantitation of the relative amounts of mRNA, the autoradiographs were scanned using a Shimadzu CS 910 chromatograph scanner, and areas under the curves were integrated using a data processor (Shimadzu Corp. Kyoto, Japan). The mRNA levels were quantitated by using three different amounts of total RNA, giving a linear relation between the specific mRNA signal and the amount of RNA applied.

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

Nuclear Run-On Studies

These were conducted essentialy as described by Groudine et al (42), with minor modifications.

Isolation of nuclei- Cells were washed, scraped using a rubber policeman, and collected by centrifugation at 500 x g at 4°C for 5 min. They were resuspended in NP40-lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 1 mM PMSF, 1 mM DTT), and after being left on ice for 5 min, homogenized in a Potter Elvehjem tube with pestle B for 15 strokes at 4°C. Resulting nuclei were again centrifuged at 500 x g and resuspended in NP40-lysis buffer. This procedure was repeated until the nuclei were free of cellular debris. They were then taken up in glycerol storage buffer (50 mM Tris-HCl pH 8.3, 40% glycerol, 5 mM MCl_2, 0.1 mM EDTA, 1 mM PMSF, 5 mM DTT), counted, and aliquoted at approximately $2*10^7$/ 200μl before being frozen ah -80°C.

RNA labeling and isolation- An aliquot of frozen nuclei was added to 200μl of transcription

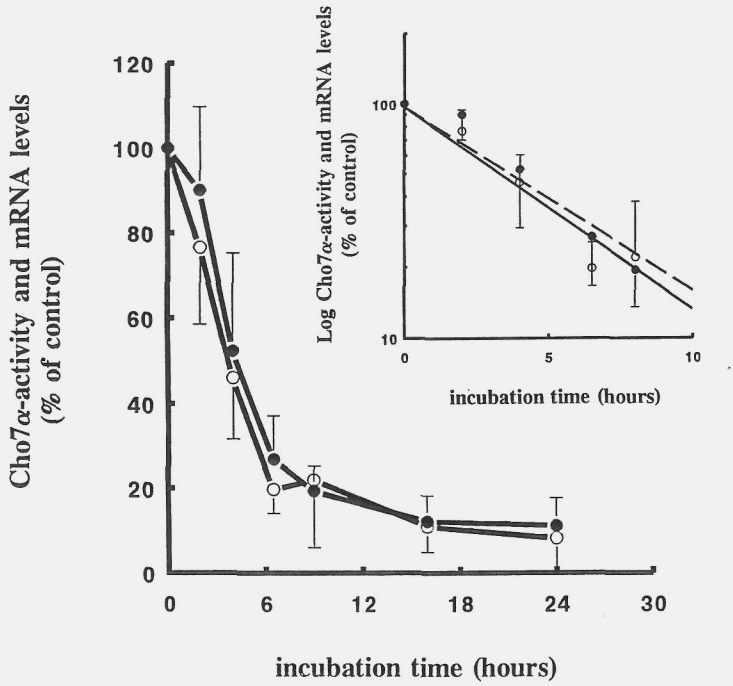
buffer (10 mM Tris-HCl pH 7.9, 140 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM of dGTP, dATP, dCTP, 0.1 mM s-adenosyl-l-methionine, 14 mM β-mercaptoethanol, 1 mg/ml heparin sulfate, 1.7 mM spermidine, 10 mM creatine phosphate, 40 μg/ml creatine kinase, 25% glycerol and 100 μCi of [α32P]UTP), and incubated while shaking at 30°C for 30 min. 600 μl of a buffer containing 0.5 M NaCl, 50 mM MgCl2, 2 mM $CaCl_2$, 10 mM Tris-HCl pH 7.4 and 200 U/ml DNAseI (Bethesda Research Laboratories) was added, and the mixture was incubated for an additional 5 min. at 30°C. 200 μl of SDS/Tris (5% w/v SDS, 0.5 M Tris-HCl pH 7.4, 0.125 M EDTA) with 200 μg/ml Proteinase K (Boehringer Mannheim) was added, and the mixture was incubated for 30 min at 42°C. RNA was extracted with 1 volume of phenol/chloroform/isoamylalcohol (50:49:1), precipitated with 2.5 volumes of ethanol and 10 μg/ml tRNA, washed, and taken up in 50 μl of Tris/EDTA (10 mM Tris-HCl pH 7.4, 1 mM EDTA). Labeled RNA was separated from free nucleotides by passage over a Sephadex G50(fine)-column (Boehringer Mannheim). The RNA was mildly degraded by a 10 min incubation on ice in 0.25 M NaOH, and the mixture was neutralized by addition of a half volume of 1 M HEPES (free acid) and precipitated with 2 volumes of ethanol and 0.1 volume of 3 M NaAc. Incorporation of label was measured by liquid scintillation counting, and equal amounts of labeled RNA were added to the filters.

Hybridization- Target DNA, being 5 μg of plasmid material containing cDNA sequences of rat cholesterol 7α-hydroxylase and hamster actin (see the above), and rat GAPDH (43) were slotblotted onto 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 as described previously, and hybridized with the labeled RNA for 36 hours in the same buffer. The various filters were washed once for 5 min and twice for 30 min in 2 x SSC/1% SDS at 65°C, and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantitation of relative amounts of mRNA was conducted as described in the above.

Statistical Analysis

Data were analyzed statistically using Student's paired t-test with the level of significance selected to be $P < 0.05$. In Figure 1- inset (shown later), the lines were fitted to the points by method of least squares. Values are expressed as means ± SD.

A.



B.

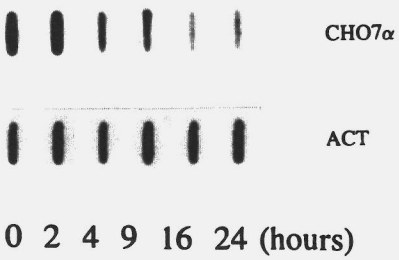


Figure 1. Time course of inhibition of cholesterol 7α-hydroxylase activity and mRNA by taurocholic acid. (A) Cells were exposed to taurocholic acid (50 μM) for different lenghts of time, between 18-42 hours of culture, and were harvested simultaneously at 42 hours of culture time. Cholesterol 7α-hydroxylase activity (-●-) and mRNA (-○-) were assessed as described under "Experimental Procedures". The absolute values for cholesterol 7α-hydroxylase activity were 343 ± 130 $pmol.hr^{-1}.mg^{-1}$ cell protein. Values shown are expressed as percentages of those incubations without taurocholic acid, and are a means ± SD of duplicate incubations, using hepatocytes of 3 to 12 rats. Inset: same data plotted semilogarithmically (dashed line and closed symbols: cholesterol 7α-hydroxylase; normal line and open symbols: cholesterol 7α-hydroxylase mRNA). The lines were fitted to the points by the method of the least squares. (B) The amount of cholesterol 7α-hydroxylase mRNA (CHO7α) was assessed by slotblotting and densitometric scanning of resulting autoradiographs, using the actin mRNA (ACT) as an internal standard to correct for differences in the amount of RNA applied.

RESULTS

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

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

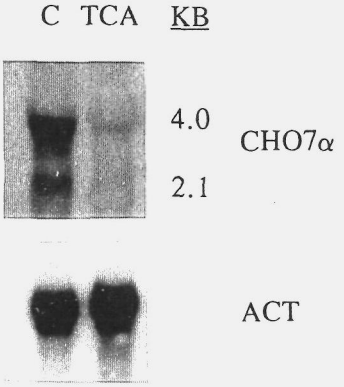


Figure 2. Northern blot hybridization of cholesterol 7α-hydroxylase mRNA. Total RNA of cells exposed to 50 μM taurocholic acid (TCA) for 24 hrs, between 18-42 hours of culture, as opposed to untreated cells (C), was electrophoresed in a 0.8% agarose/ 1M formaldehyde gel, transferred to Hybond-N and subsequently hybridized with probes for cholesterol 7α-hydroxylase (CHO7α) and actin mRNA (ACT), as described under "Experimental Procedures". 10 μg of total RNA was applied in both lanes.

Detection of cholesterol 7α-hydroxylase mRNA was done using the PCR probe directed against it. Northern blot hybridization of total RNA isolated from cultured hepatocytes revealed the existence of at least three distinct messengers (Fig. 2), of which the two most abundant ones are approximately 2.1 and 4.0 kb in length. These results on the relative amounts and lengths of cholesterol 7α-hydroxylase mRNAs in cells correspond well with previous findings observed in the rat liver in vivo (9,10). Quantitation of the amount of mRNA by slotblotting experiments (Fig. 1B) in total RNA from cells treated with taurocholate, showed that cholesterol 7α-hydroxylase mRNA levels follow the same pattern as the enzyme activity; i.e. 91 ± 8% inhibition after 24 hours of incubation with 50 μM taurocholate (Fig. 1A). The apparent halflife of the mRNA is estimated to be 4 hours (inset). The actin mRNA, used as a control, exhibited no significant changes in level when taurocholic acid was present in the medium. Northern blot hybridization of these same samples demonstrated that all three detectable cholesterol 7α-hydroxylase mRNAs were susceptible to inhibition (Fig. 2).

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

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

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

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

Rat hepatocytes were incubated for 24 hours, from 18-42 hours of culture, with various amounts of taurocholic acid (0-70 μM). Cells were harvested after 42 hours of culture, for determination of cholesterol 7α-hydroxylase activity and mRNA. Bile acid synthesis was measured after an 8-hour preincubation period, from 26 to 50 hours of culture time, as described under "Experimental Procedures". Data are expressed as a percentage of control (no bile acids added) and are means ± SD of independent experiments using hepatocytes from n rats. The amount of cholesterol 7α-mRNA was assessed by slotblotting and densitometric scanning of resulting autoradiographs, using the actin mRNA as an internal standard to correct for differences in the amount of total RNA applied to the filter. The absolute values for cholesterol 7α-hydroxylase activity were 343 ± 130 pmol.hr^{-1}.mg^{-1} cell protein, and for bile acid synthesis 2.72 ± 0.90 μg.24 h^{-1}.mg^{-1} cell protein. * Indicates a significant difference ($p < 0.05$) compared to control values.

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

Nuclear run-on studies were conducted using nuclei isolated from hepatocytes which had been incubated with 50μM of taurocholic acid for 24 hours, between 18 and 42 hours of culture time. [α^{32}P]-Labeled total RNA was hybridized to rat cholesterol 7α-hydroxylase cDNA, and to hamster actin- and rat GAPDH cDNA. The latter two served as transcriptional activity controls between the different samples, and specific transcriptional activity of cholesterol 7α-hydroxylase is expressed relative to that of actin.

Figure 3 shows that addition of 50μM taurocholic acid lowers the transcription rate of cholesterol 7α-hydroxylase of cells by 43.5 ± 5%, whereas it has no effect on the transcription of the GAPDH-gene.

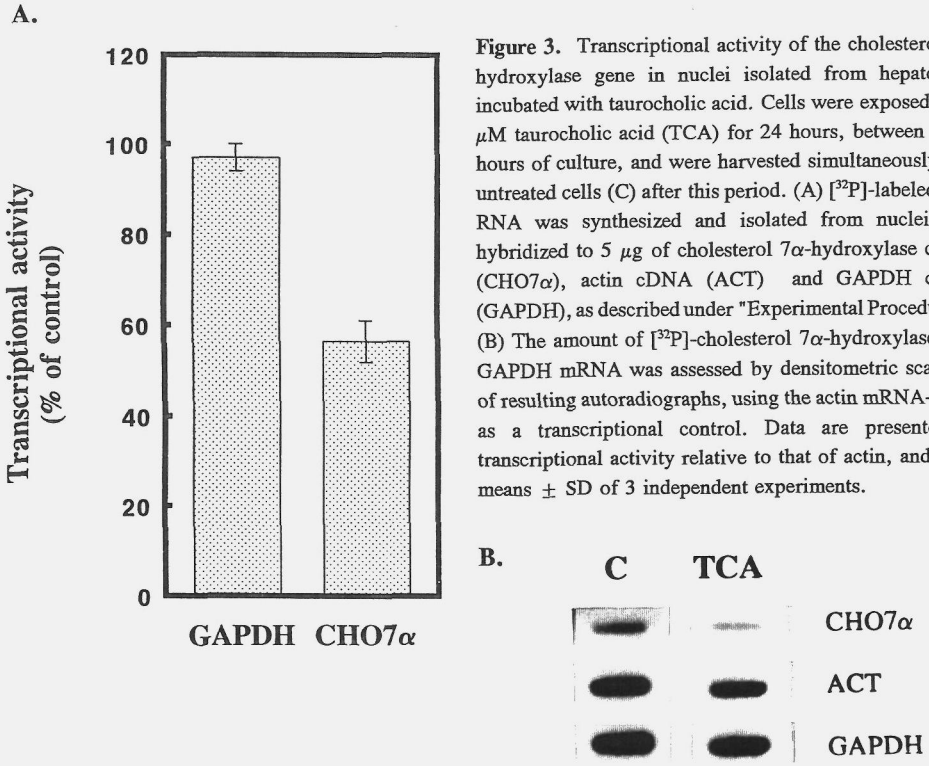


Figure 3. Transcriptional activity of the cholesterol 7α-hydroxylase gene in nuclei isolated from hepatocytes incubated with taurocholic acid. Cells were exposed to 50 μM taurocholic acid (TCA) for 24 hours, between 18-42 hours of culture, and were harvested simultaneously with untreated cells (C) after this period. (A) [^{32}P]-labeled total RNA was synthesized and isolated from nuclei, and hybridized to 5 μg of cholesterol 7α-hydroxylase cDNA (CHO7α), actin cDNA (ACT) and GAPDH cDNA (GAPDH), as described under "Experimental Procedures". (B) The amount of [^{32}P]-cholesterol 7α-hydroxylase- and GAPDH mRNA was assessed by densitometric scanning of resulting autoradiographs, using the actin mRNA-signal as a transcriptional control. Data are presented as transcriptional activity relative to that of actin, and are a means ± SD of 3 independent experiments.

Effect of Different Bile Acids on Cholesterol 7α-Hydroxylase mRNA in Cultured Rat Hepatocytes

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

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

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

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

Rat hepatocytes were incubated with different bile acids (50 μM) from 18 to 42 hours of culture. Cells were harvested at 42 hours, and the cholesterol 7α-hydroxylase mRNA level was determined. Data are expressed as a percentage of control (no bile acids added) and are means ± SD of independent experiments using hepatocytes from n rats. The amount of cholesterol 7α-hydroxylase mRNA was assessed by slotblotting and densitometric scanning of resulting autoradiographs, using the actin mRNA as an internal standard to correct for differences in the amount of total RNA applied to the filter.

* Indicates a significant difference ($p < 0.05$) between control and treated cells.

DISCUSSION

The present study shows that bile acid synthesis in cultured rat hepatocytes is subject to negative feedback regulation by bile acids through a direct effect of bile acids on the hepatocytes. Failure to observe such suppression of bile acid synthesis in vitro by exogenously added bile acids, as has been reported, using hepatocytes of both rat (18-20) and rabbit (21), has challenged the idea that bile acids inhibit their own synthesis directly when

returning to the liver via the enterohepatic circulation. It has also led to the suggestion that an extrahepatic repressor, possibly originating from the intestine in the presence of bile acids, is responsible for the actual inhibition (46). Our results show unequivocally that a possible repressor system must originate in the hepatocyte. These data are in good agreement with those obtained by Kwekkeboom et al (28,29) using cultured pig hepatocytes, indicating that this regulation is not a species-specific effect.

The reason that we find negative feedback regulation in our in vitro systems is most probably due to the fact that the hepatocytes of both species were cultured under conditions that have been shown to maintain bile acid biosynthetic capacity and cholesterol 7α-hydroxylase activity during culture (29,47,48). Elevation of the initial levels of cholesterol 7α-hydroxylase activity and mRNA by feeding rats with chow supplemented with 2% cholestyramine, prior to isolation of the hepatocytes, was not found to be obligatory to observe feedback regulation. Although bile acid synthesis was 2.3-fold lower in hepatocytes from rats fed on control chow (1.17 ± 0.62 $\mu g.24h^{-1}.mg^{-1}$ cell protein (49) versus $2,72 \pm 0.90$ $\mu g.24h^{-1}.mg^{-1}$ cell protein, this study), similar results as reported in this paper were obtained using rats fed on control chow (data not shown). We conclude that rat hepatocytes are a suitable model for studies on negative feedback regulation by bile acids, provided that a proper maintenance of cholesterol 7α-hydroxylase is ensured during culture.

An earlier suggestion that rat hepatocytes do not show feedback regulation of bile acid synthesis in vitro as a result of loss of capacity to take up exogenous bile acids from the medium (29) can not be upheld. The hepatocytes are still capable to take up sufficient bile acids to be able to downregulate bile acid synthesis as well as cholesterol 7α-hydroxylase, notwithstanding the gradual loss of capacity to accumulate bile acids intracellularly during culture (45). Even low concentrations (10 μM) of bile acids are already sufficient to exhibit a marked decrease in cholesterol 7α-hydroxylase activity and mRNA levels. The magnitude of inhibition is comparable to that reported using cultured pig hepatocytes (29).

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

cholesterol 7α-hydroxylase will be suppressed predominantly in these cells. Corresponding with this, we found a 7.9-fold higher enzyme activity in periportal hepatocytes as compared with periportal hepatocytes from rats fed on normal chow (34).

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

Our data clearly demonstrate that bile acids regulate cholesterol 7α-hydroxylase at the mRNA level, in agreement with observations made in vivo by others (9,10,58). We found a 91% suppression of steady-state mRNA levels, whereas transcriptional activity was lowered by 43%. In vivo in cholestyramine-fed rats we have also observed a considerably stronger increase in cholesterol 7α-hydroxylase mRNA than in transcriptional activity (data not shown). These results suggest that, although there is a substantial effect of bile acids on transcription rates of the cholesterol 7α-hydroxylase gene, the mRNA levels may be determined for a major part at the post-transcriptional level, e.g. by stability of the mRNAs. It is tempting to speculate that the multiple AU-rich sequences in the 3'-non-coding region of cholesterol 7α-hydroxylase mRNA are somehow involved in this regulation. Our data correspond in broad outline with those reported by Pandak et al. (58), who in vivo in rat also found that cholesterol 7α-hydroxylase is regulated at the level of mRNA, however in contrast to our results predominantly at the level of transcription.

Considerable differences between the bile acids most common to rat, with respect to their inhibitory effect, were observed. Both primary (cholic- and chenodeoxycholic acid) and secondary (deoxycholic acid) bile acids had a profound effect on cholesterol 7α-hydroxylase. This finding is in contrast with in vivo data, in which a strong inhibition of bile acid synthesis was found only after intravenous infusions of (tauro)deoxycholic acid (24), or lithocholic or 3β-hydroxy-5-cholenoic acids (27) but not when taurocholic acid was used. It was postulated that primary bile acids had to be converted to secondary ones in order to

become active. Judging from our results, this is not necessary. The trihydroxy bile acids α- and β-muricholic acid, which account for 30% of total bile acid pool size in rat in vivo (51), had only marginal effects. The potency of suppression was shown to be dependent on the bile acid structure and linked to hydrophobicity of the particular bile acid, as has been postulated by Heuman et al (50). Cholic acid does not entirely fit this model, however, as it is less hydrophobic than deoxy- or chenodeoxycholic acid, but nonetheless just as active an inhibitor. Additionally, there were no differences between the conjugated and unconjugated forms of cholic acid with respect to inhibitory action, while there are major differences in hydrophobicity of these bile acids (50). Apparantly conjugation is of no importance for regulating potency, but much more so the structure of the free bile acid. The cultured rat hepatocyte is an attractive model to further elucidate structure-function relationships with respect to differential effects of bile acids on bile acid synthesis.

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CHAPTER 3

SUPPRESSION OF STEROL 27-HYDROXYLASE mRNA AND TRANSCRIPTIONAL ACTIVITY BY BILE ACIDS IN CULTURED RAT HEPATOCYTES

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ABSTRACT

In previous work we have demonstrated suppression of cholesterol 7α-hydroxylase by bile acids at the level of mRNA and transcription, resulting in a similar decline of bile acid synthesis in cultured rat hepatocytes (Twisk J, Lehmann EM, Princen HMG (1993) Biochem. J. 290: 685-691). In view of the substantial contribution of the "alternative" or "27-hydroxylase" route to total bile acid synthesis, as has been demonstrated in cultured rat hepatocytes and in vivo in humans, we evaluated the effects of various bile acids, commonly found in bile of rats, on the regulation of sterol 27-hydroxylase in cultured rat hepatocytes.

Addition of taurocholic acid, being the most predominant bile acid in rat bile, to the culture medium of rat hepatocytes resulted in a 72% inhibition of sterol 27-hydroxylase activity. The effect was exerted at the level of sterol 27-hydroxylase mRNA, showing a time- and dose-dependent decline with a maximal suppression (-75%) at 50 μM taurocholic acid after 24 h of culture. The decline of the mRNA followed first-order kinetics with an apparent half-life of 13 h. Under these conditions the cholesterol 7α-hydroxylase mRNA (-91%), and bile acid synthesis (i.e. chenodeoxycholic and β-muricholic acids, -81%) were also maximally suppressed. In contrast, no change was found in the level of lithocholic acid 6β-hydroxylase mRNA.

Assessment of the transcriptional activity of a number of genes involved in routing of cholesterol towards bile acids showed similar suppressive effects of taurocholate on gene expression of sterol 27-hydroxylase and cholesterol 7α-hydroxylase (-43% and -42%, respectively), while the expression of the lithocholic 6β-hydroxylase gene remained unaffected. Taurocholic acid, as well as unconjugated cholic acid, were equally effective in suppressing sterol 27-hydroxylase mRNA. The more hydrophobic bile acids chenodeoxycholic and deoxycholic acids also showed a strong inhibition of 57% and 76% respectively, whereas the hydrophilic β-muricholic acid was not active.

We conclude that a number of bile acids, in physiological concentrations, suppress sterol 27-hydroxylase by down-regulation of sterol 27-hydroxylase mRNA and transcriptional activity and that coordinate suppression of both sterol 27-hydroxylase and cholesterol 7α-hydroxylase results in inhibition of bile acid synthesis in cultured rat hepatocytes.

INTRODUCTION

The synthesis of bile acids is the most important pathway for the metabolism and excretion of cholesterol from the mammalian body (1). From investigations in the 1970s, predominantly with rats, it was established that in this sequence of reactions nuclear transformations of cholesterol involving 7α-hydroxylation by cholesterol 7α-hydroxylase as initial step, precede the side chain degradation, leading to formation of both cholic and

chenodeoxycholic acids. Cholesterol 7α-hydroxylase is considered to be the major control point in regulation of bile acid synthesis (1). However, an alternative pathway may exist, specifically in the formation of chenodeoxycholic acid. Human and rat liver mitochondria are able to convert cholesterol to 27-hydroxycholesterol (2,3). It has been shown that this intermediate can be further metabolized to 3β-hydroxy-5-cholenoic acid in rat liver peroxisomes (4), and to 7α-hydroxy intermediates by human liver microsomes and mitochondria (5,6), ultimately leading to preferential formation of chenodeoxycholic acid in rat and man (7-12). Introduction of a 27-hydroxyl group has been reported to almost completely prevent a subsequent introduction of a 12α-hydroxyl group (1).

There is accumulating evidence that in normal human subjects both pathways, i.e. the 7α-hydroxylase or neutral pathway and the 27-hydroxylase or acidic pathway, are involved in biosynthesis of bile acids (11). The bile acid intermediates 27-hydroxycholesterol and 3β-hydroxy-5-cholenoic acid are present in relative high concentrations in human blood (11,13,14). The 27-hydroxylase pathway becomes the major route to chenodeoxycholic acid in patients with liver disease, in whom the activity of cholesterol 7α-hydroxylase is low (11). Recent work of our group supports an important contribution of the 27-hydroxylase pathway to total bile acid synthesis in both rat and human hepatocytes (12). The immunosuppressive drug cyclosporin A has been shown to selectively inhibit 27-hydroxylation of cholesterol, leading to a strong decrease in the production of chenodeoxycholic acid, ultimately resulting in a decreased bile acid formation in cultured rat and human hepatocytes (12,15). In agreement with this finding, a defect in sterol 27-hydroxylase, as is the case in patients suffering from the rare autosomal recessive disease cerebrotendinous xanthomatosis (CTX) (16), causes reduced levels of bile acids and particularly of chenodeoxycholic acid (17).

27-Hydroxylation of cholesterol is catalysed by an ω-hydroxylase in mammalian liver, which is located in the inner mitochondrial membrane and a member of the cytochrome P-450-superfamily. It almost exclusively attacks the C27-methyl group and is therefore denoted as "27-hydroxylase", though it has been referred to as "26-hydroxylase" in the majority of publications on this enzyme. The enzymes from rabbit (18,19) as well as rat (20) and pig (21) liver have been characterized. cDNA sequences of sterol 27-hydroxylase from rabbit (22), rat (23,24) and human (25) have been reported, and mRNA measurements showed that the enzyme is expressed in several organs and tissues other than liver (22).

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

We have shown previously that physiological concentrations of bile acids suppress bile acid synthesis and cholesterol 7α-hydroxylase activity in cultured pig hepatocytes (27,28). Recently we reported a profound, dose-dependent inhibition of bile acid synthesis by bile acids, accompanied by a reduction in cholesterol 7α-hydroxylase mRNA and transcriptional activity in cultured rat hepatocytes (29,30). The magnitude of inhibition of bile acid synthesis

was found to be similar to that of suppression of cholesterol 7α-hydroxylase. In view of the significant contribution of the 27-hydroxylase pathway to total bile acid synthesis in these cells (12), it is concievable that regulation at the level of cholesterol 7α-hydroxylase alone would result in a less pronounced effect of bile acids on total bile acid synthesis than is actually observed. Therefore, we evaluated the effect of various bile acids on bile acid synthesis and sterol 27-hydroxylase activity in cultured rat hepatocytes. Using a recently available rat 27-hydroxylase cDNA clone (22,24), we were able to monitor sterol 27-hydroxylase mRNA levels and to measure transcription rates using nuclear run-off assays. This study shows that, in addition to feedback inhibition of cholesterol 7α-hydroxylase, bile acids also suppress sterol 27-hydroxylase activity by down-regulation of sterol 27-hydroxylase mRNA and transcriptional activity.

EXPERIMENTAL PROCEDURES

Materials

Materials used for isolation and culturing of rat hepatocytes, determination of mass production of bile acids, and assaying sterol 27-hydroxylase activity were obtained from sources described previously (31-33). Taurocholic acid, cholic acid and deoxycholic acid were obtained from Sigma Chemicals (St. Louis, MO, USA), chenodeoxycholic acid was from Serva (Heidelberg, Germany) and β-muricholic acid was obtained from Steraloids (Wilton, NH, USA). [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (400 Ci/mmol) and [4-^{14}C]-cholesterol (60 mCi/mol) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, UK.

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

Rat Hepatocyte Isolation and Culture

Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously (31-33). Viability, as determined by trypan blue exclusion, was higher than 90%. The cells were seeded on 60-mm diameter plastic tissue culture dishes or 6-well cluster plates (Costar, Cambridge, MA, USA) at a density of 1.5×10^5 cells/cm^2 in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 140 nM insulin, 50 nM dexamethasone, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37°C in a 5% CO_2/95% air atmosphere (31,32). After a 4-

hour attachment period, medium was refreshed with 1.0 ml (6-well plates) or 2.5 ml (dishes) of culture medium with hormones as described above. Bile acids were added to the hepatocytes at various times between 18 and 48 hours of culture age as indicated in the results section. Cells were harvested at the same time for measurement of sterol 27-hydroxylase activity, mRNA and transcriptional activity. Cell viability, after culturing with the various bile acids, was assessed by ATP measurements (34) and MTT-assays[2] (35). The latter is dependent on the cellular reduction of MTT (Sigma Chemical Co., St. Louis, MO, USA) by the mitochondrial dehydrogenase of viable cells, to a blue formazan product which can be measured spectrophotometrically. The assay was performed essentially as described by De Vries et al. (35). In short, parallel with the various incubations, cells on 12-well plates ($5x10^5$ cells/well) in 0.5 ml medium were incubated with bile acids. At the end of the incubation period, 55 μl of MTT solution (5 mg MTT/ml PBS) was added to each well for 2 hours. The medium was aspirated, and 1 ml 100% DMSO was added to solubilize the formazan crystals. Absorbance at 545 nm was measured immediately.

Quantitation of Mass Production of Bile Acids

Mass production of bile acids by rat hepatocytes was measured by gas liquid chromatography after a preincubation period of 8 hours (from 18-26 hours of culture age), during the following 24-hour culture period from 26 to 50 hours as described previously (33), in the presence or absence of bile acids. Rat hepatocytes in monolayer culture synthesize predominantly cholic acid (20-30% of total bile acid synthesis) and β-muricholic acid, together with minor amounts of chenodeoxycholic and α-muricholic acids (70-80%) (33,36). After addition of taurocholic acid as inhibiting agent, synthesis of cholic acid can not be measured. Values reported are, therefore, expressed as a percentage of the production of α- and β-muricholic acids and chenodeoxycholic acid.

Assay of Sterol 27-Hydroxylase

Sterol 27-hydroxylase activity in homogenates of cultured hepatocytes was measured as mentioned in ref. 37. [^{14}C]-labelled products were analyzed by thin layer chromatography, and the amount of [^{14}C]-27-hydroxycholesterol was quantitated by scraping off and counting of the spots containing this product, using the [^{14}C]-cholesterol input as a recovery standard. Blank values, determined by running parallel incubations without a NADPH-generating system, were subtracted before calculating enzyme activity. Protein and cholesterol were assayed as previously described (32).

RNA Isolation, Blotting and Hybridization Procedures

Isolation of total RNA, and subsequent electrophoresis, blotting and hybridization techniques, were performed as described previously (29,38). Quantitation of mRNA levels was performed using slot-blotting techniques as described in ref. 39.

The following DNA fragments were used as probes in hybridization experiments: a 1.6 kb HindIII/XbaI fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. J. Strauss (24), the 1.6 kb PCR-synthesized fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region (29), a 0.7 kb EcoRI fragment of pFR29-3 containing the cDNA for hamster lithocholic acid 6β-hydroxylase (CYP3A10), kindly provided by Dr. G. Gil (40). The sterol 27-hydroxylase cDNA was isolated from a rat liver cDNA library (24) using the rabbit sterol 27-hydroxylase cDNA, previously isolated by the group of Russell, as a probe (22). As controls a 1.2 kb PstI fragment of hamster actin cDNA (41), a 1.2 kb PstI fragment of GAPDH-cDNA (42), and a 0.8 kb PstI/BamH1 fragment of rat cyclophylin cDNA (43) were used. For all probes used in slot-blotting experiments, a linear relationship between areas under the curves and mRNA concentration was shown on an autoradiograph, using concentrations between 2 and 8 μg of total RNA. The hamster actin cDNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter.

Nuclear Run-Off Studies

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

Hybridization - Target DNA, being 5 μg of plasmid material containing cDNA sequences of rat sterol 27-hydroxylase, rat cholesterol 7α-hydroxylase, hamster lithocholic acid 6β-hydroxylase, hamster actin, rat GAPDH (see the above) and the empty vector pUC18, were slot-blotted onto strips of Hybond-N$^+$ filter (Amersham), and crosslinked with 0.4 N NaOH for 30 min. The filters were preincubated for 30 min at 65°C in a sodium phosphate buffer as described above, and hybridized with the labelled RNA for 36 hours in the same buffer. [^{32}P-UTP had been incorporated into nascent RNA, using isolated nuclei from cells which had been cultured with or without bile acids for 24 hours, between 18 and 42 hours of culture time. After hybridization, the various filters were washed once for 5 min and twice for 30 min in 2 x SSC/1% SDS at 65°C, and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantitation of relative amounts of mRNA was conducted as described (29).

Statistical Analysis

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

RESULTS

Effect of Taurocholic Acid on Bile Acid Synthesis

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

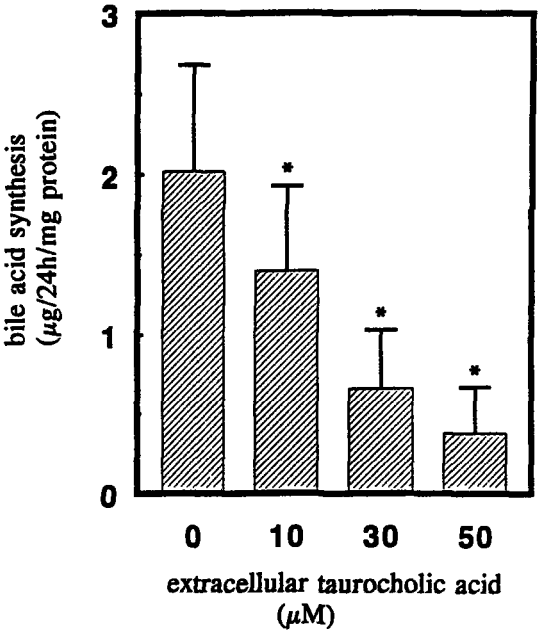

Figure 1. Effect of taurocholic acid on mass production of bile acids
Rat hepatocytes were incubated for 24 hours with various amounts of taurocholic acid (0-50 μM). Bile acid synthesis was measured after an 8-hour preincubation period (18-26 h), from 26-50 hours of culture time, as described under "Experimental Procedures", in the presence or absence of taurocholic acid. Values are expressed as means $\pm$ S.D. of independent experiments using hepatocytes from 5-6 rats. The absolute value for chenodeoxycholic acid and β-muricholic acid synthesis was 2.02 $\pm$ 0.66 $\mu g.24^{-1}.mg^{-1}$ cell protein. * Indicates a significant difference ($p < 0.05$) compared with control values.

Effect of Taurocholic Acid on Sterol 27-Hydroxylase Activity and mRNA

To assess a possible role of sterol 27-hydroxylase in downregulation of bile acid biosynthesis by bile acids, sterol 27-hydroxylase activity towards cholesterol and mRNA levels were measured. Sterol 27-hydroxylase activity amounted to 73 $\pm$ 29 $pmol.h^{-1}.mg^{-1}$ cell protein in control cells, and was significantly suppressed (-72 $\pm$ 20%) upon addition of 50 μM of taurocholic acid to the culture medium, for 24 hours. Northern blotting of total RNA isolated from cultured hepatocytes revealed the existence of a single 2.4 kb mRNA for this enzyme (Fig. 2), in agreement with the previously reported size of the 27-hydroxylase mRNA in rat liver in vivo (24). Figure 2 also shows a strong suppression of the sterol 27-hydroxylase

mRNA in the presence of 50 μM of taurocholic acid, concomitant with a profound decrease in cholesterol 7α-hydroxylase mRNA under these circumstances. In contrast, the lithocholic acid 6β-hydroxylase mRNA did not change significantly upon addition of 50 μM taurocholic acid. Taurocholic acid therefore does not show similar effects on all mRNAs coding for cytochrome P-450 enzymes involved in bile acid biosynthesis, and inhibition by the compound is thus not a reflection of a general effect on these messengers. Taurocholic acid had no effect on the mRNAs of the housekeeping genes actin and GAPDH, nor on cyclophylin mRNA, used as internal standards (Fig. 2).

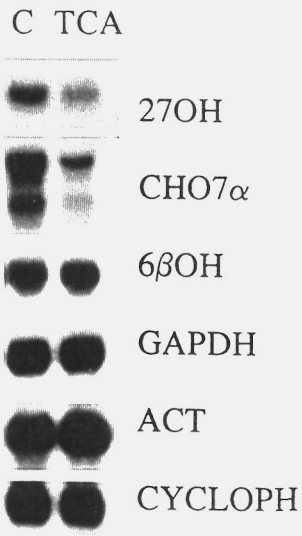

Figure 2. Effect of taurocholic acid on mRNA of enzymes involved in bile acid synthesis
Total RNA of cells exposed to 50 μM taurocholic acid (TCA) for 24 hrs, between 18-42 hours of culture, as opposed to untreated cells (C), was electrophoresed in a 0.8% agarose/1 M formaldehyde gel, transferred to Hybond-N^+ and subsequently hybridized with probes for sterol 27-hydroxylase (27OH), cholesterol 7α-hydroxylase (CHO7α), lithocholic acid 6β-hydroxylase (6βOH), and GAPDH, actin (ACT) and cyclophilin (CYCLOPH) mRNA, as described under "Experimental Procedures". 10 μg of total RNA was applied in both lanes.

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

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

Extracellular taurocholate (μM)	MTT-assay (% of control)	Cellular ATP (% of control)
0	100	100
30	98 ± 5	92 ± 13
50	96 ± 14	96 ± 12
70	84 ± 5*	97 ± 10

Rat hepatocytes were incubated for 24 hours, from 18-42 hours, with various amounts of taurocholic acid (0-70 μM) to assess the effect on cell viability. After 42 hours of culture, reduction of MTT by mitochondrial dehydrogenase and ATP content of these cells were measured as described in "Experimental Procedures". Data are expressed as a percentage of control (no bile acids added), and are means ± S.D. of independent experiments using hepatocytes from 4-6 rats. The absolute values for cellular ATP content were 20.1 ± 4.3 nmol.mg^{-1} cell protein. * Indicates a significant difference ($p < 0.05$) compared to control values.

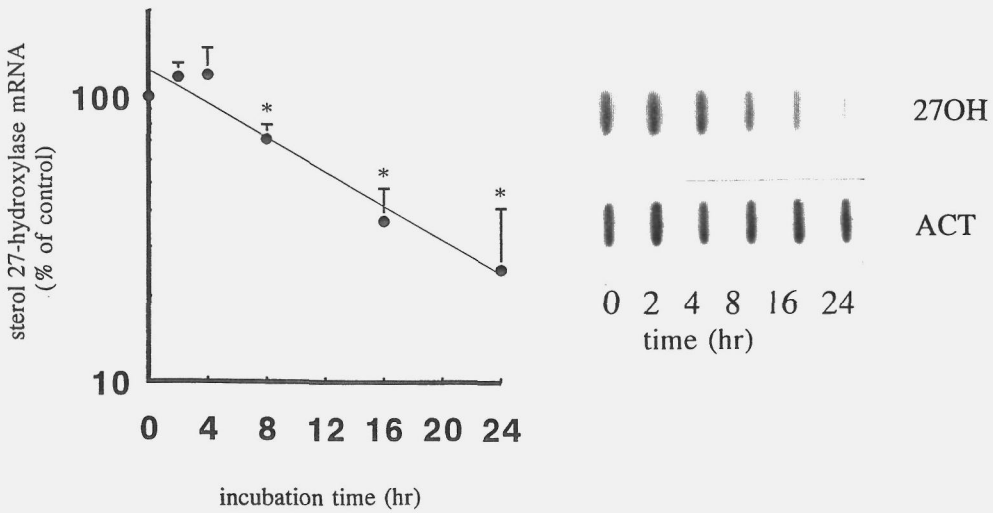

Figure 3. Time course of suppression of sterol 27-hydroxylase mRNA by taurocholic acid
Cells were exposed to taurocholic acid (50 μM) for different lengths of time, between 18-48 hours of culture, and were harvested simultaneously at 48 hours of culture time. The amount of sterol 27-hydroxylase mRNA (27OH) was assessed by slot-blotting and densitometric scanning of resulting autoradiographs (inset), using the actin mRNA (ACT) as an internal standard to correct for differences in the amount of RNA applied. Values shown are expressed as percentages of incubations without taurocholic acid, and have been plotted semi-logarithmically, as a means ± S.D. of incubations using hepatocytes from 3-8 rats. * Indicates a significant difference ($p < 0.05$) compared to control values.

Time and Dose Dependency of the Effect of Taurocholic Acid on Sterol 27-Hydroxylase mRNA Level

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

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

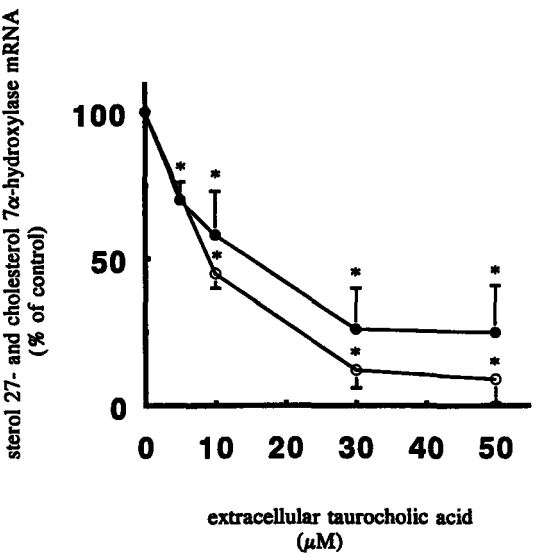

Figure 4. Dose-dependency of suppression of sterol 27-hydroxylase and cholesterol 7α-hydroxylase mRNA by taurocholic acid
Rat hepatocytes were incubated for 24 hours, from 18-42 hours of culture, with various amounts of taurocholic acid (0-50 μM). Cells were harvested after 42 hours of culture, for determination of sterol 27-hydroxylase (closed circles) and cholesterol 7α-hydroxylase mRNA (open circles); the latter is shown for comparison. The amount of sterol 27-hydroxylase and cholesterol 7α-hydroxylase mRNA was assessed by slot-blotting and densitometric scanning of resulting autoradiographs, using the actin mRNA as an internal standard to correct for differences in the amount of total RNA applied to the filter. For details see the "Experimental Procedures" section. Data are expressed as a percentage of control (no bile acids added) and are means $\pm$ S.D. of independent experiments using hepatocytes from 3-8 rats. * Indicates a significant difference ($p < 0.05$) compared to control values.

Effect of Taurocholic Acid on Bile Acid Synthesis and Sterol 27-Hydroxylase Activity and mRNA in Hepatocytes from Rats Fed on Normal Chow

To exclude the possibility that the effect of taurocholic acid on sterol 27-hydroxylase activity and mRNA results from a suppression of a derepressed bile acid synthesis after supplementation of the diet of the intact animal with 2% cholestyramine prior to isolation of the hepatocytes, part of the experiments described in the above were repeated using hepatocytes from rats fed normal chow. Bile acid synthesis (i.e. chenodeoxycholic acid and β-muricholic acid synthesis) in these hepatocytes (1.03 ± 0.51 $\mu g.24h^{-1}.mg^{-1}$ cell protein) was suppressed by $67 \pm 12\%$ (n=3) after incubation with 50 μM taurocholic acid. Addition of 50 μM of taurocholic acid to the medium of non-induced hepatocytes for a period of 24 hours led to a strong suppression of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities and mRNA levels ($-85 \pm 11\%$ and $-81 \pm 3\%$ for cholesterol 7α-hydroxylase activity and mRNA, respectively, and $-69 \pm 16\%$ and $-74 \pm 7\%$ for sterol 27-hydroxylase activity and mRNA, respectively). These values are comparable to those obtained with hepatocytes from cholestyramine-treated rats, indicating that taurocholic acid also inhibits sterol 27-hydroxylase activity and mRNA in hepatocytes from rats fed on normal chow.

Effect of Different Bile Acids on Sterol 27-Hydroxylase mRNA in Cultured Rat Hepatocytes

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

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

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

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

Rat hepatocytes were incubated with different bile acids (50 μM) from 18 to 42 hours of culture. Cells were harvested at 42 hours, and the sterol 27-hydroxylase mRNA level was determined as described in "Experimental Procedures". Data are expressed as a percentage of control (no bile acids added) and are means ± S.D. of independent experiments using hepatocytes from 3-8 rats. The amount of sterol 27-hydroxylase mRNA was assessed by slot-blotting and densitometric scanning of resulting autoradiographs, using the actin mRNA as an internal standard to correct for differences in the amount of total RNA applied to the filter. * Indicates a significant difference ($p < 0.05$) between control and treated cells.

Effect of Taurocholic Acid on the Transcriptional Activity of the Sterol 27-Hydroxylase Gene in Cultured Rat Hepatocytes

To further examine the mechanism of suppression of sterol 27-hydroxylase activity and mRNA levels, nuclear run-off studies were conducted using nuclei isolated from hepatocytes which had been incubated with 50 μM of taurocholic acid for 24 hours, between 18 and 42 hours of culture time. [α^{32}P]-labelled total RNA was hybridized to rat sterol 27-hydroxylase cDNA, rat cholesterol 7α-hydroxylase cDNA, hamster lithocholic acid 6β-hydroxylase cDNA, hamster actin and rat GAPDH cDNA. The latter two served as transcriptional activity controls between the different samples, and specific transcriptional activity of the genes is expressed relative to that of actin. The empty vector pUC18 shows that there is no non-specific hybridization of the gene transcripts (Fig. 5).

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

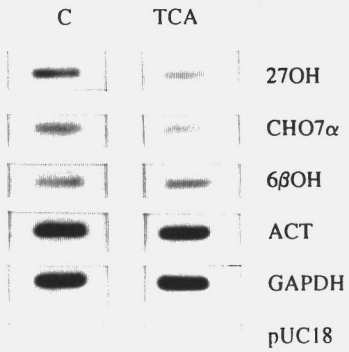

Figure 5. Transcriptional activity of the sterol 27-hydroxylase gene in response to taurocholic acid

Autoradiograph of run-off transcripts for sterol 27-hydroxylase (27OH), cholesterol 7α-hydroxylase (CHO7α), lithocholic acid 6β-hydroxylase (6βOH), actin (ACT), and GAPDH isolated from nuclei from rat hepatocytes incubated with (TCA) or without (C) 50 μM of taurocholic acid for 24 h. [^{32}P]-labelled total RNA was hybridized to immobilized cDNAs as indicated in the "Experimental Procedures" section, and the resulting blot was exposed to Hyperfilm for 48-120 h. Non-specific hybridization was checked using the empty vector pUC18. The relative amounts of transcribed [^{32}P]-mRNA were assessed by densitometric scanning, as described, using the actin mRNA as an internal standard.

DISCUSSION

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

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

The concentration of 27-hydroxycholesterol is considerably lower in the circulation of rat than in man (26), suggesting that the pathway involving initial 27-hydroxylation of cholesterol is of less importance in the former species. This contention is supported by the fact that cholic acid is the predominant bile acid in bile of rats (50). Stimulation of bile acid synthesis via interruption of the enterohepatic circulation will further increase the contribution of the 7α-hydroxylase pathway.

Nevertheless, there are indications that sterol 27-hydroxylase is subject to regulation in

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

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

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

hydroxylase promoter may be located in the region between nucleotides -49 and -79 (30). Comparison of these sequences with DNA sequences of the human sterol 27-hydroxylase promoter (59)- the sequence of the rat 27-hydroxylase promoter is still unknown- revealed a high degree of homology in the region between nucleotides -254 and -280, suggesting that similar elements in the cholesterol 7α-hydroxylase and sterol 27-hydroxylase promoter may be involved in negative feedback regulation by bile acids.

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

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

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CHAPTER 4

STRUCTURAL ASPECTS OF BILE ACIDS INVOLVED IN REGULATION OF 7α-HYDROXYLASE AND STEROL 27-HYDROXYLASE

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Submitted.

SUMMARY

We have recently reported that coordinate down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase by bile acids, commonly found in rat bile, results in suppression of bile acid synthesis in cultured rat hepatocytes (Twisk, De Wit, and Princen (1994) Biochem J: in press). In the current study, we have assessed the effects of a large group of different bile acids, both naturally occurring and synthetic, on these two key-enzymes, to elucidate structural features which render bile acids potent as a regulator of bile acid synthesis in cultured rat hepatocytes.

Addition of 50μM deoxycholate or cholate, two relatively hydrophobic bile acids, to the culture medium of hepatocytes resulted in strong suppression of cholesterol 7α-hydroxylase (-75% and -88%) and sterol 27-hydroxylase activity (-76% and -72%). On the other hand, two hydrophilic bile acids, β-muricholate and ursocholate, yielded no effect. These differential effects were also reflected in mRNA levels and transcriptional activity for the two enzymes, as assessed by nuclear run-off assays, showing a parallel suppression of both parameters in response to cholate (-78% and -43%, respectively for cholesterol 7α-hydroxylase mRNA and transcription, and -76% and -42%, respectively for sterol 27-hydroxylase mRNA and transcription), and yielding no effect when ursocholate was added. Transient expression analysis, using a promoter-reporter construct containing the proximal part of the cholesterol 7α-hydroxylase promoter, in cultured rat hepatocytes, demonstrated a reduction of transcriptional activity by cholate (-72%), but not by ursocholate. In contrast, transcription of the gene encoding the key-enzyme in cholesterol synthesis, HMG-CoA reductase, was not affected by bile acids, while mRNA levels for this enzyme were slightly up-regulated by cholate (+47%), but not by ursocholate.

Assessment of the effects of 27 different bile acids, varying in number, position and orientation (α/β) of hydroxyl groups on the steroid nucleus of the molecule, on cholesterol 7α-hydroxylase mRNA showed only a weak correlation with the hydrophobicity index of the bile acid involved ($r = 0.61$). Analysis of the three-dimensional structure of a number of these bile acids suggests that hydroxyl groups situated in close proximity of each other within the molecule, creating a hydrophilic environment as in the case of cholate, may be a prerequisite for strong inhibitory potency. Deviation from this situation leads to a markedly lesser effect on suppression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase.

INTRODUCTION

Hepatic conversion of cholesterol into bile acids is a major route for elimination of cholesterol from the mammalian body (1,2). Based upon information obtained *in vivo* in rat

(3-8) and man (9,10), and *in vitro* using cultured pig (11,12) and rat (13-15) hepatocytes, it was concluded that cholesterol 7α-hydroxylase, the major rate-limiting enzyme in the bile acid biosynthetic pathway, is repressed by the flux of bile acids returning to the liver in portal blood. In a recent paper, however, we have shown that not only cholesterol 7α-hydroxylase, but also sterol 27-hydroxylase is down-regulated by bile acids (16). The latter enzyme is involved in the alternative routing of cholesterol to bile acids, a route which has been shown to contribute considerably to bile acid synthesis both *in vivo* in man (17), and *in vitro* in cultured human and rat hepatocytes (18,19). Both enzymes are coordinately down-regulated by taurocholate, one of the major bile acids in rat bile, at the level of mRNA and transcription, leading ultimately to suppression of bile acid synthesis (16).

With regard to the functional structure of a bile acid in terms of its potency to inhibit bile acid synthesis, it has been postulated that repressional activity is directly correlated to the hydrophobicity of a given bile acid (20,21). However, studies *in vivo* involving feeding of bile acids are difficult to interpret due to the many types of conversion taking place in the mammalian body. In addition, distinct views exist, originating from *in vivo* studies in rat and rabbit, reporting taurocholate not active, whereas taurodeoxycholate and taurolithocholate were strong inhibitors of bile acid synthesis. It was suggested that primary bile acids first have to be converted to secondary ones to become regulatory (22-24). Alternatively, specifically monohydroxy bile acids derived from either intestinal or hepatic sources, were reported to effectively down-regulate bile acid synthesis in the rabbit, indicating that the hydroxylation status *per se* is important for inhibitory potency (24). Shefer and co-workers (25) reported strong suppression of bile acid synthesis by taurocholate, but not by tauroursocholate, at the level of cholesterol 7α-hydroxylase *in vivo* in rat. Epimerization of the 7-hydroxyl group, yielding such drastic differences in effect on this enzyme, suggests that specific three-dimensional structure of a bile acid might also be important, rather than hydrophobicity alone, in determining the ability to regulate. The latter is in agreement with previous results obtained with cultured pig and rat hepatocytes, in which cholate, hyodeoxycholate, chenodeoxycholate, and deoxycholate showed equal suppressive effects on bile acid synthesis and cholesterol 7α-hydroxylase, while differing significantly in hydrophobicity index (11-13).

In this study, we have assessed the effects of a large number of bile acids, both naturally-occurring and synthetic, and differing highly in number, position, and orientation of hydroxyl groups in the basic steroid structure, on cholesterol 7α-hydroxylase, to elucidate whether there exists a basic structural requirement for a bile acid to be able to exert suppressive effects on the expression of this enzyme. For reasons of comparison, a subset of these bile acids was also tested for effects on sterol 27-hydroxylase. In these experiments, measures were taken to prevent or reduce interconversions of bile acids by the hepatocyte, facilitating interpretation of results.

Results presented indicate that, in general, both cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities are coordinately down-regulated by similar bile acids at the level of mRNA and gene transcription. The correlation of the potency of bile acids to inhibit cholesterol 7α-hydroxylase, versus their hydrophobicity index (HI_r), however, is a weak one ($r = 0.61$), suggestive of additional important structural aspects involved in down-regulation.

EXPERIMENTAL PROCEDURES

Materials and bile acids

Materials used for isolation and culturing of rat hepatocytes, and assaying cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities, were obtained from sources described previously (26-28). Taurocholate, glycocholate, cholic acid and deoxycholic acid were obtained from Sigma Chemicals (St. Louis, MO, USA), chenodeoxycholic acid was from Serva (Heidelberg, Germany) and β-muricholic acid was obtained from Steraloids (Wilton, NH, USA). All other bile acids used in this study were obtained from and synthesized by Prof.Dr. Takashi Iida and Dr. Tamaaki Tamaru, from the Department of Industrial Chemistry, College of Engineering, Nihon University, Japan, as described previously (29-32). Ketoconazole was obtained from Janssen Life Sciences Products (Beerse, Belgium).

Radiochemicals ([α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (400 Ci/mmol) and [4-^{14}C]-cholesterol (60 mCi/mol)) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, UK.

Male Wistar rats weighing 250-350 g were used throughout and were maintained on standard chow and water ad libitum. Two days before isolation of hepatocytes, rats were fed a diet supplemented with 2% cholestyramine (Questran, Bristol Myers B.V. Weesp, The Netherlands) (13). For preparation of hepatocytes, animals were killed between 9 and 10 a.m. Institutional guidelines for animal care were observed in all experiments.

Rat Hepatocyte Isolation and Culture

Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously (26-28). Viability, as determined by trypan blue exclusion, was higher than 90%. The cells were seeded on 60-mm diameter plastic tissue culture dishes or 6-well cluster plates (Costar, Cambridge, MA, USA) at a density of 1.5×10^5 cells/cm^2 in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 140 nM insulin, 50 nM dexamethasone, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and were maintained at 37°C in a 5% CO_2/95% air atmosphere (12,13,27). After a 4-hour attachment period, medium was refreshed with 1.0 ml (6-well plates) or 2.5 ml (dishes) of culture medium as described in the above. Bile acids, dissolved in 10% (v/v) DMSO, were added to the hepatocytes for 16 hours, during two consecutive 8-hour periods,

between 26-42 hours of culture age. The final concentration of DMSO in the medium was 0.1% (v/v). Cells were harvested at the same time for measurement of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities, mRNA levels and transcriptional activities.

To prevent the effect of conversion of added bile acids by the hepatocytes, and to prolong the period of time that the bile acids could be effective, medium containing bile acids was refreshed after the first 8-hour period and incubated for another 8-hour period. Alternatively, hydroxylation of bile acids by the hepatocytes was inhibited by simultaneous incubation of bile acids with 10 μM ketoconazole over a similar period as described above, in a separate set of experiments. The latter substance has been shown to effectively inhibit hydroxylation reactions catalyzed by NADPH-cytochrome P-450 dependent monooxygenases (33), among them cholesterol 7α-hydroxylase (up to 94% at 10 μM) (26). Culturing of cells in the presence of ketoconazole thus made assessment of effects of bile acids on cholesterol 7α-hydroxylase activity impossible, but had no effect on expression of cholesterol 7α-hydroxylase mRNA levels (data not shown). Conversion of bile acids by the hepatocytes, either in the presence or absence of ketoconazole, was determined by gas liquid chromatography after incubation of cells with a given bile acid for an 8-hour period. Hepatocytes incubated with bile acids and ketoconazole were used for determination of mRNA levels.

Cell viability, after culturing with the various bile acids, or ketoconazole, was assessed by ATP measurements (34) and MTT-assays (35). The latter is dependent on the cellular reduction of MTT (Sigma Chemical Co., St. Louis, MO, USA) by the mitochondrial dehydrogenase of viable cells, to a blue formazan product which can be measured spectrophotometrically. The assay was performed essentially as described by De Vries et al. (35). In short, parallel with the various incubations, cells on 12-well plates (5×10^5 cells/well) in 0.5 ml medium were incubated with bile acids. At the end of the incubation period, 55 μl of MTT solution (1 mg MTT/ml Williams E medium) was added to each well and incubated for 2 hours at 37°C. The medium was aspirated, and 1 ml 100% DMSO was added to solubilize the formazan crystals. Absorbance at 545 nm was measured immediately.

Assay of Cholesterol 7α-hydroxylase and Sterol 27-Hydroxylase

Cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities in homogenates of cultured hepatocytes were measured as mentioned in references 27 and 36, respectively. [^{14}C]-labelled products were separated by thin layer chromatography, and the amount of [^{14}C]-7α-hydroxycholesterol and [^{14}C]-27-hydroxycholesterol was quantitated by scraping off and counting of the spots containing these products, using the [^{14}C]-cholesterol input as a recovery standard. Blank values, determined by running parallel incubations without a NADPH-generating system, were subtracted before calculating enzyme activity. Protein and cholesterol were assayed as previously described (27).

RNA Isolation, Blotting and Hybridization Procedures

Isolation of total RNA, and subsequent electrophoresis, blotting and hybridization techniques were performed as described previously (13). Quantitation of mRNA levels was performed using slot-blotting techniques as described in ref. 37.

The following DNA fragments were used as probes in hybridization experiments: the 1.6 kb PCR-synthesized fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region (13), a 1.6 kb HindIII/XbaI fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. J. Strauss (38), and a 773 bp HindIII fragment of hamster HMG-CoA reductase cDNA (39). The sterol 27-hydroxylase cDNA was isolated from a rat liver cDNA library (38) using the rabbit sterol 27-hydroxylase cDNA, previously isolated by the group of Russell, as a probe (40). As controls a 1.2 kb PstI fragment of hamster actin cDNA (41), and a 1.2 kb PstI fragment of GAPDH-cDNA (42) were used. For all probes used in slot-blotting experiments, a linear relationship between areas under the curves and mRNA concentration was shown on an autoradiograph, using concentrations between 2 and 8 μg of total RNA. The hamster actin cDNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter.

Nuclear Run-Off Studies

Nuclear run-off studies were conducted essentially as described by Groudine et al. (43), with minor modifications (13).

Hybridization - Target DNA, being 5 μg of plasmid material containing cDNA sequences of rat cholesterol 7α-hydroxylase, rat sterol 27-hydroxylase, hamster HMG-CoA reductase, hamster actin, rat GAPDH (see the above) and the empty vector pUC18, were slot-blotted onto strips of Hybond-N^+ filter (Amersham), and crosslinked with 0.4 N NaOH for 30 min. The filters were preincubated for 30 min at 65°C in a sodium phosphate buffer as described (13), and hybridized with the labelled RNA for 36 hours in the same buffer. [^{32}P]-UTP had been incorporated into nascent RNA, using isolated nuclei from cells which had been cultured with or without bile acids for 16 hours, between 26 and 42 hours of culture time. After hybridization, the various filters were washed once for 5 min and twice for 30 min in 2 x SSC/1% SDS at 65°C, and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantitation of the relative amounts of mRNA synthesized was conducted as described (13).

Transfection experiments and CAT assays

At 22 hours after their isolation, cells were subjected to transfection (14). Recombinant plasmids to be used for transfection were purified by centrifugation to equilibrium in CsCl-Ethidium-bromide-density gradients (44). In transient-expression assays, 3 μg of test plasmid and 1 μg of standard LacZ plasmid (45), used as internal control for transfection efficiency, were cotransfected as a CaP_i precipitate (46). After 4 hours, the precipitate was removed,

and cells were treated with Williams E medium containing 15% glycerol for 1 min, and supplied with fresh culture medium with or without bile acids (50 μM). At 42 hours after transfection, cells were harvested and cell extracts were prepared. Preparation of cell extracts and CAT assays were performed essentially as described by Gorman et al (47). Protein concentrations were determined with BCA protein-assay reagent (Pierce). The amounts of acetylated product were quantitated, after thin layer chromatography and autoradiography, with a Phosphor-imager 400B (Molecular Dynamics). Data were corrected for protein-content and transfection efficiency.

Determination of the hydrophobicity index (HI_x) of bile acids

HI_x of all bile acids, used in this study, was determined by methods described by Heuman et al. (21), using a Hitachi L-6000 High Performance Liquid Chromatograph. The HI_x of an individual bile acid is based on its capacity factor (k_x) in C18 reversed phase HPLC, expressed relative to the capacity factors of taurocholate and taurolithocholate; column used being a Capcell Pak C18 AG 120 (25 cm x 4.6 mm I.D.; 5 μm; Shiseido, Tokyo, Japan). The eluent consisted of a gradient of methanol-0.025M potassium phosphate buffer (pH = 7.0), ranging from 4:1 to 7:3 (v/v) at 1 ml/min, with U.V.-detection set at 210 nm.

Molecular modelling of bile acids

The software package QUANTA/CHARm, implemented on a Silicon Graphics 4000 XZ workstation, was used to model the structures of bile acids. As a starting point, the crystal structure of methylcholate (48) was retrieved from the Cambridge Crystallographic Database (Cambridge Crystallographic Data Center, Lensfield Road, Cambridge, U.K.). Polar hydrogen atoms were added, and the resulting structure was energy minimized using default values as provided by the program. The structure of allo-methylcholate was built by inverting the hydrogen atom at position 5, and subsequent energy minimization. Likewise, other bile acids were analyzed.

Statistical Analysis

Data were analyzed statistically using Student's paired t-test with the level of significance selected to be $P < 0.05$. Values are expressed as means $\pm$ SD. In Fig. 2, the line was fitted to the points by the method of least squares. Pearson correlation coefficients were calculated to determine the correlation between hydrophobicity index and suppression of cholesterol 7α-hydroxylase mRNA levels.

RESULTS

Effects of different bile acids on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, mRNA and transcriptional activity

Since hydrophobicity has been suggested to be a major determinant for the potency of a given bile acid to inhibit bile acid synthesis (12,13,15,20,21), the effects of the hydrophobic bile acids deoxycholate (HI_x = +0.60) and cholate (HI_x = +0.01) were compared with those of β-muricholate (HI_x = -0.76) and ursocholate (HI_x = -1.04), two hydrophilic bile acids.

Table 1 shows that 50 μM of deoxycholate added to medium of cultured hepatocytes for a period of 16 hours resulted in -75 ± 15% suppression of the cholesterol 7α-hydroxylase activity level. Previous data from our group (13,16) has shown that this dosage and culture period for bile acids yields maximal effects. Addition of cholate resulted in comparable suppression of enzyme activity (-88 ± 6%), while there was no effect of the 7β-epimer of cholate, ursocholate, nor of β-muricholate. Assessment of sterol 27-hydroxylase activity levels revealed similar effects of these bile acids on this enzyme, showing strong suppression by deoxycholate (-76 ± 12%) and cholate (-72 ± 20%), and no significant effects of ursocholate or β-muricholate. Cell viability was not affected by the concentration of the bile acids used (data not shown). Addition of the different bile acids had no effect on intracellular cholesterol levels (49 ± 10 nmol per mg cell protein for control cells), neither could the effects observed be explained by differences in uptake by the hepatocyte in terms of accumulation as determined by gas liquid chromatography, which were similar for all bile acids concerned (data not shown). It should also be noted that interconversion by the hepatocyte does not play a role in the effects described in the above, as the bile acids cholate, ursocholate, and β-muricholate are not or only marginally metabolized. Only deoxycholate is partly converted into cholate (49), but these two bile acids have equal suppressive effects.

The differential effects of the bile acids were also reflected by the steady-state levels of mRNA for both cholesterol 7α-hydroxylase and sterol 27-hydroxylase. Northern-blotting and hybridization with various cDNA-probes of total RNA isolated from hepatocytes incubated with 50μM of cholate or ursocholate, as compared with control cells, showed a parallel down-regulation of all three mRNAs for cholesterol 7α-hydroxylase (2.1, 3.6, and 4.0 kb) in size (13,50,51), and of the single mRNA for sterol 27-hydroxylase (2.4 kb in rat liver) (16,38) by cholate, and not by ursocholate (Fig. 1). β-Actin and GAPDH, used as internal standards, showed no response to the bile acids added.

Table 2 summarizes the effects of the different bile acids tested on the levels of mRNA for the enzymes mentioned. Deoxycholate and cholate inhibited cholesterol 7α-hydroxylase (-75 ± 16% and -78 ± 10%, respectively) and sterol 27-hydroxylase (-75 ± 16% and -76 ± 12%, respectively) mRNA levels considerably, while there was no effect by either β-muricholate or ursocholate. The level of both HMG-CoA reductase mRNAs together was increased (+47 ± 21%) by cholate, but remained unaltered upon addition of ursocholate.

Analysis of transcriptional activity (Table 3) in cells incubated for 16 hours with 50μM cholate or ursocholate, showed suppression of transcription of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes (-43 ± 8 and -42 ± 9%, respectively) relative to that of β-actin, used as an internal standard. There was no effect on HMG-CoA reductase gene transcription, suggesting that the observed up-regulation at the level of mRNA for this particular enzyme represents post-transcriptional changes. There was also no effect on the expression of the house-keeping gene GAPDH. Ursocholate showed no effect on transcription of any of the genes analyzed.

Table 3. Effect of cholate and ursocholate on transcriptional activity of the cholesterol 7α-hydroxylase, sterol 27-hydroxylase and HMG-CoA reductase genes.

Gene	relative transcriptional activity (% of control)	
Bile acid added:	cholate	ursocholate
cholesterol 7α-hydroxylase	57 ± 8*	92 ± 10
sterol 27-hydroxylase	58 ± 9*	110 ± 18
HMG-CoA reductase	90 ± 23	94 ± 12
GAPDH	89 ± 24	100 ± 16

Cells were exposed to bile acids (50 μM) for 16 hours, from 26-42 hours of culture, in two 8-hour periods, as opposed to untreated cells, as described in the legends to table 1. Cells were harvested and transcriptional activity for cholesterol 7α-hydroxylase, sterol 27-hydroxylase, HMG-CoA reductase and GAPDH was assessed relative to that of actin, used as an internal standard. [^{32}P]-labeled total RNA was hybridized to immobilized corresponding cDNAs as indicated in the "Experimental Procedures" section, and the resulting blots were exposed to Hyperfilm for 48-120 hours. Non-specific hybridization was checked using the empty vector pUC18. Results shown are expressed as transcriptional activity relative to that of actin, and as a percentage of control (0.1% DMSO). Data are a means ± SD of independent experiments using hepatocytes from 3-4 rats. * Indicates a difference ($p < 0.05$) compared with control values.

Table 4. CAT-activity of the cholesterol 7α-hydroxylase promoter-reporter construct -348*cat* in response to different bile acids in transfected cultured rat hepatocytes.

Bile acid added		Relative CAT-activity (% of control)
control (0.1% DMSO)		100
cholate	(3α7α12α)	28 ± 7*
ursocholate	(3α7β12α)	104 ± 10

Primary rat hepatocytes were transfected with the CaP_i-method, as described in the "Experimental Procedures" section. Transfected cells were subsequently incubated with 50 μM cholate or ursocholate for 42 hours, and CAT activity was assessed afterwards. The cholesterol 7α-hydroxylase promoter activity is expressed relative to that of control (0.1% DMSO), and as a mean of 4 independent experiments. * Indicates a significant difference ($P < 0.05$) compared with control values.

Effect of cholate and ursocholate on CAT-activity in cells transfected with a cholesterol 7α-hydroxylase promoter-CAT-reporter construct

Recent results obtained by Hoekman et al (14), using transient expression assays, indicatedthat the region between nucleotides -79 and -49 of the rat cholesterol 7α-hydroxylase promoter is essential for a bile acid induced response. We wanted to assess whether, using this system, a differentiation is made between bile acids. The -348R*cat*-construct, consisting of the proximal 348 nucleotides of the cholesterol 7α-hydroxylase promoter, fused to the CAT-reporter gene (14), was used in transient-expression experiments. Table 4 shows that, while cholate had a strong inhibitory effect on promoter activity of the -348R*cat*-construct (-72 ± 8%), ursocholate did not. The SV40*cat*-signal, showing a strong basal expression originating from the SV40-promoter, and used as an external standard in parallel incubations, showed no response to either bile acid (data not shown). These results confirm the conclusions drawn from nuclear run-off assays, showing that, at least in part, down-regulation of cholesterol 7α-hydroxylase mRNA levels is a result of decreased transcription. Furthermore, suppression of gene expression via a distinct DNA-element within the cholesterol 7α-hydroxylase promoter appears to be specific in terms of bile acid structure.

Structure-function relationships

Previous studies have indicated that cholesterol 7α-hydroxylase mRNA levels decline rapidly in parallel with enzyme activity levels, in response to bile acids ($T_{½}$ = 4h for both parameters), and the mRNA is therefore considered a good parameter in this respect (13). Twenty-seven different bile acids were therefore tested on their ability to down-regulate levels of cholesterol 7α-hydroxylase mRNA. Metabolism of bile acids by hepatocytes was reduced or prevented by short-term incubations and by renewing of medium and bile acids.

Hydroxylation of bile acids by the hepatocytes was lowered by simultaneous incubation of bile acids in the presence of 10 μM ketoconazole, as demonstrated by the decrease in metabolism of chenodeoxycholate, a bile acid specifically subject to conversion (49). Without additional measures, 89% of chenodeoxycholate was converted to β-murichолate, as determined by gas liquid chromatography, over an 8-hour period by cultured rat hepatocytes. Simultaneous incubation with 10μM ketoconazole lowered conversion to 25% over this period of time. Nonetheless, culturing cells for two 8-hour periods with 50 μM chenodeoxycholate in the absence of ketoconazole yielded a 45 $\pm$ 8% suppression of cholesterol 7α-hydroxylase mRNA, despite conversion of this bile acid to a non-active metabolite under these circumstances. The latter finding indicates a rapid effect of bile acids on mRNA levels for this enzyme, prior to appreciable conversion, in agreement with short-term down-regulation by bile acids as reported before (13). Simultaneous addition of both chenodeoxycholate and ketoconazole led to a further reduction (-85 $\pm$ 8%) of cholesterol 7α-hydroxylase mRNA (Table 5).

The bile acids used for analysis, differed in hydrophobicity index, and in number, position and orientation (α/β) of hydroxyl groups present on the ringstructure of the bile acid. Additional changes concerned the presence of a keto-group at the 7-, 12- or both positions instead of the normal hydroxyl-entity. In a few cases there was also variation in the orientation of the hydrogen atom located at the 5-position in the ring-structure (5α or 'allo' as opposed to the common 5β-epimers). Table 5 summarizes the characteristics of each bile acid tested, and their inhibitory potency in terms of effect on cholesterol 7α-hydroxylase mRNA level. Both synthetic and naturally-occurring monohydroxy bile acids were powerful suppressors of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA levels. These bile acids, however, had a very negative impact on cell-viability, which was decreased by approximately 50%, as judged by measurement of mitochondrial dehydrogenase activity (MTT-assay). These compounds were hence excluded from further analysis.

In general, hydrophobic bile acids were potent inhibitors, as demonstrated by the strong suppression of cholesterol 7α-hydroxylase mRNA by cholate (3α7α12α), be it conjugated or not (-91 $\pm$ 8%, -86 $\pm$ 5%, and -78 $\pm$ 10% for tauro-, glyco- and unconjugated cholate, respectively), chenodeoxycholate (3α7α; -85 $\pm$ 8%), and deoxycholate (3α12α; -79 $\pm$ 14%). Nonetheless, Fig. 2, in which cholesterol 7α-hydroxylase mRNA levels are plotted versus the hydrophobicity index of the bile acid in question, demonstrates that the effects of a marked number of bile acids can not be explained by hydrophobicity alone, resulting in a moderate correlation coefficient (r-value = 0.61). Evident exceptions were lagodeoxycholate (3α12β; -7 $\pm$ 21%) and allo-cholate (allo-3α7α12α; -2 $\pm$ 20%), in comparison with cholate (-78 $\pm$ 10%), which differ only very little in hydrophobicity. On the other hand, as stated in the above, cholate (3α7α12α), chenodeoxycholate (3α7α), and deoxycholate (3α12α) were equally active in suppressing cholesterol 7α-hydroxylase mRNA levels, allthough they differ significantly in their respective HI_x. Among the more hydrophilic bile acids there were also

clear exceptions, as demonstrated by the strong suppressive capacity of isohyodeoxycholate (3β,6α; -64 ± 10%), and murocholate (3α,6β; -52 ± 16%). Other examples within this group were the synthetic bile acids 3α7k12α (-43 ± 16%), and 3α7k12k (-45 ± 16%), both of which are hydrophilic compounds, nevertheless active as a suppressor of cholesterol 7α-hydroxylase. Taken together, these data indicate that apart from hydrophobicity as a general characteristic of a bile acid, other aspects of its structure may be important in determining inhibitory potency.

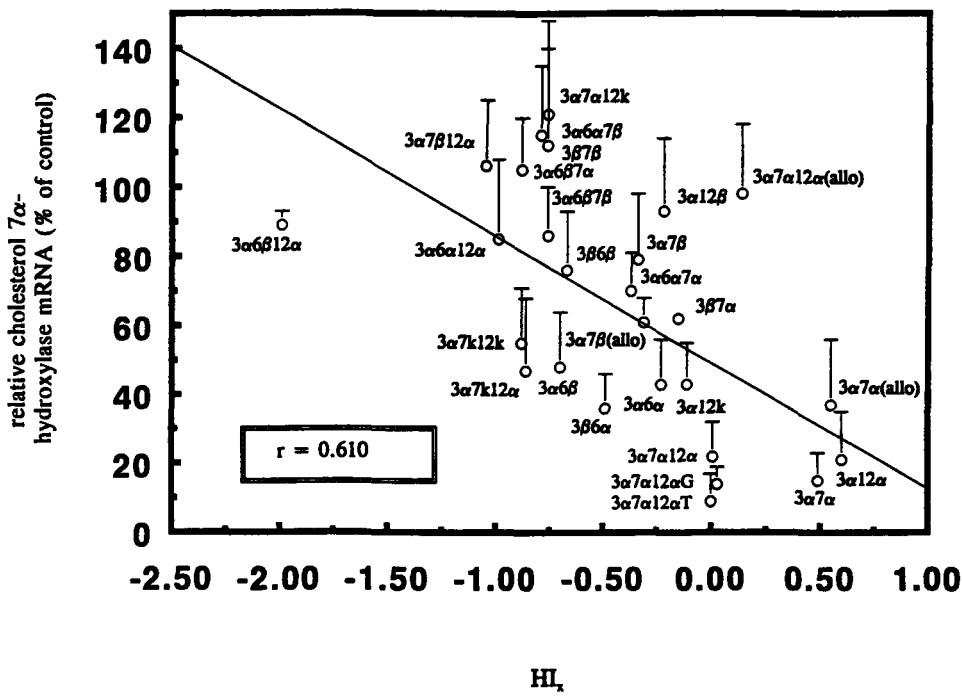

Figure 2. Effect of different bile acids on cholesterol 7α-hydroxylase mRNA levels versus their hydrophobicity index. Rat hepatocytes were incubated with different bile acids (50 μM) from 26 to 42 hours of culture, for two 8-hour periods, in the presence of 10 μM ketoconazole. Medium of cells was refreshed in the second 8-hour period, and fresh bile acids and ketoconazole were added. Cells were harvested at 42 hours, and the cholesterol 7α-hydroxylase mRNA levels were assessed as described in the legends to table 5. Hydrophobicity indices (HI_x) were determined by HPLC and converted to scale by graphic interpolation as proposed by Heuman et al. (21). Cholesterol 7α-hydroxylase is plotted versus HI_x. Structural characteristics depicted refer to bile acids as presented in Table 5.

Table 5. Effects of different bile acids (50 μM) on cholesterol 7α-hydroxylase mRNA in primary cultures of rat hepatocytes.

Bile acid	hydroxyl substituents	HI_x	Cholesterol 7α-hydroxylase mRNA (% of control)	n
I. conjugated bile acids				
1. glycocholate	3α7α12α	0.03	14 ± 5*	3
2. taurocholate		0.00	9 ± 8*	12
II. unconjugated bile acids				
3. hyodeoxycholate	3α6α	-0.23	43 ± 13*	4
4. murocholate	3α6β	-0.70	48 ± 16*	4
5. isohyodeoxycholate	3β6α	-0.49	36 ± 10*	3
6. isomurocholate	3β6β	-0.67	76 ± 17*	4
7. chenodeoxycholate	3α7α	0.49	15 ± 8*	3
8. allo-chenodeoxycholate	3α7α(allo)	0.55	37 ± 19*	4
9. ursodeoxycholate	3α7β	-0.34	79 ± 19*	5
10. allo-ursodeoxycholate	3α7β(allo)	-0.31	61 ± 7*	4
11. isochenodeoxycholate	3β7α	-0.15	62 ± 2*	3
12. isoursodeoxycholate	3β7β	-0.76	112 ± 28	4
13. deoxycholate	3α12α	0.60	25 ± 16*	4
14. lagodeoxycholate	3α12β	-0.22	93 ± 21	3
15. hyocholate	3α6α7α	-0.37	70 ± 11*	5
16. ω-muricholate	3α6α7β	-0.79	115 ± 20	3
17. α-muricholate	3α6β7α	-0.88	105 ± 15	2
18. β-muricholate	3α6β7β	-0.76	86 ± 14	3
19. -	3α6α12α	-0.99	85 ± 23	3
20. -	3α6β12α	-1.99	89 ± 4*	3
21. cholate	3α7α12α	0.01	22 ± 10*	3
22. allo-cholate	3α7α12α(allo)	0.14	98 ± 20	3
23. ursocholate	3α7β12α	-1.04	106 ± 19	7
III. bile acids with keto-substituents				
24.	3α12k	-0.11	43 ± 12*	3
25.	3α7k12α	-0.86	47 ± 21*	5
26.	3α7α12k	-0.76	121 ± 27	4
27.	3α7k12k	-0.88	55 ± 16*	4

Rat hepatocytes were incubated with different bile acids (50 μM) from 26 to 42 hours of culture, for two 8-hour periods in the presence of 10 μM ketoconazole. Medium was refreshed in the second 8-hour period, and fresh bile acids and ketoconazole were added. Cells were harvested at 42 hours, and the cholesterol 7α-hydroxylase mRNA levels were assessed by slot-blotting and densitometric scanning of resulting autoradiographs, using the actin mRNA as an internal standard to correct for differences in the amount of RNA applied, as described in "Experimental Procedures". Data are expressed as a percentage of control (0.1% DMSO) and are a means ± SD of independent experiments using hepatocytes from n rats. HI_x for each bile acid used was determined by HPLC and converted to scale by graphic interpolation, as proposed by Heuman et al. (21), and further described in "Experimental Procedures". * Indicates a significant difference ($P < 0.05$) compared with control values.

DISCUSSION

The present study shows hydrophobic bile acids to be powerful inhibitors of bile acid synthesis at the level of cholesterol 7α-hydroxylase and sterol 27-hydroxylase. However, apart from hydrophobicity, other structural features are important for determining inhibitory potency.

The results presented show down-regulation by the bile acids deoxycholate (3α12α) and cholate (3α7α12α) of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, as a result of an effect of these compounds on mRNA expression and transcriptional activity of the corresponding genes. In contrast, there was no effect of β-muricholate (3α6β7β) or of ursocholate (3α7β12α), two hydrophilic bile acids, on either enzyme. A simple 7β-epimerization, as is the case for ursocholate versus cholate, renders the bile acid ineffective, in agreement with previous *in vivo* studies in rats showing a decrease in bile acid synthesis and similar decline of cholesterol 7α-hydroxylase activity after infusion of taurocholate, while yielding no effects after infusion of tauroursocholate (25). The current paper shows that the key-enzymes, involved in both major and alternative routing of cholesterol to bile acids, are sensitive to similar bile acids.

Analysis of the rat cholesterol 7α-hydroxylase promoter via the transient expression system described, revealed a preference for cholate over ursocholate in terms of inhibition of CAT-activity of the cholesterol 7α-hydroxylase promoter construct -348R*cat*. In a previous paper, Hoekman et al. (14) described the localization of a specific DNA sequence, responsive to bile acids, between -79 and -49 nucleotides preceding the transcriptional initiation site. Apparently, this regulatory element differentiates, directly or indirectly, between bile acids in accordance with their differential effects *in vivo* and *in vitro*. Promoter regions of both cholesterol 7α-hydroxylase (nucleotides -49 to -79 in rat) and sterol 27-hydroxylase (nucleotides -254 to -280 in human) show similarities limited to a region subject to interaction with several known transcriptional factors (14,57). For instance, a putative binding-site for the liver-specific transcription factor HNF-1a is present within these regions. In accordance with the observed coordinate regulation of both enzymes by similar bile acids (16, and this study), this suggests that specific bile acids may interact in some way with a DNA sequence (bile acid response element, or BARE) common to the promoter region of both enzymes.

Several views have been expressed with respect to the nature of bile acids involved in suppression of cholesterol 7α-hydroxylase. It has been suggested that specifically monohydroxylated bile acids are active as inhibitor of the enzyme (24). Use of this particular subgroup of bile acids, however, yielded toxic effects, in accordance with reported hepatotoxicity of these compounds *in vitro* (58). Absence of an effect of infusion of cholate *in vivo* in rabbit, as opposed to an effect of either deoxycholate or lithocholate, has led to the

suggestion that primary bile acids have to be converted intestinally in order to become active (22-24). Equal effects of both primary (e.g. cholate) and secondary (deoxycholate) bile acids (Table 5) rules out this possibility.

The generally accepted idea is that hydrophobicity determines inhibitory potency of bile acids (15,20,21). Nevertheless, in a previous paper we clearly show equal inhibitory effects of cholate, chenodeoxycholate, and deoxycholate, despite substantial differences in their respective hydrophobicity indices (12,13). We therefore determined the effects of a much larger group of bile acids on cholesterol 7α-hydroxylase, differing highly in number, position and orientation of the OH-groups present on the basic molecule. Other changes made concerned bile acids with a keto-group instead of a hydroxyl group, and isomerisation of the 5β-hydrogen atom, resulting in 5α- or allo-forms. Figure 2 shows that the correlation between inhibition of cholesterol 7α-hydroxylase mRNA and the hydrophobicity index of the bile acid involved is a weak one ($r = 0.61$). The low correlation coefficient cannot be attributed to conversion of bile acids by the hepatocyte. Only a few of the bile acids that do not follow the 'hydrophobicity rule' are subject to conversion, and even so are converted into bile acids that cannot explain observed effects. Chenodeoxycholate (3α7α) is converted into β-muricholate (3α6β7α), a non-active bile acid (Tables 1,2 and 5) for 89% within 8 hours. Nevertheless it is a potent suppressor of cholesterol 7α-hydroxylase mRNA levels (down to 55 $\pm$ 8%). Efforts to prevent conversion by refreshing medium and adding new bile acids within the 16-hour period, in combination with suppression of conversion using ketoconazole, lowered this value to 15 $\pm$ 8%. Taken together, it is apparent that the effects of the various bile acids tested take place very rapidly, preceding appreciable conversion by the hepatocyte. Addition of taurocholate to rat hepatocytes leads to a 50% reduction in cholesterol 7α-hydroxylase mRNA levels within 4 hours (13), in agreement with this assumption. The short half-life of the messenger RNA is consistent with the reported half-life for the cholesterol 7α-hydroxylase enzyme *in vivo* in rat (59,60). In this respect, discrepancies between our results and those obtained by Stravitz and coworkers (15), can be explained by differences in time-curves in response to taurocholate. The latter work, in which primary rat hepatocytes were cultured with various bile acids in serum-free medium supplemented with thyroxine and dexamethasone, showed a 50% reduction of cholesterol 7α-hydroxylase mRNA only after 24 hours of incubation. Under the latter conditions, conversion of bile acids by the hepatocyte may play an important role in the effects observed.

Strong examples of effects of bile acids that can not be explained by hydrophobicity are cholate (3α7α12α) versus allo-cholate ((5α)3α7α12α) and lagodeoxycholate (3α12β), and murocholate (3α6β) versus β-muricholate (3α6β7β). In both cases, the bile acids differ only slightly in hydrophobicity index, but show marked differences in inhibitory potency. Two bile acids were therefore analyzed with molecular modelling techniques, using the crystal structure of methylcholate as a starting point (48). Based on the molecular structure

described, it was evident that the three hydroxyl groups present on methylcholate are in close proximity of each other, and that they are grouped together within the molecule to form a hydrophilic component within an otherwise relatively hydrophobic molecule (Fig. 3a). Several bile acids were analyzed by changing the basic cholate structure, and subsequent remodelling into an energetically minimized conformation. This analysis elucidated that allo-cholate, having a proton in the 5α-position, as opposed to cholate (5β), did not have the hydroxyl groups in proximity within the molecule. Calculation of the approximate distances between the three hydroxyl groups shows large differences, particularly between the 3α- and 12α-OH (8.4Å in allo-cholate as opposed to 6.4Å in cholate), and between 3α- and 7α-OH (6.4Å and 5.0Å, respectively). This particular epimerization causes drastic changes in the conformation of the steroid backbone (Fig. 3b), as a result of which the three hydroxyl groups are directed away from each other. Allo-cholate is a very poor regulator of cholesterol 7α-hydroxylase mRNA. In other cases, small or large changes in the relative position of the hydroxyl groups disrupting the hydrophilic microenvironment within the molecule, could be correlated to the effect of the particular bile acid on cholesterol 7α-

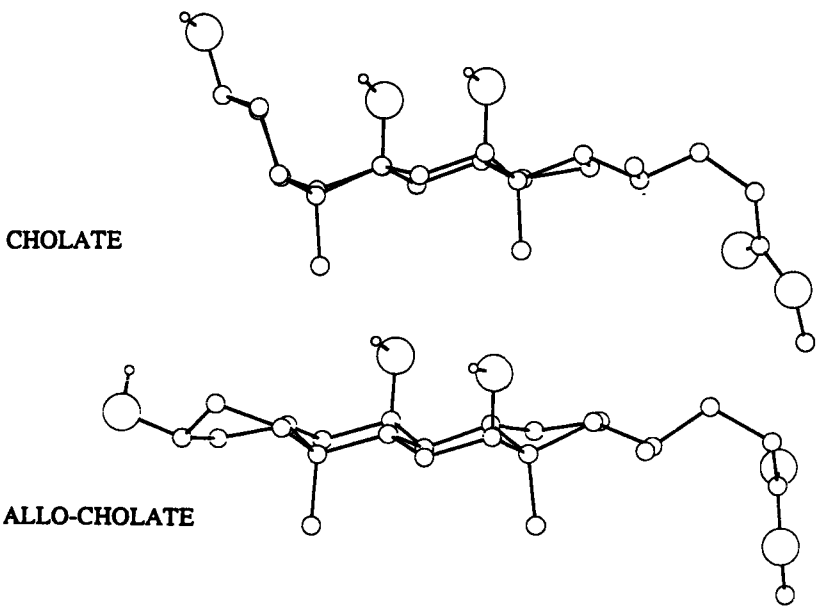

Figure 3. Three-dimensional structure of cholate versus allo-cholate. Three-dimensional depiction of cholate, based on the crystallographical studies of methylcholate by Miki et al. (48). The structure of allo-cholate was derived from the previous one by computer-modelling according to methods described in "Experimental Procedures". Small circles: hydrogen atoms (shown only when connected to oxygen atoms); intermediate circles: carbon atoms; large circles: oxygen atoms.

hydroxylase. Epimerization of the hydroxyl group in the 7-position, as is the case for cholate (3α7α12α) versus ursocholate (3α7β12α), chenodeoxycholate (3α7α) versus ursodeoxycholate (3α7β), and in the 12-position deoxycholate (3α12α) versus lagodeoxycholate (3α12β), weakened hydrophobic/hydrophilic compartimentalization within the molecule. In each case, this resulted in a decreased ability to down-regulate cholesterol 7α-hydroxylase mRNA.

The intramolecular organisation of cholate could be suggestive of some factor binding to the hydrophilic component of the molecule, acting perhaps as an anchor for interaction. It is feasible that binding of a protein factor regulating cholesterol 7α-hydroxylase gene transcription to a presumed BARE sequence (11,61), may be dependent on the relative orientation of the hydroxyl groups present on the bile acid molecule. As in the case of allocholate, the hydroxyl groups protrude from the steroid base, and may interfere sterically with binding to the factor. Presence of two hydroxyl groups or more requires them to be in close proximity three-dimensionally (3α7α12α, 3α7α, 3α12α), and any deviation therefrom leads to intermediate (3α7k12k, 3α7k12α, 3α6β, 3β6α, 3α6α or 3α12k) or no capacity at all (allo-3α7α12α, 3α7β12α, 3α12β, 3α7β) to down-regulate cholesterol 7α-hydroxylase.

Hydrophobicity, as determined by assessing relative distribution of a given bile acid within a two-phase system as described, is dependent on the number, position and orientation of hydroxyl groups within the molecule. It is therefore difficult to segregate hydrophobicity *per se* from other structural aspects of a bile acid, with respect to its potency to affect cholesterol 7α-hydroxylase and sterol 27-hydroxylase. Nevertheless, from studying effects of a large group of bile acids, differing significantly in hydroxylation-status and hydrophobicity index, it can be concluded that the effects observed can not be explained by differences in hydrophobicity alone. We postulate that coordinate down-regulation of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase at the level of gene transcription by bile acids requires specific structuralization of the bile acid molecule. Compartimentalization, in which the hydroxyl groups present on the bile acid are in close proximity and hence form a clear hydrophilic environment, may be a prerequisite for binding to a putative factor involved in interaction with regulatory sequences within the cholesterol 7α-hydroxylase and sterol 27-hydroxylase promoter.

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CHAPTER 5

HETEROGENEOUS EXPRESSION OF CHOLESTEROL 7α-HYDROXYLASE AND STEROL 27-HYDROXYLASE GENES IN THE RAT LIVER LOBULUS

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ABSTRACT

We investigated the lobular localization and molecular level of expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase, two key-enzymes in bile acid synthesis, in isolated periportal and pericentral hepatocytes, and by *in situ* hybridization of rat liver. Enzyme activity, mRNA, and gene transcription of cholesterol 7α-hydroxylase were predominant in pericentral hepatocytes of control rats, being 7.9-fold, 9.9-fold, and 4.4-fold higher than in periportal hepatocytes, respectively. Similar localization was found for sterol 27-hydroxylase; 2.9-fold, 2.5-fold, and 1.7-fold higher enzyme activity, mRNA, and gene transcription, respectively, in pericentral hepatocytes. Interruption of the enterohepatic circulation with colestid showed up-regulation of these parameters for both enzymes, as a result of stimulated gene expression mainly in the periportal zone. In contrast, mRNA levels and gene transcription of HMG-CoA reductase, showed opposite lobular distribution. Selective periportal expression for the latter was enhanced, but remained local, after colestid-treatment. *In situ* hydridization showed unambiguously that cholesterol 7α-hydroxylase mRNA is localized exclusively in the pericentral zone and that sterol 27-hydroxylase mRNA is expressed preferentially in the pericentral region, all be it less pronounced. Administration of colestid led to expression of both genes within a larger area of the liver lobulus. In conclusion, we suggest that cholesterol 7α-hydroxylase and sterol 27-hydroxylase are coordinately regulated by the bile acid gradient over the lobulus, resulting in predominant expression in the pericentral zone. Opposite lobular localization of cholesterol and bile acid synthesis provides an alternative view to interregulation of these metabolic pathways.

INTRODUCTION

The liver plays an important role in the homeostatic maintenance of a large number of nutrients in the blood, such as carbohydrates, amino acids and lipids, and is the main site of intermediary metabolism thereof. It has become increasingly clear that not all hepatocytes contribute equally in this task. In contrast, contribution of hepatocytes to uptake, storage, interconversion and release of various compounds shows a large degree of heterogeneity along the portocentral axis, even up to a point that only a few cells are involved in a given function (1-4). The concept of "metabolic zonation" dictates that the heterogeneous expression of enzymes in the liver lobulus (or acinus) is a major determinant for the proper execution and regulation of various liver functions (5,6). Opposite metabolic pathways like gluconeogenesis and glycolysis are carried out simultaneously by hepatocytes in periportal and pericentral zones, respectively, and are separately localized within the liver (1,3). Importantly, the distribution may be dynamic under different physiological and pathological

conditions, in the sense that the liver may adapt to certain requirements by changes in distribution patterns. Liver-cell heterogeneity thus provides the basis for effective regulation and adaptation to different metabolic states.

It has previously been shown that cholesterol synthesis is predominantly localized in the periportal hepatocytes, as judged from the positive immunohistochemical staining for HMG-CoA synthase and HMG-CoA reductase protein in only 20% of the periportal cell fraction (7,8). Excretion of cholesterol into bile, either as free cholesterol or following its conversion into bile acids, is the predominant pathway for elimination of cholesterol from circulation in mammals (9,10). Rate of bile acid formation is therefore considered an important determinant for cholesterol homeostasis. On the other hand, bile acid synthesis and major key-enzyme in routing of cholesterol to bile acids, cholesterol 7α-hydroxylase, are mainly localized pericentrally (11,12). Opposite lobular localization of cholesterol synthetic and metabolic pathways poses the interesting question how the two are interregulated to achieve homeostasis.

Another aspect of bile acid synthesis concerns the different pathways of bile acid formation. According to current views, the initial and rate-determining step in routing of cholesterol to bile acids is catalyzed by cholesterol 7α-hydroxylase (9). However, accumulating evidence has led to suggest that an alternative pathway exists, involving initial 27-hydroxylation of cholesterol via sterol 27-hydroxylase (13). Based both on *in vivo* studies in humans (13), and studies using cultured human and rat hepatocytes (14) it was concluded that this alternative pathway may contribute considerably to total bile acid synthesis (13,14). Hitherto, lobular expression of sterol 27-hydroxylase has not been assessed.

One of the major regulatory processes affecting bile acid biosynthesis is bile acid-induced feedback inhibition, which is exerted at the level of cholesterol 7α-hydroxylase by the flux of bile acids returning to the liver via portal blood (9). Bile acids are taken up efficiently by the periportal hepatocytes (15-19), thereby creating decreasing concentration gradients along the sinusoids (20). Consequently, periportal hepatocytes are exposed to a 6-fold higher concentration of bile acids, as compared with those in the pericentral area (15,16). In line with this, a concentration-dependent and direct down-regulation of bile acid synthesis, at the level of cholesterol 7α-hydroxylase, was found *in vitro* when cultured pig (21) and rat (22,23) hepatocytes were incubated with bile acids. In a recent paper, our group demonstrated that sterol 27-hydroxylase is regulated in parallel with cholesterol 7α-hydroxylase in cultured rat hepatocytes, resulting in coordinate down-regulation of both enzymes at the mRNA and transcriptional level by similar bile acids. Thus it was postulated that efficient down-regulation of bile acid synthesis by bile acids is accomplished by coordinate regulation of both key-enzymes (24).

In the present study, we have assessed the distribution patterns for both cholesterol 7α-hydroxylase and sterol 27-hydroxylase within the liver lobulus. In addition, we established

the molecular level at which these expression patterns are imposed, by measuring mRNA levels in freshly isolated periportal and pericentral hepatocytes, and transcriptional activity levels for these enzymes using nuclear run-off assays. Dynamic aspects of lobular distribution of these cholesterol-metabolizing enzymes, and relationship of their expression with lobular bile acid concentrations, were determined by treatment of rats with colestid. The bile-acid-sequestrant, like cholestyramine, has been shown to lead to up-regulation of bile acid synthesis (25), as a result of lowering of the bile acid concentration in portal blood (26). Lobular distribution in control and stimulated rats was also assessed by *in situ* hybridization of sections of rat liver.

The current study shows heterogeneous distribution of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase. Heterogeneity resulted from coordinate differential transcriptional activity of both genes, and even more so of steady-state mRNA levels for the enzymes, residing primarily in the pericentral zone of the liver lobulus. Opposite expression was found for the HMG-CoA reductase gene. Treatment of rats with colestid led to a more overall recruitment of hepatocytes within the lobulus for bile acid synthetic capacity, resulting from increased expression of the cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene within a large part of the liver lobule.

METHODS

Materials used for isolation of rat hepatocytes, determination of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, and determination of mRNA and transcriptional activity levels, have been described previously (22,27-30).

Animals

Male Sprague-Dawley rats (200-280 gm) were used for isolation of hepatocytes. Animals were kept in a strictly controlled 12-hr light and dark cycle (lights on from 06:00-18:00 h) on standard chow (Alma H 1003, Botzenhardt, Kempten, F.R.G.) and tap water *ad libitum*. A separate group of animals was fed a similar diet supplemented with 5% (w/w) Colestid (Upjohn, Belgium), for a 7-day period prior to isolation (31). Time of isolation was between 08:00-9:00 h.

Isolation of hepatocytes

Total liver parenchymal cells were isolated by the two-step collagenase perfusion technique, modified as described (28).

Periportal and pericentral subfractions of hepatocytes were isolated by the digitonin/collagenase perfusion technique, as described by Quistorff (32) and Lindros &

Penttilä (33), with modifications described elsewhere (27). Viability, assessed by trypan blue exclusion, was higher than 90% and 80% for normal and isolated periportal and pericentral hepatocytes, respectively. Cell suspensions from digitonin/collagenase perfusions with a viability index of less than 70% were discarded.

The efficiency of enrichment of periportal and pericentral hepatocytes was monitored by measurements of glutamine synthetase, alanine aminotransferase and pyruvate kinase activity (Table 1).

Table 1. Activity of marker-enzymes in pericentral and periportal hepatocytes.

Enzyme	activity (nmoles/min per mg cell protein)		PC:PP ratio
	pericentral	periportal	
glutamine synthetase	632 ± 167 (6)	13 ± 6 (6)	48.6
alanine aminotransferase	189 ± 94 (6)	293 ± 105 (6)	0.65
pyruvate kinase	168 ± 72 (5)	137 ± 41 (4)	1.22

Pericentral and periportal hepatocytes were isolated as described in "materials and methods". Purity of hepatocyte preparations was assessed by determination of activities for various marker enzymes. Data are expressed as absolute values ± SD of enzyme activities, using hepatocytes from (n) rats, or as a ratio of PC:PP activity.

Enzyme assays

The activity of glutamine synthetase was determined by the glutamyltransferase assay with modifications reported previously (34). Alanine aminotransferase and pyruvate kinase were determined according to Bergmeyer (35). Cholesterol 7α-hydroxylase and sterol 27-hydroxylase were assessed using homogenates as described in detail (14,29,30). Protein and cholesterol were assayed according to methods described (30).

RNA isolation, blotting and hybridization procedures

Total RNA was isolated from whole livers or freshly isolated periportal and pericentral hepatocytes, and quantitation thereof was performed as previously described (22). Probes used in hybridization experiments were labelled by the random-primer method (Mega-prime, Amersham) to approximately 6 x 10^8 cpm/μg DNA. After hybridization and washing, the filters were exposed to Hyperfilm MP (Amersham) together with an intensifying screen (Eastman-Kodak Co.) for 48-120 h at -80°C. For quantitation of the relative amounts of mRNA, the autoradiographs were scanned using a Shimadzu CS 910 chromatograph scanner, and areas under the curves were integrated using a data processor (Shimadzu Corp. Kyoto,

Japan). 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 as described in detail in ref. 22; a 1.6 kb HindIII/XbaI fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. Jerome Strauss (36), and isolated from a rat liver cDNA library using the rabbit sterol 27-hydroxylase cDNA, previously isolated by Russell and coworkers (37), as a probe; a 700 bp EcoRI fragment of hamster lithocholic acid 6β-hydroxylase cDNA (38); a 773 bp HindIII fragment of hamster HMG-CoA reductase cDNA (39); a 1.5 kb PstI fragment of rat glutamine synthetase cDNA (40,41); a 1.2 kb PstI fragment of hamster actin cDNA; and a 1.1 kb PstI fragment of rat GAPDH. The latter two served as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter.

Nuclear run-off studies

Nuclear run on studies were conducted essentially as described in ref. 22. Whole livers, or freshly isolated periportal and pericentral hepatocytes, from control and colestid-treated rats served as material for the isolation of nuclei. For whole liver preparations, livers were perfused with saline-solution (0.9% NaCl), cut into small fragments, washed and homogenized mechanically (10 strokes, 180 rpm) in NP40-lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 1 mM PMSF, 1 mM DTT), at 4°C. The resulting suspension was filtered (100 μm) prior to an additional homogenizing step. Alternatively, directly after isolation, periportal and pericentral hepatocytes were washed and resuspended in NP40-lysis buffer. Both preparations were further homogenized, after being left on ice for 5 min, in a Potter Elvehjem tube with pestle B for 15 strokes at 4°C. Resulting nuclei were centrifuged at 500 x g and resuspended in NP40-lysis buffer. Washing procedures in NP40-lysis buffer were repeated until the nuclei were free of cellular debris. They were then taken up in glycerol storage buffer (50 mM Tris-HCl pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 5 mM DTT), counted, and aliquoted at approximately 2 x 10^7/500 μl before being frozen at -80°C.

RNA labelling and isolation was performed as described (22). Target DNA, being 5 μg of plasmid material containing cDNA sequences of rat cholesterol 7α-hydroxylase, rat sterol 27-hydroxylase, hamster HMG-CoA reductase, hamster lithocholic acid 6β-hydroxylase, hamster actin and rat GAPDHs, or the empty vector pUC19, were slotblotted onto strips of Hybond-N^+ filter (Amersham) and crosslinked. The filters were hybridized with the [^{32}P]-labelled RNA for 36 hours, washed and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantitation of relative amounts of mRNA synthesized was conducted using a Phosphor-imager 400B (Molecular Dynamics).

***In situ* hybridization experiments**

Liver-tissue was fixed in 4% formaldehyde-solution and quickly frozen in liquid Freon 22, for preparation of sections (41). The *in situ* hybridization experiments were performed on closely adjacent sections to allow easy comparison of the patterns of hybridization. [^{35}S]-Labelled probes for *in situ* hybridization were prepared using the multiprime DNA labeling method, to a specific activity of 5-10 x 10^8 cpm/μg of DNA. Prehybridization treatments, hybridization and autoradiography were carried out precisely as described previously (41,42). Negative controls included RNAse-treated sections, and hybridizations with an empty vector (pBR322).

RESULTS

Heterogeneous distribution of cholesterol 7α-hydroxylase and sterol 27-hydroxylase

Pericentral and periportal hepatocytes were isolated by digitonin/collagenase perfusion as described (43), and purity of the different preparations was determined in terms of enrichment of specific marker enzymes known to be differentially expressed. Table 1 shows strong predominant expression of glutamine synthetase in the pericentral fraction (pericentral (PC):periportal (PP) ratio being 48.6), in agreement with expression of this enzyme within the most distal pericentral hepatocytes surrounding the central venules of rat liver (43,44). Other marker enzymes, alanine aminotransferase and pyruvate kinase, showed PC:PP ratio's of 0.65 and 1.22, respectively, in agreement with data reported by others (33). Taken together, the perfusion technique allows for a good separation of hepatocytes into a pericentral and periportal fraction, keeping differential expression of several known marker enzymes intact.

Northern-blotting of total RNA isolated from periportal and pericentral hepatocytes (Fig. 1) shows the typical expression pattern for cholesterol 7α-hydroxylase in both cell preparations (mRNAs 2.1, 3.6, and 4.0 kb in size, as reported before (22,45,46)). Clearly, expression of cholestrol 7α-hydroxylase mRNA is particularly strong in the pericentral area, as is that of glutamine synthetase (two mRNAs of 1.6 and 2.8 kb, as described (47)), assessed for reasons of comparison. The latter is in agreement with exclusive expression of both mRNA and protein (41,47) in a very limited fraction of pericentral cells. Sterol 27-hydroxylase (2.4 kb in rat liver) (24,36) is expressed less abundantly in rat liver, as compared with the messengers described in the above. Nevertheless, this mRNA was also localized predominantly pericentrally. Figure 2 summarizes the distribution of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and mRNA levels, the latter relative to β-actin mRNA. β-Actin mRNA was used as an internal standard, and did not vary between different cell-preparations, neither from control (Fig. 1)- nor from colestid-treated rats (data not

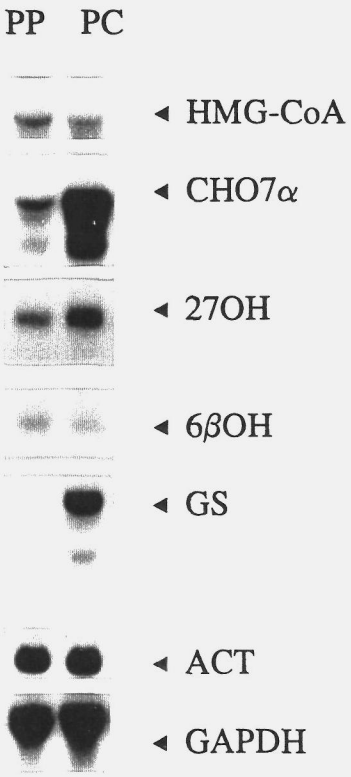
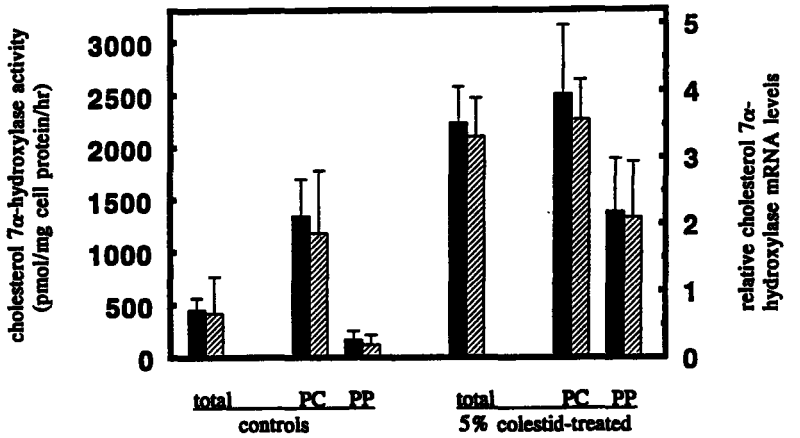


Figure 1. Distribution patterns for cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA in periportal and pericentral hepatocytes. Periportal (PP) and pericentral (PC) hepatocytes were isolated by digitonin/collagenase perfusion, as described in "material and methods". Immediately after isolation of hepatocytes, total RNA was isolated for mRNA analysis. 10 μg of total RNA was electrophoresed in a 0.8% agarose/1 M formaldehyde gel, transferred to Hybond-N^+, and subsequently hybridized with [^{32}P]-labelled cDNA probes for HMG-CoA reductase (HMG-CoA), cholesterol 7α-hydroxylase (CHO7α), sterol 27-hydroxylase (27OH), lithocholic acid 6β-hydroxylase (6βOH), glutamine synthetase (GS), β-actin (ACT), and GAPDH. The latter two served as an internal standard. Resulting filters were subjected to autoradiography for 2-5 days, and specifically for detection of HMG-CoA reductase mRNA: 2-3 weeks.

shown). Both cholesterol 7α-hydroxylase activity and mRNA levels are predominant in hepatocytes from the pericentral area, showing respective PC:PP-ratio's of 7.9 and 9.9 (Fig. 2a). For sterol 27-hydroxylase, heterogeneity of expression is less extreme, showing a 2.9 and 2.5-fold higher activity and mRNA-level, respectively, within the pericentral hepatocytes (Fig. 2b).

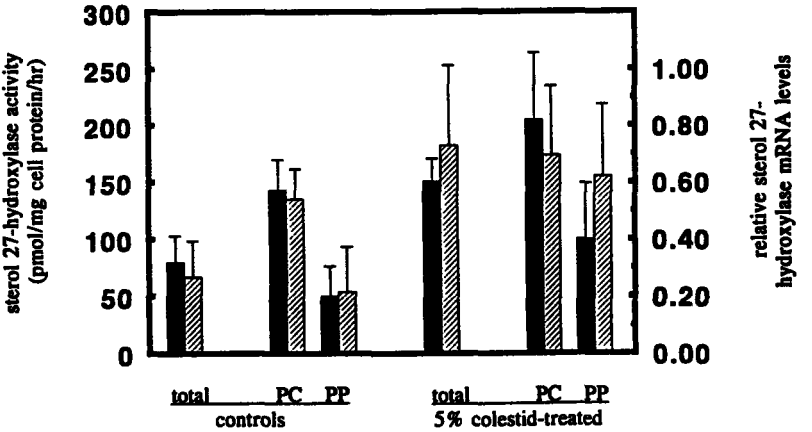
Figure 2. Distribution patterns for cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and mRNA levels in isolated pericentral and periportal hepatocytes from control- and colestid-treated rats
Enzyme activity for cholesterol 7α-hydroxylase and sterol 27-hydroxylase in isolated pericentral and periportal hepatocytes was assessed as described in "materials and methods". RNA samples were prepared and mRNA analysis was performed, as described in the legends to Fig. 1. Rats used for hepatocyte preparations were either fed normal chow, or a diet supplemented with 5% colestid (w/w). Values are expressed in terms of absolute enzyme activity (solid bars), or in arbitrary units of mRNA relative to expression of β-actin (hatched bars), and are means ± SD using hepatocytes from 4-7 rats. PC:PP-ratio's are indicated at the bottom of the figure, as is the extent of stimulation in total hepatocyte preparations by the colestid-treatment. (A), cholesterol 7α-hydroxylase; (B), sterol 27-hydroxylase.

A.



	PC:PP-ratio		stimulation by 5% colestid
	controls	5% colestid	
activity	7.9	1.8	4.9x
mRNA-levels	9.9	1.7	5.0x

B.



	PC:PP-ratio		stimulation by 5% colestid
	controls	5% colestid	
activity	2.9	1.9	1.9x
mRNA-levels	2.5	1.1	2.7x

In view of reports on the periportal localization of HMG-CoA reductase, a key-enzyme in the cholesterol biosynthetic route (7,8), it was of interest to assess the relative mRNA levels for this enzyme within the liver lobulus as well, thus acting as an additional internal control for the identity of periportal hepatocytes. HMG-CoA reductase mRNA levels were very low, both in periportal and pericentral hepatocytes, as indicated by the longer exposure time required (legend to Fig. 1). Low levels of HMG-CoA reductase mRNA in livers of control rats were reported by others (48,49) as well. Nevertheless, a mean PC:PP-ratio of 0.5 (n=3) was detected, in agreement with positive immunohistochemical staining for the HMG-CoA reductase protein of hepatocytes located in the periportal zone (7,8).

Expression of lithocholic acid 6β-hydroxylase, primarily involved in metabolism of secondary bile acids returning to the liver via portal blood (i.e. lithocholic acid; ref. 38), was found preferentially in the periportal zone (Fig. 1; PC:PP-ratio of 0.4, n=3). The latter indicates that not all mRNAs of enzymes involved in bile acid biosynthesis are similarly localized.

Effect of colestid-treatment of rats on the heterogeneity of mRNA patterns for different enzymes

Rats were treated with 5% colestid for 7 days prior to isolation of hepatocytes. The bile-acid-sequestering property of this agent, like that of cholestyramine, leads to up-regulation of bile acid synthesis in man (25) and rat (11,12), as a result of diminished bile acid concentrations in portal blood (26). Assessment of activity for marker enzymes, within different hepatocyte preparations from these rats, revealed PC:PP ratio's similar to those found in control rats (data not shown). As shown in Fig. 2a, feeding rats a colestid-supplemented diet resulted in overall stimulation of both enzyme activity and mRNA levels for cholesterol 7α-hydroxylase. The strong increase in cholesterol 7α-hydroxylase activity (4.9-fold) and mRNA (5.0-fold) in whole liver preparations of stimulated rats is mainly due to stimulation of both parameters in the periportal hepatocytes. Levels for cholesterol 7α-hydroxylase activity within this zone rose from 170 $\pm$ 81 to 1386 $\pm$ 504 pmol/hr per mg of cell protein (8.2-fold), as did mRNA levels for this enzyme (11.1-fold), while both parameters were only stimulated 1.9-fold in the pericentral hepatocytes. Consequently, PC:PP-ratio's for cholesterol 7α-hydroxylase activity and mRNA were lowered to 1.8 and 1.7, respectively, in livers of stimulated rats.

Sterol 27-hydroxylase activity and mRNA (Fig. 2b) were also stimulated in colestid-treated rats (1.9-fold and 2.7-fold, respectively, as compared with control rats), be it less marked. Sterol 27-hydroxylase mRNA was specifically up-regulated in the portal zone (2.9-fold), thereby lowering the PC:PP-ratio from 2.5 in control rats to 1.1 in colestid-treated animals. Sterol 27-hydroxylase activity was mildly stimulated in hepatocytes from both zones, particularly in the portal fraction (2-fold).

HMG-CoA reductase mRNA levels were also increased in colestid-treated rats (4.5-fold, data not shown), in agreement with up-regulation of mRNA (48,49) and activity levels

(49,50) for this enzyme by bile acid sequestrants. In contrast to cholesterol 7α-hydroxylase, however, this particular increase is not a result of overall expression of the messenger in livers from stimulated rats, but rather of selective up-regulation in the portal zone. HMG-CoA reductase mRNA was stimulated 7.8-fold in the portal region, and only 2.7-fold in the pericentral area. The PC:PP-ratio for mRNA of this enzyme was hence lowered even further, from 0.5 in control livers to 0.1 in livers from colestid-treated rats.

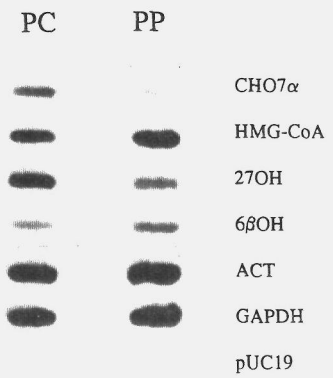


Figure 3. Transcriptional activity of the cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes in isolated pericentral and periportal hepatocytes. Nuclei were prepared from freshly isolated pericentral (PC) and periportal (PP) hepatocytes, as described in "materials and methods". [^{32}P]-labelled total RNA was synthesized *in vitro* using these nuclear preparations, and hybridized to different cDNA-probes. Resulting filters were subjected to autoradiography. Probes used were cDNAs for cholesterol 7α-hydroxylase (CHO7α), HMG-CoA reductase (HMG-CoA), sterol 27-hydroxylase (27OH), lithocholic acid 6β-hydroxylase (6βOH), β-actin (ACT), and GAPDH. The latter two served as internal standards. Non-specific hybridization was checked using an empty vector (pUC19).

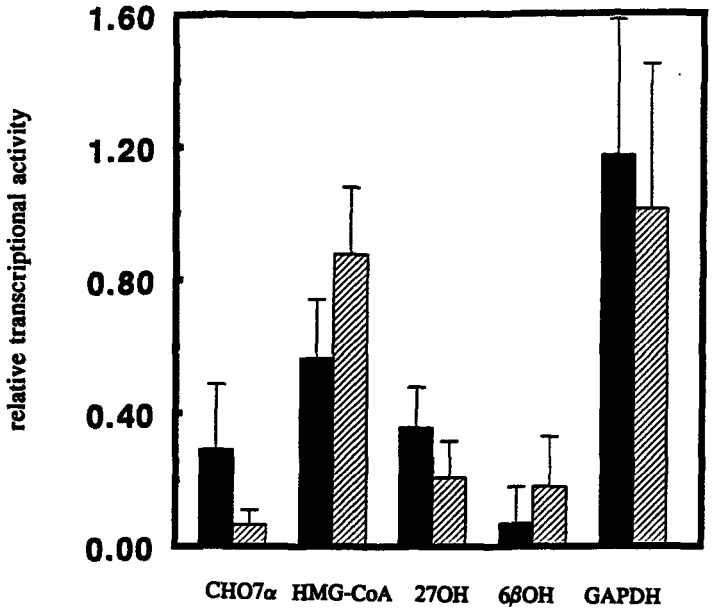
Transcriptional activity of the cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes in different zones of the liver lobulus

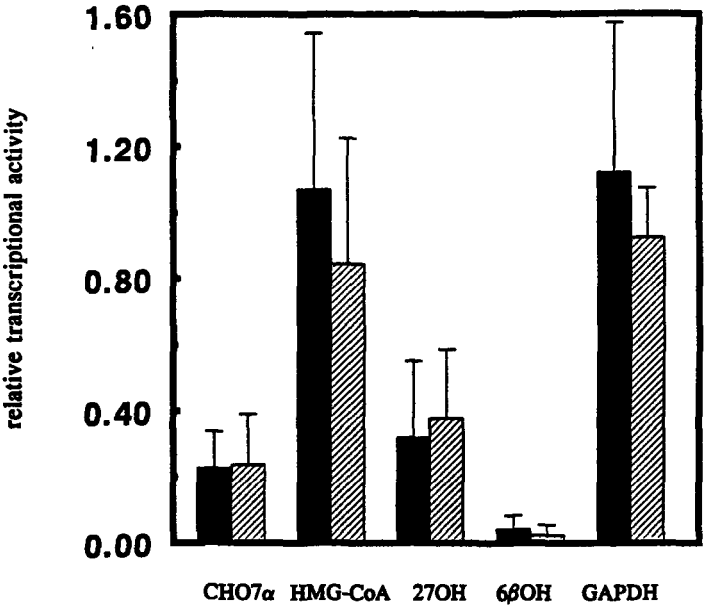
Nuclei from freshly isolated pericentral and periportal hepatocytes were used in nuclear run-off assays. Fig. 3 shows a typical autoradiograph of a hybridization experiment, in which [^{32}P]-labelled RNA from pericentral and periportal nuclei was hybridized to cDNAs specific for cholesterol 7α-hydroxylase, HMG-CoA reductase, sterol 27-hydroxylase, and lithocholic acid 6β-hydroxylase. As internal standards, transcriptional activities of β-actin and GAPDH genes were also assessed in these nuclear preparations. While no difference was found in expression patterns of the latter two, expression of the cholesterol 7α-hydroxylase gene is clearly pericentral (PC:PP-ratio amounting to 4.4, Fig. 4a). Similarly, transcriptional activity of the sterol 27-hydroxylase gene was found to be highest in the pericentral region (PC:PP-ratio of 1.7). In contrast, HMG-CoA reductase gene expression was preferentially localized in periportal cells (PC:PP-ratio of 0.7), in agreement with opposite lobular localization of mRNA for this enzyme. The overall transcriptional activity is high for HMG-CoA reductase, reaching levels somewhat lower than expression of β-actin, and GAPDH, indicating that posttranscriptional processes may be responsible for the low mRNA levels

observed for this enzyme. Lithocholic acid 6β-hydroxylase gene expression was also localized predominantly periportally (PC:PP-ratio being 0.4), in agreement with expression of mRNA levels for this enzyme (Fig. 1).

Treatment of rats with 5% colestid resulted in enhanced transcriptional activity in whole liver preparations of the cholesterol 7α-hydroxylase (3.6-fold), sterol 27-hydroxylase (2.2-fold) and HMG-CoA reductase (5.5-fold) genes. In contrast, lithocholic acid 6β-hydroxylase gene transcription was down-regulated (3.6-fold), while house-keeping genes β-actin and GAPDH were not affected by colestid-treatment. Analysis of gene expression in isolated pericentral and periportal cells from stimulated rats (Fig. 4b) showed marked expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes over the entire portocentral axis. Up-regulation of transcriptional activity in the pericentral zone only was found for the HMG-CoA reductase gene, while gene expression for lithocholic acid 6β-hydroxylase was specifically down-regulated in the portal zone.

Figure 4. Transcriptional activity of the cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes in isolated pericentral and periportal hepatocytes from control- and colestid-treated rats. Nuclear preparations were prepared from control rats, and animals fed chow supplemented with 5% colestid, and run-off assays were performed as described in the legends to Fig. 3, and in "materials and methods". Levels of transcriptional activity in the different hepatocyte sub-fractions was calculated relative to expression of β-actin, used as an internal standard, and presented as a means $\pm$ SD using hepatocytes from 4-7 rats. (4a and b), freshly isolated pericentral (solid bars) and periportal hepatocytes (hatched bars) served as material for the isolation of nuclei, using control (a) and colestid-treated (b) rats. cDNA probes used were as described in the legends to Fig. 3. PC:PP-ratio's are indicated at the bottom of the figure.





	PC:PP-ratio				
controls	4.4	0.7	1.7	0.4	1.2
5% colestid	1.0	1.3	0.9	1.8	1.2

In situ hybridization of livers from control and colestid-treated rats

Fig. 5 shows heterogeneous expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA, as detected by *in situ* hybridization of liver sections from control and colestid-treated rats. Cholesterol 7α-hydroxylase (Fig. 5a) is allmost exclusively and abundantly expressed in a limited fraction of hepatocytes surrounding the terminal venules. Particular pericentral expression was also detected for glutamine synthetase (Fig. 5c), as reported previously (41,47), and assessed for reasons of comparison and positive zonal identification. *In situ* hybridization of control livers with the rat sterol 27-hydroxylase cDNA-probe also showed positive staining of pericentral hepatocytes only, be it far less abundantly, and less discretely, in agreement with mRNA analysis shown previously for this enzyme (Fig. 2b). Rather, a slight gradient of weak positive staining, declining towards the periportal zone, was found (Fig. 5b).

Treatment with colestid caused a more abundant expression of cholesterol 7α-hydroxylase mRNA throughout a large section of the lobulus (Fig. 5d), concomitant with observed expression patterns of cholesterol 7α-hydroxylase activity and mRNA. Sterol 27-hydroxylase mRNA showed a similar, but less marked up-regulation by colestid-treatment, resulting in positive staining of a large fraction of hepatocytes within each lobular unit (Fig. 5e). Glutamine synthetase showed no colestid-induced effect, and remained pericentrally localized (Fig. 5f). The latter agrees well with reported rigid heterogeneity of this enzyme in rat liver (51,52).

Figure 5. Localization of cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA on serial liver sections by *in situ* hybridization. Liver sections were made from control (a-c) and colestid-treated rats (d-f), according to methods described in "materials and methods", and hybridized *in situ* with [^{35}S]-labelled probes for cholesterol 7α-hydroxylase (a and d), sterol 27-hydroxylase (b and e), and glutamine synthetase (c and f). The latter was assessed as a positive identification of the pericentral zone. Sections depicted are of a central vein with surrounding hepatocytes.

DISCUSSION

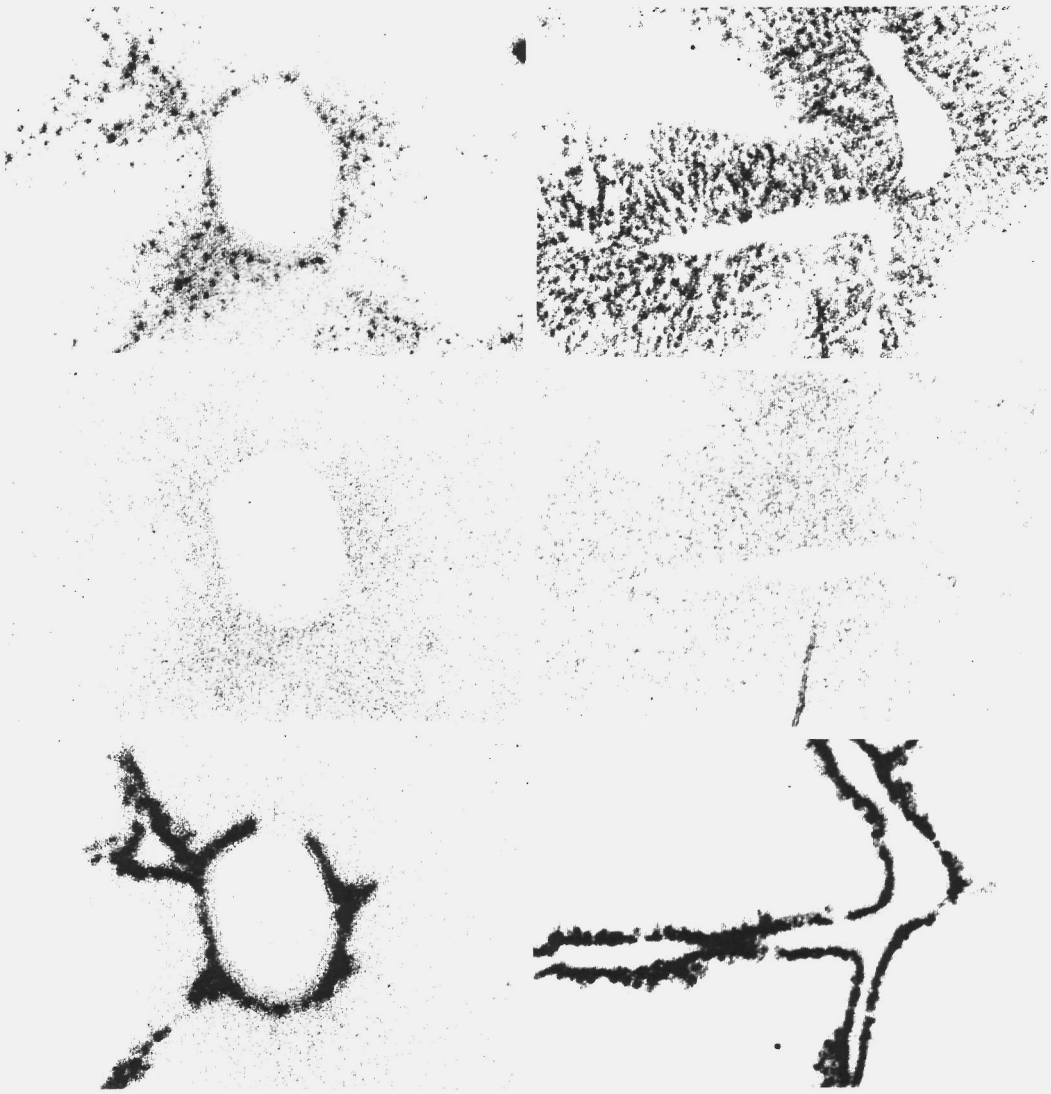
The current study shows that key-enzymes involved in bile acid biosynthesis, cholesterol 7α-hydroxylase and sterol 27-hydroxylase, predominate in the pericentral area of the rat liver lobulus. Under normal feeding conditions, the distribution of both enzymes is accomplished by parallel expression of mRNA levels and transcriptional activity of the corresponding genes. The localization is dynamic, and responds to reduced portal bile acid concentrations after colestid-treatment by changing distribution patterns for transcriptional activities, and particularly mRNA levels of these enzymes, to a more overall expression, resulting in recruitment of a larger portion of hepatocytes within the liver lobulus for bile acid synthetic purposes.

control

5% colestid-treated

a-c

e-f



Parallel lobular distribution of cholesterol 7α-hydroxylase and sterol 27-hydroxylase.
A strong preferential expression of cholesterol 7α-hydroxylase activity, mRNA and gene transcription was detected within the pericentral zone (PC:PP[1]-ratio's of 7.9, 9.9 and 4.4, respectively). These results provide the molecular basis for the reported predominantly pericentral expression of bile acid synthesis and cholesterol 7α-hydroxylase activity (11,12). In addition, the present data show that enzyme activity and mRNA level for sterol 27-hydroxylase are also higher in the pericentral zone (PC:PP-ratio's of 2.9 and 2.5, respectively), as a result of a higher transcriptional activity of the corresponding gene (1.7-fold) in this area. The latter enzyme is involved in alternative routing to bile acids via initial 27-hydroxylation of cholesterol, a pathway which has been shown to contribute considerably to bile acid synthesis, both *in vivo* in man (13) and in cultured human and rat hepatocytes (14).

Coordinate bile acid-induced feedback of bile acid synthetic enzymes.
Almost nothing is known about regulation of sterol 27-hydroxylase. It has been suggested that the enzyme is of minor importance for regulation of bile acid synthesis and composition of bile acids formed in rat (53). However, recent in vitro studies showed that sterol 27-hydroxylase may be regulated by factors known to also affect cholesterol 7α-hydroxylase. Both enzymes were inhibited to a similar extent by bile acids (24) and insulin (54). Thus it is conceivable that the alternative pathway is also subject to regulation *in vivo*, and in maintenance of cholesterol homeostasis. The co-localized expression of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities and mRNA levels, further confirmed with *in situ* hybridization experiments, strongly suggests the involvement of both enzymes in determining expression of total bile acid synthesis within the lobulus.

The data presented support the concept that bile acid synthesis in vivo is regulated by the flux of bile acids returning to the liver through enterohepatic circulation. Allthough in principle all hepatocytes have equal uptake capacity for bile acids (19,55), the microanatomy of the liver lobule results in a lobular concentration gradient during blood-flow through the liver (15,16,55). We have suggested that high bile acid concentrations are responsible for major down-regulation of bile acid synthesis specifically in the portal area, whereas the route is hardly affected in the pericentral zone (11,12,56). A relationship between portal bile acid concentrations and bile acid biosynthetic capacity of hepatocytes has been assumed previously (57,58). Further evidence for direct regulation of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase by bile acids was obtained recently with *in vitro* studies, showing a concentration-dependent down-regulation of enzyme activities and mRNA by bile acids in cultured rat hepatocytes, manifest at the transcriptional level (24).

Treatment of rats with bile acid sequestrants has been shown to result in lowering of the bile acid gradient over the liver lobulus, as a consequence of diminished bile acid

concentrations in portal blood (26). Subsequent up-regulation of bile acid synthesis as a result of colestid-treatment may be caused by a reduced down-regulation within the lobulus (11,12). In addition, as a result of diversion of bile acids in this way, loss of zonal heterogeneity of bile acid excretion and cell polarity, in terms of cellular translocation of bile acids has been demonstrated (67). The current study shows that stimulation of bile acid synthesis by colestid-treatment may result from de-repression of transcriptional activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes, and particularly mRNA levels of these enzymes, in the portal zone. The latter results provide further evidence that bile acids are directly involved in inducing distribution patterns for cholesterol 7α-hydroxylase and sterol 27-hydroxylase within the liver lobulus. Furthermore, the colestid-treatment shows that heterogeneity for these enzymes is not rigid, but responds to changing metabolic requirements.

The molecular level of regulation by bile acids

The heterogeneous expression of mRNA levels for both enzymes is induced at the level of gene transcription. Nevertheless, allthough relative levels for sterol 27-hydroxylase mRNA and transciptional activity within the lobulus are closely linked, the stronger PC:PP-ratio for cholesterol 7α-hydroxylase mRNA, as compared with transcriptional activity of the gene (PC:PP-ratio's of 9.9 and 4.4, respectively), suggests that ultimate expression of mRNA and activity for this enzyme may be regulated at multiple levels. The distinct expression of cholesterol 7α-hydroxylase mRNA in only a few pericentral cells (Fig. 5a), whereas transcriptional activity for this gene is low, but not absent, in the periportal region, substantiates this view. This becomes even more apparent after colestid-treatment, resulting in up-regulated levels of cholesterol 7α-hydroxylase mRNA in both zones of the lobulus (11-fold and 2-fold, repectively, for PP and PC-zones), while the transcriptional activity of the gene is affected only in the portal area, and to a modest degree (2-fold). In line with this thought, it has been suggested that stability of the cholesterol 7α-hydroxylase messenger is an important determinant of steady-state mRNA levels for this enzyme with respect to regulation by bile acids (22,60). AU-rich sequences present in the 3'-noncoding region of cholesterol 7α-hydroxylase mRNA may be involved in such a regulatory scheme (61).

mRNA levels and transcriptional activity analysis for lithocholic acid 6β-hydroxylase and HMG-CoA reductase showed that not all enzymes involved in maintenance of cholesterol homeostasis are co-localized. Portal expression of lithocholic acid 6β-hydroxylase is conceivable in view of involvement of this enzyme in metabolism of secondary bile acids. Periportal hepatocytes, subject to the highest bile acid concentrations, are expected to be most active in conversion of lithocholic acid to murideoxycholic acid. Additionally, it has been demonstrated that the enzyme is up-regulated by feeding bile acids to hamsters (38). In agreement with this finding, we have shown that expression of the enzyme can be down-regulated at the level of transcription, by diversion of bile acids from the circulation (3.6-fold

in whole liver preparations, and Fig. 4a and b). Consequently, high expression of lithocholic acid 6β-hydroxylase in the portal area, where blood rich in bile acids enters the liver, may reflect a protective mechanism of the liver to minimize hepatotoxic effects.

The link between cholesterol synthesis and bile acid formation

Opposite lobular localization of mRNA and transcriptional activity for HMG-CoA reductase is interesting, since it poses questions regarding interregulation of cholesterol synthetic- and bile acid synthetic routes, and how each may contribute to liver cholesterol homeostasis. Specifically with regard to functional pools of cholesterol contributing to bile acid formation, controversy exists. It has been postulated that newly synthesized cholesterol is the preferred substrate for cholesterol 7α-hydroxylase (62,63). Based on the results presented in this and other reports (7,8,11,12), enzymes involved in *de novo* cholesterol synthesis and bile acid synthesis are strictly separated, and therefore newly-synthesized cholesterol can not be the preferred substrate under normal physiological circumstances. In line with this assumption, Robins and coworkers have shown that liver-synthesized cholesterol is preferentially secreted into bile, without being metabolized (64). Furthermore, Scheibner and coworkers recently demonstrated that the bulk of bile acids synthesized in the first short period following bile duct ligation originates from preformed cholesterol (65).

Stimulation of *de novo* cholesterol synthesis in control rats by treatment with mevalonate did not result in an effect on cholesterol 7α-hydroxylase, while HMG-CoA reductase activity was profoundly inhibited (66). Similarly, administration of HMG-CoA reductase inhibitor to hypercholesterolemic patients had no impact on synthesis of acidic sterols in bile (67), nor did administration to gallstone patients have an effect on cholesterol 7α-hydroxylase activity (68). These data demonstrate that under normal circumstances, manipulation of the *de novo* cholesterol pool does not result in alteration of bile acid synthesis levels.

In this and previous studies (11,12), we have shown that treatment of rats with colestid leads to expansion of bile acid synthetic capacity within the lobulus. These results indicate a direct linkage between cholesterol synthesis and bile acid synthesis, as a result of diversion of bile acids, and may explain the increased use of *de novo* cholesterol under these circumstances (63,65,69,70). Interestingly, HMG-CoA reductase mRNA, though up-regulated by colestid, remained predominantly periportally localized. In agreement with these findings, it has been shown that activity of HMG-CoA reductase remains periportal after treatment of rats with cholestyramine, but that expression of the enzyme up to the pericentral area is accomplished when this treatment is combined with mevinolin-administration to rats (7,8).

In conclusion, the present study demonstrates that heterogeneous localization of bile acid synthesis is accomplished by preferential transcriptional activity and mRNA levels of key-enzymes for this route, cholesterol 7α-hydroxylase and sterol 27-hydroxylase, in the pericentral zone of the liver lobulus. Co-localization of the two enzymes provides insights

on how feedback regulation of bile acid synthesis by bile acids is achieved, and how the total cholesterol pool within the liver is regulated to meet different metabolic demands. The concept of metabolic zonation provides the basis for this understanding, and may shed further light on interregulation of pathways involved in maintainance of cholesterol homeostasis in the liver.

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CHAPTER 6

SELECTIVE UPREGULATION OF CHOLESTEROL 7α-HYDROXYLASE BY βVLDL, AND NOT BY LDL OR HDL IN CULTURED RAT HEPATOCYTES

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ABSTRACT

Lipoproteins may supply substrate for the formation of bile acids. However, the role of cholesterol in regulation bile acid biosynthesis is not well understood. We therefore studied the effect of different lipoproteins on two key-enzymes in bile acid synthesis, i.e. cholesterol 7α-hydroxylase and sterol 27-hydroxylase, in cultured rat hepatocytes. Hepatocytes were cultured in the presence of lipoprotein deficient serum, and incubated with various lipoprotein fractions isolated from 0.5% cholesterol-fed rabbits. Both β-migrating very low density lipoprotein (βVLDL, 40 μg/ml) and low density lipoprotein (LDL, 100 μg/ml) caused a significant increase in intracellular cholesteryl ester content of cells (2-fold and 1.7-fold, respectively), while the high density lipoprotein fraction (HDL, 50% v/v) showed little or no effect, after a 24 hour incubation period. Bile acid synthesis was increased by βVLDL (1.8-fold) and to a lesser extent by LDL (1.6-fold), with no effect upon addition of HDL. Formation of bile acids was further increased in response to βVLDL in combination with ACAT-inhibitor CI-976. However, only βVLDL stimulated cholesterol 7α-hydroxylase activity (1.5-fold), while with both LDL and HDL no stimulation was observed. Sterol 27-hydroxylase was not affected by any of the lipoproteins added. βVLDL caused a dose-dependent elevation of the cholesterol 7α-hydroxylase mRNA levels, reaching maximal stimulation at 40 μg/ml βVLDL (3.2-fold). Again, no such elevation was observed with LDL or HDL. The increase in cholesterol 7α-hydroxylase mRNA by βVLDL was paralleled by a time-dependent and rapid up-regulation of transcriptional activity (1.9-fold after 3 hours), as determined by nuclear run-off assays. Transient expression experiments in hepatocytes, transfected with a promoter-reporter construct containing the proximal 348 nucleotides of the cholesterol 7α-hydroxylase promoter, showed an enhanced gene transcription (2-fold) with βVLDL. This particular region of the cholesterol 7α-hydroxylase promoter has also been shown to harbor recognition sequences for a bile acid induced response (Hoekman FM, Rientjes JMJ, Twisk J, Princen HMG, Mager WH (1993) Gene 130: 217-223), suggesting that a composite element plays a major role in transcriptional regulation of the cholesterol 7α-hydroxylase gene by various physiological mediators.

We conclude that specifically βVLDL increases bile acid synthesis by stimulating cholesterol 7α-hydroxylase gene transcription. The enzyme is not affected by other lipoprotein fractions, nor did any of the lipoproteins have an effect on sterol 27-hydroxylase. While both βVLDL and LDL-cholesterol may form substrate for bile acid synthesis, different effects of these lipoproteins on expression of cholesterol 7α-hydroxylase suggest a different intracellular processing and coupling to the bile acid biosynthetic route.

INTRODUCTION

In mammals cholesterol homeostasis in the liver is maintained by the inter-regulation of different pathways. On the one hand cholesterol is synthesized endogenously from acetate precursors, a pathway in which the rate-limiting enzyme and major regulatory point is considered to be 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase. On the other hand exogenous cholesterol can be directed to liver cells by receptor-mediated uptake. The excess of cholesterol can be stored as cholesteryl ester, a process which is controlled by the activity of acyl CoA:cholesterol acyltransferase (ACAT). However, the major pathway for actual elimination of cholesterol from the body is by conversion to bile acids, or via bile acid dependent excretion of cholesterol. The rate of bile acid synthesis is therefore considered an important regulator of cholesterol homeostasis.

The primary route to bile acids is initiated by 7α-hydroxylation of cholesterol. The rate-limiting step in this pathway is catalysed by cholesterol 7α-hydroxylase, a microsomal cytochrome P450-dependent enzyme (1-3). There is accumulating evidence for the existence of an alternative pathway towards bile acid synthesis (4), involving initial 27-hydroxylation of cholesterol by sterol 27-hydroxylase, also a cytochrome P450-dependent enzyme, but located in the inner mitochondriondrial membrane (5). It has been shown that this alternative pathway can contribute substantially to bile acid synthesis in vivo in humans (4), and in cultured human and rat hepatocytes (6,7).

Cholesterol 7α-hydroxylase displays a response to various physiological signals, the most important of which is considered to be exerted through the enterohepatic circulation of bile acids (8). In rats it has been demonstrated that bile acid synthesis is down-regulated in vivo after feeding or intravenous administration of bile acids, at the level of cholesterol 7α-hydroxylase activity (2,9), mRNA (10-12), and transcription (12). In vitro studies, using cultured pig (13) and rat (14) hepatocytes demonstrated that bile acids act directly on the hepatocyte, and exert an effect both at a transcriptional and post-transcriptional level (14,15). Similar bile acids also act as regulator of sterol 27-hydroxylase mRNA and gene expression (16), demonstrating that both enzymes may be coordinately regulated by similar physiological signals. This assumption was recently corroberated with in vitro studies in cultured rat hepatocytes, showing a dose-dependent down-regulation both cholesterol 7α-hydroxylase and sterol 27-hydroxylase by insulin, manifest at the transcriptional level. These results provided the molecular basis for normalization of bile acid synthesis by this hormone after treatment of diabetes mellitus in humans (17).

A regulatory role in the formation of bile acids has also been ascribed to cholesterol, all be it controversial. A mode of regulation of bile acid biosynthesis by cholesterol has been suggested involving saturation of the enzyme cholesterol 7α-hydroxylase (18-20). However, Einarsson et al (21,22) showed that various feeding conditions led to fluctuations in cholesterol 7α-hydroxylase activity, while having no, or opposite effects on the saturation

index of the enzyme. In vivo studies in rats have focussed on the role of *de novo* cholesterol in regulation of bile acid synthesis. Inhibition of endogenous cholesterol synthesis by a bolus administration of lovastatin, an inhibitor of HMG-CoA reductase, in rats with a chronic- or short-term bile fistula, has been shown to suppress cholesterol 7α-hydroxylase activity (23-25). This suppression could be prevented by simultanous infusion of mevalonate, indicating the high rate of bile acid synthesis under these conditions to be dependent on *de novo* cholesterol synthesis for supply of sufficient substrate. These experiments are not conclusive with respect to a possible role of cholesterol as a regulator of bile acid synthesis, as administration of mevalonate alone to chronic biliary diverted rats had no impact on cholesterol 7α-hydroxylase (24), yet when rats were subjected to short-term biliary diversion, however, a stimulatory effect of mevalonate was found (25). Furthermore, experiments using control animals treated with mevalonate or, alternatively, inhibitors of HMG-CoA reductase, have yielded divergent results. Long-term administration of mevinolin to control rats had no impact on cholesterol 7α-hydroxylase (26), while intraperitoneal injection of mevalonate led to a rapid up-regulation of cholesterol 7α-hydroxylase activity and mRNA (27). In a later paper by Vlahcevic and coworkers (25), such up-regulation by mevalonate could not be demonstrated in control rats.

Alternatively, a role for dietary cholesterol in regulating bile acid synthesis, and cholesterol 7α-hydroxylase has been demonstrated. Several groups have shown that feeding rats a 2% cholesterol-diet led to an increased cholesterol 7α-hydroxylase activity (12,28), mRNA level (10,11,12,29), and transcriptional activity (12). Transport of dietary cholesterol to the liver does not seem necessary, however, to observe an increase in cholesterol 7α-hydroxylase, as lymph fistulation of cholesterol-fed rats still showed up-regulation of the enzyme (30,31). Feeding rats a diet rich in cholesterol led to a decreased half-life of circulating cholic acid, and a concomittant increase in feacal excretion of bile acids, indicating that dietary cholesterol may exert stimulatory effects on bile acid synthesis indirectly (28). In agreement with this view, intravenous administration of cholesterol-enriched Intralipid failed to stimulate cholesterol 7α-hydroxylase (28,32). Similarly, infusion of rat liver with cholesterol-rich lipoproteins did not have any effect on cholesterol 7α-hydroxylase (33). A mechanism was 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 reflux of bile acids to the liver (28). Hence, according to this concept, bile acids remain the major regulator of bile acid synthesis of cholesterol 7α-hydroxylase.

In the current study, we have re-assessed the role of cholesterol as a possible regulator of bile acid biosynthesis, using monolayer cultures of rat hepatocytes, providing the advantage of being able to discriminate between direct and indirect events. The effects of different lipoproteins as a source of exogenous cholesterol, were assessed on bile acid formation and key-enzymes of this metabolic route. Our results show that exogenous

cholesterol, specifically the βVLDL fraction, stimulates bile acid synthesis at the level of cholesterol 7α-hydroxylase by up-regulation of transcriptional activity of this enzyme, while sterol 27-hydroxylase remains unaffected. Possible links to differential intracellular sorting of lipoprotein-derived cholesterol are discussed.

MATERIALS AND METHODS

Materials

Materials used for isolation and culturing of rat hepatocytes, and assaying cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity were obtained from sources described previously (34-36). [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (400 Ci/mmol) and [4-^{14}C]-cholesterol (60 mCi/mol)) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, UK. Mevalonate, supplied as mevalonolactone (Sigma Chemicals, St.Louis, MO, USA), was prepared by treatment with 1 volume 0.2 N NaOH for 30 min at 37°C, and subsequent neutralization with 1 volume 0.2 N HCl, before addition to the medium. CI-976 was obtained from Parke Davis Pharmaceutical Research Division, Michigan, USA.

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

Rat Hepatocyte Isolation and Culture

Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously (34-36). Viability, as determined by trypan blue exclusion, was higher than 90%. The cells were seeded on 60-mm diameter plastic tissue culture dishes or 6-well cluster plates (Costar, Cambridge, MA, USA) at a density of $1.5*10^5$ cells/cm^2 in Williams E medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 140 nM insulin, 50 nM dexamethasone, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37°C in a 5% CO_2/95% air atmosphere (34,35). After a 4-hour attachment period, medium was refreshed with 1.0 ml (6-well plates) or 2.5 ml (dishes) of culture medium as described above, supplemented with 10% lipoprotein deficient serum (LPDS) instead of 10% FCS, and cells were incubated for a further 14 hours. Lipoproteins, or mevalonate, were added to the medium of cells after this period, between 18-42 hours of culture age, unless otherwise stated. Cells were harvested at the same time after a 42 h culture period for measurement of intracellular lipid content, cholesterol 7α-hydroxylase and

sterol 27-hydroxylase activity, mRNA and transcriptional activity.

Isolation of lipoprotein deficient serum; lipoprotein isolation and characterization
Lipoprotein deficient serum was isolated from Fetal Calf Serum (Boehringer Mannheim) by ultracentrifugation at 4°C for 48 h after a density adjustment with solid KBr (37). The LPDS fraction (d > 1.21 g/ml) was dialysed at 4°C against 10 mM sodium phosphate, 0.15 M NaCl (pH 7.4) for 24 h, and subsequent dialysis against Williams E medium for an additional 24 h. Williams E medium was added up to the initial volume (100% LPDS). Before addition to the culture medium, the LPDS was filtered through a 0.22 μm membrane.

For the isolation of lipoproteins, blood was obtained from rabbits fed a diet supplemented with 0.5% (w/w) cholesterol for 7 days. Lipoproteins were isolated from serum by ultracentrifugation according to Redgrave et al. (38) at 4°C for 24 h. The different lipoprotein fractions were chosen based on determination of density (density measuring cell DMA 602M Mettler/Paar, Graz, Austria) and cholesterol-distribution profile. The βVLDL-fraction (d < 1.006) contained most of the lipoprotein-associated serum cholesterol (10.27 mg/mg protein), was rich in phospholipids (2.16 mg/mg protein), and contained relatively low amounts of triglycerides (0.65 mg/mg protein; CHOL:TG = 15.8), as a consequence of displacement by cholesterol, rendering this particle of β-migrating mobility. SDS-PAGE of the isolated lipoproteins showed βVLDL to be especially rich in apolipoprotein (apo) E, with some apoB100 and apoB48. LDL (d = 1.017) contained less cholesterol (4.09 mg/mg protein), phospholipid (1.11 mg/mg protein) and triglycerides (0.26 mg/mg protein), and was particularly rich in apoB100, somewhat less in apoB48, and also contained some apoE. The HDL-fraction (d = 1.095) contained low amounts of cholesterol (0.61 mg/mg protein), and somewhat lower levels of phospholipids (0.42 mg/mg protein), and triglycerides (0.11 mg/mg protein), was rich in apoAI and contained some apoAII and apoAIV. All fractions had similar CE:total cholesterol ratio's (approximately 0.70- 0.75). Fractions of similar densities were isolated from rat serum, originating from rats fed a 0.5% cholesterol-enriched diet as described. The different fractions contained somewhat less cholesterol per mg protein, but displayed similar distribution patterns for apolipoproteins, as assessed by SDS-PAGE. The various fractions were stabilized by addition of 10% LPDS, before dialysis for 24 h against sodium phosphate buffer, as described above, and for another 24 h against Williams E medium. Before being used for incubations, the lipoprotein fractions were filtrated through a 0.45 μm membrane (βVLDL) or a 0.22 μm membrane (LDL and HDL). Various determinations were performed as follows: protein (39), and total cholesterol (CHOD-PAP kit nr. 236.691, Boehringer-Mannheim, Mannheim, Germany), free cholesterol (CHOD-PAP kit nr. 310.328, Boehringer-Mannheim), phospholipid (Phospholipase D Cholin-oxidase PAP kit nr. 990-54009, Wako Chemicals, Neuss, Germany) and triglyceride content (GPO-PAP kit nr. 701.904, Boehringer-Mannheim), as described by the manufacturer. SDS-PAGE gel electrophoresis was performed as described in ref. (40).

Measurement of the mass of intracellular triglycerides, cholesterol and cholesteryl esters of cultured rat hepatocytes

After a 24 h incubation period, with or without different lipoproteins or mevalonate, cells were washed 3 times with cold phosphate buffer saline (pH 7.4). Thereafter cells were harvested by scraping, and homogenized by sonication (Branson, 60 W, 20 s). Samples were taken for measurement of protein content. Lipids were extracted from the cell suspension as described by Bligh & Dyer (41), after addition of cholesterol acetate (2 μg per sample) as an internal standard. The neutral lipids were separated by high performance thin layer chromatography (h.p.t.l.c) on silica-gel-60 precoated plates as described (42). Quantification of the amounts was done by scanning the plates with a Shimadzu (Kyoto, Japan) CS910 chromatograph scanner at 380 nm, and areas under the curves were integrated by using a data processor (Shimadzu).

Quantification of mass production of bile acids

Mass production of bile acids by rat hepatocytes was measured by g.l.c. after a preincubation period of 8 h (from 18 to 26 h of culture age), during the following 16 h culture period from 26 to 42 h in the absence or presence of lipoproteins, or mevalonate, as described previously (14,36).

Assay of Cholesterol 7α-hydroxylase and Sterol 27-Hydroxylase

Cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity in homogenates of cultured hepatocytes was measured as described previously (6,35). [^{14}C]-labelled products were analyzed by thin layer chromatography, and the amount of [^{14}C]-7α-hydroxycholesterol and [^{14}C]-27-hydroxycholesterol was quantitated by scraping off and counting of the spots containing these products, using the [^{14}C]-cholesterol input as a recovery standard. Blank values, determined by running parallel incubations without a NADPH-generating system, were subtracted before calculating enzyme activity. Protein and cholesterol were assayed as previously described (35,39).

RNA Isolation, Blotting and Hybridization Procedures

Isolation of total RNA, and subsequent electrophoresis, Northern-blotting or slot-blotting, and hybridization techniques were performed as described previously (14). 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 (14); a 1.6 kb HindIII/XbaI fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. J. Strauss (43), and isolated from a rat liver cDNA library using the rabbit sterol 27-hydroxylase cDNA, previously isolated by Russell and coworkers (44), as a probe. As controls a 1.2 kb PstI fragment of hamster actin cDNA (45), and a 1.2 kb PstI fragment of rat GAPDH-cDNA (46) were used. For all probes used in slot-blotting experiments, a linear

relationship between areas under the curves and mRNA concentration was shown on an autoradiograph, using concentrations between 2 and 8 μg of total RNA. The actine- or GAPDH-cDNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter.

Nuclear Run-Off Studies

Nuclear run-off studies were conducted essentially as described in ref. 14. *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 the above) and the empty vector pUC19, were slot-blotted onto strips of Hybond-N^+ filter (Amersham), and crosslinked with 0.4 N NaOH for 30 min. The filters were preincubated for 30 min at 65°C in a sodium phosphate buffer as described above, and hybridized with the labelled RNA for 36 hours in the same buffer. [^{32}P]-UTP had been incorporated into nascent RNA, using isolated nuclei from cells which had been cultured with or without βVLDL for a different lenghts of time between 24 and 48 hours of culture time. After hybridization, the various filters were washed once for 5 min and twice for 30 min in 2 x SSC/1% SDS at 65°C, and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantification of relative amounts of transcribed mRNA was performed using a Phosphor-imager 400B (Molecular Dynamics).

Transfection experiments and CAT assays

At 22 hours after their isolation, cells were subjected to transfection, using plasmid -348R*cat*, and CAT-assays were performed, as described previously (15). -348R*cat* contains the proximal 348 nucleotides of the cholesterol 7α-hydroxylase promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene, used as a reporter. The amounts of acetylated product as represented by autoradiography were quantitated with a Phosphor-imager 400B (Molecular Dynamics). Data were corrected for protein-content and transfection efficiency.

Statistical Analysis

Data were analyzed statistically using Student's paired t-test. Values are expressed as means $\pm$ SD.

RESULTS

Effect of different lipoproteins and mevalonate on the intracellular lipid levels of cultured rat hepatocytes

To investigate whether the different lipoproteins, added to the medium of cells, were taken up by the hepatocytes, intracellular lipid levels were determined by h.p.t.l.c, as described in "Materials and Methods". For reasons of comparison, intracellular lipid levels were also determined of hepatocytes incubated with 10mM mevalonate, as a source for endogenous cholesterol synthesis.

Hepatocytes were cultured for 24 h in medium containing 10% LPDS, in the absence of a cholesterol source, or in the presence of 40 μg/ml βVLDL (protein), 100 μg/ml LDL (protein), 50% (v/v) HDL, or 10 mM mevalonate. Based on protein and cholesterol determinations, described amounts of βVLDL and LDL provide the cells with approximately equal amounts of cholesterol (legends to Table 1). The described amount of mevalonate also theoretically accounts for an equivalent amount of cholesterol, to be synthesized endogenously by the hepatocyte, as compared with uptake of βVLDL and LDL.

Addition of βVLDL ($d < 1.006$) or LDL ($d = 1.017$) increased the cholesteryl ester (CE) amounts in the hepatocytes significantly to 207 ± 52% and 166 ± 36% respectively ($p < 0.05$), compared with incubations in 10% LPDS only (Table 1), indicating that both lipoprotein fractions are taken up efficiently by the cultured hepatocyte. With HDL ($d = 1.095$) no significant increase was observed (118 ± 11%). Addition of 10 mM mevalonate to the culture medium of the hepatocytes resulted in a significant increase in CE-content (265 ± 89%), which was in the same order of magnitude as observed with βVLDL or LDL.

The increase in CE content by βVLDL was prevented by simultaneous culturing in the presence of the ACAT inhibitor CI-976 (50 μM) (88 ± 1%), while addition of CI-976 alone did not affect intracellular CE levels (data not shown). Additionally, the amounts of free cholesterol and triglycerides were also assessed in cells cultured in absence or presence of different lipoprotein fractions, or mevalonate. These parameters remained unchanged, irrespective of the source of cholesterol, as compared with incubations in 10% LPDS only. Taken together, these results indicate that both exogenously delivered (βVLDL- and LDL-) cholesterol and endogenously synthesized cholesterol are primarily directed to the cholesterol ester pool, in agreement with similar observations in Hep G2 cells (42). This process is inhibited by simultaneous inhibition of ACAT.

Identical experiments performed with lipoproteins isolated from rat serum, yielded similar results (Table 1), indicating that the hepatocyte does not differentiate between these species with respect to uptake of lipoproteins from different animal species. All further experiments were performed with lipoproteins isolated from rabbit serum.

Table 1. Effect of different lipoproteins and mevalonate on the intracellular lipid levels of cultured rat hepatocytes.

cholesterol source:	CE	FC	TG
control (10% LPDS)	100	100	100
rabbit			
+ 40 μg/ml βVLDL	207 ± 52*	103 ± 9	105 ± 7
+ 40 μg βVLDL + 50 μM CI-976	88 ± 1	111 ± 14	111 ± 21
+ 100 μg/ml LDL	166 ± 36*	104 ± 9	105 ± 10
+ 50% HDL	118 ± 11	105 ± 6	99 ± 7
rat			
+ 40 μg/ml βVLDL	280 ± 9*	102 ± 4	109 ± 10
+ 100 μg/ml LDL	225 ± 11*	106 ± 1	105 ± 10
+ 50% HDL	93 ± 11	99 ± 10	105 ± 7
+ 10 mM mevalonate	265 ± 89*	97 ± 10	92 ± 10

Rat hepatocytes were incubated for 24 hours, from 18 to 42 h of culture, in the absence of additional cholesterol source (10% LPDS), or presence of 40 μg/ml βVLDL, 40 μg/ml βVLDL + 50 μM CI-976, 100 μg/ml LDL, 50% v/v HDL, or 10 mM mevalonate. Amounts of βVLDL and LDL are expressed in protein/ml. Lipoproteins used were isolated from rabbits fed a 0.5% cholesterol-enriched diet, or from rats fed a similar diet, according to "Materials and Methods". Cells were harvested and cellular cholesteryl ester (CE) content was determined as described, as well as free cholesterol (FC) and triglycerides (TG). Values are expressed as a percentage of control, and as a means ± S.D. of incubations of hepatocytes from 3-6 rats. The mean absolute amounts, present in control cells, were 6.88 ± 0.38 μg/mg cellular protein (CE), 11.91 ± 1.50 μg/mg (FC) and 15.40 ± 2.67 μg/mg cellular protein (TG), n=6. Amounts of cholesterol added correspond to 455 ± 98 μg/ml (βVLDL), 451 ± 128 μg/ml (LDL) and 58 ± 53 μg/ml (HDL). * indicates a significant difference ($p < 0.05$) compared with control values.

Effect of lipoproteins on the mass production of bile acids in cultured rat hepatocytes

Bile acid synthesis was measured over a 16 h incubation period as described in "Materials and Methods", amounting to 3.68 ± 1.49 μg bile acids/mg protein/24 hr in control incubations (10% LPDS only). Main bile acids formed were cholic acid and β-muricholic acid in a ratio of approximately 20:80, with minor synthesis of deoxycholic acid, chenodeoxycholic acid, and α-muricholic acid. Incubation of cells in medium containing 40 μg/ml of βVLDL increased the total bile acid synthesis significantly ($p < 0.05$) up to 175 ± 38% of control values (Fig. 1). 100 μg/ml LDL showed a smaller increase (156 ± 51%) while no stimulation of bile acid synthesis was observed with HDL (96 ± 27%). The

stimulation of bile acid synthesis, in response to βVLDL, was further enhanced ($p < 0.05$) upon simultaneous addition of 50 μM CI-976 (235 ± 66%), indicating that the exogenous cholesterol source is increasingly directed towards the bile acid synthetic pathway under these conditions. Addition of 50 μM CI-976 alone also stimulated bile acid synthesis (142 ± 11%). Incubation of cells with medium containing 10 mM mevalonate, resulted in similar stimulation of bile acid synthesis (210 ± 30%), as compared with βVLDL, suggesting that both exogenous- and endogenously synthesized pools of cholesterol are efficiently utilized for the formation of bile acids.

The ratio between cholic acid and β-muricholic acid, the main bile acids formed, was not affected by any of the lipoprotein fractions or mevalonate. This ratio remained approximately 20:80, as found in cells cultured in 10% LPDS only.

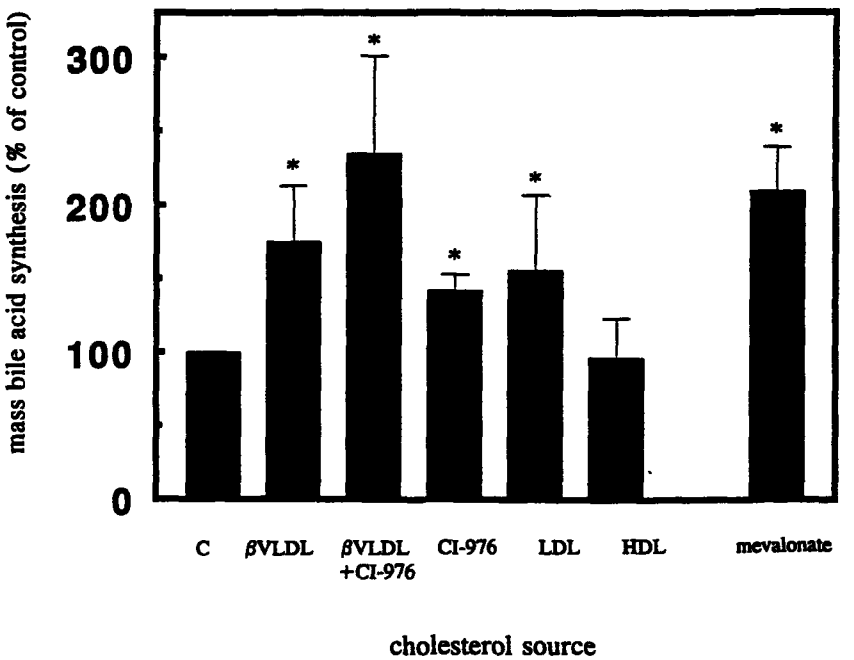


Figure 1. Effect of different lipoproteins, or mevalonate, on the mass production of bile acids. After an 8-hour preincubation period (18-26 h), rat hepatocytes were cultured for 16 hours (between 26-42 h of culture) in absence (10% LPDS) or presence of 40 μg/ml βVLDL, 40 μg/ml βVLDL + 50 μM CI-976, 50 μM CI-976, 100 μg/ml LDL, 50% v/v HDL, or 10 mM mevalonate. For details concerning absolute amounts of cholesterol added see legends to Table 1; for details concerning lipoprotein composition, and measurement of bile acid synthesis, see "Materials and Methods". Values are represented as a means ± S.D. of duplicate measurements of hepatocytes from 3-4 rats. The absolute value for total bile acid synthesis in controls cultured in 10% LPDS only was 3.68 ± 1.49 μg bile acids/ mg cellular protein/ 24 hours. * indicates a significant difference ($p < 0.05$) compared with control values, or intra-individualy.

Effect of different lipoproteins on the cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity in cultured rat hepatocytes

To further assess the level at which exogenous cholesterol enhances mass production of bile acids, enzyme activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase were determined, two key-enzymes in the bile acid synthetic pathway. Rat hepatocytes were cultured in 10% LPDS, in the absence or presence of βVLDL (40 μg/ml), LDL (100 μg/ml) or HDL (50% v/v). As can been seen in Fig. 2, only addition of βVLDL caused a significant ($p < 0.05$) increase in cholesterol 7α-hydroxylase activity (149 ± 15%), while addition of LDL showed no effect (102 ± 32%). HDL caused a decrease in cholesterol 7α-hydroxylase activity (-47 ± 23%; $p < 0.05$). Remarkably, 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.

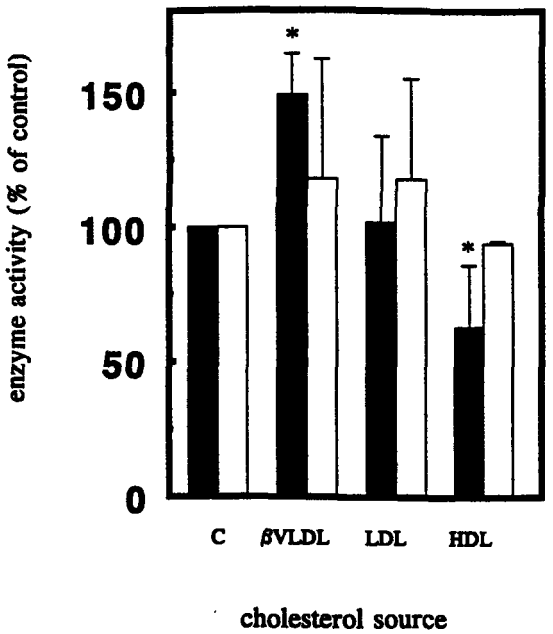


Figure 2. Effect of different lipoproteins on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity. Rat hepatocytes were incubated for 24 hours, from 18 to 42 h of culture, without (10% LPDS) or with 40 μg/ml βVLDL, 100 μg/ml LDL or 50% v/v HDL. See also legends to Table 1 and "Materials and Methods" for details concerning lipoprotein composition and absolute amounts of cholesterol added. Enzyme activity was determined as described in "Materials and Methods". Values are expressed as a means ± S.D. of duplicate incubations of hepatocytes from 3 rats. The absolute values for enzyme activity in controls cultured in 10% LPDS only were 352 ± 38, and 79 ± 18 pmol/ mg cellular protein per hour, respectively, for cholesterol 7α-hydroxylase and sterol 27-hydroxylase. * indicates a significant difference ($p < 0.05$) compared with control values.

Effect of different lipoproteins on the mRNA levels in cultured rat hepatocytes

Figure 3a shows the slot-blotting analysis of total RNA isolated from cells incubated with medium containing 10% LPDS only, or supplemented with 40 μg/ml βVLDL, 100 μg/ml LDL or 50% v/v HDL. From the autoradiograph it can be seen that βVLDL selectively up-regulates the cholesterol 7α-hydroxylase mRNA levels, while both LDL and HDL have no effect. Neither sterol 27-hydroxylase mRNA, nor that of β-actin, used as an internal

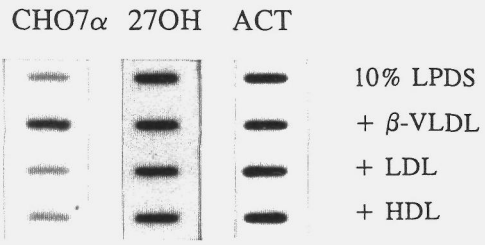
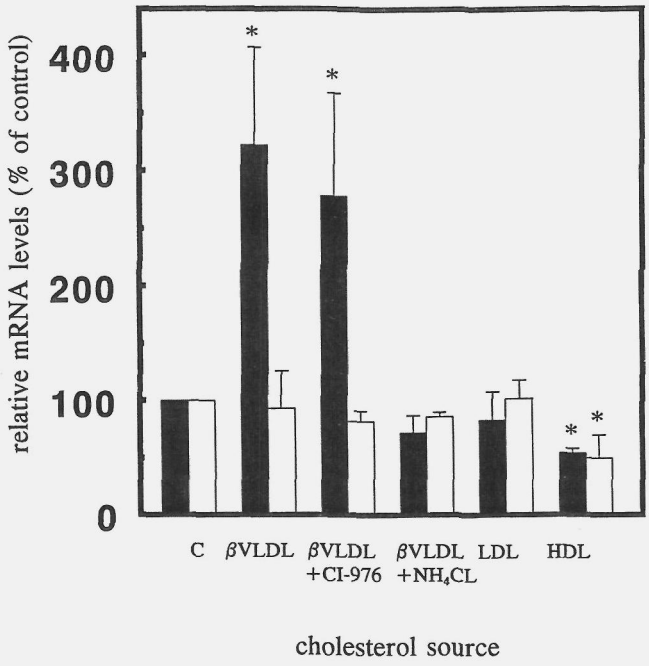


Figure 3. Effect of different lipoproteins on cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA levels. Rat hepatocytes were cultured for 24 hours, between 18 and 42 h of culture, in absence (10% LPDS) or presence of different lipoproteins.

A: The amounts of cholesterol 7α-hydroxylase (CHO7α) and sterol 27-hydroxylase (27OH) mRNA, and β-actin (ACT) mRNA were assessed by slot-blotting and densitometric scanning of resulting autoradiographs, using the β-actin mRNA as an internal standard to correct for differences in the amounts of total RNA applied to the filter. For details see the "Materials and Methods" section. Controls, 10% LPDS only; βVLDL, 40 μg protein/ml; LDL, 100 μg protein/ml; HDL, 50% v/v.



B: Amounts of cholesterol 7α-hydroxylase mRNA (7α-OHse, closed bars) and sterol 27-hydroxylase mRNA (27OHse, open bars) were quantitated by densitometric scanning of autoradiographs, using the β-actin mRNA signal as an internal standard. Values are expressed as a percentage of control (10% LPDS only) and are a means ± S.D. of independent experiments using hepatocytes from 3-8 rats. * indicates a significant difference ($p < 0.05$) compared with control values.

standard, were affected by any of the lipoproteins. Figure 3b summarizes the effects of various lipoproteins added, after quantitating the mRNA signals from autoradiographs, relative to expression of β-actin. βVLDL strongly up-regulated cholesterol 7α-hydroxylase mRNA (322 ± 85%), while with LDL present in the medium, mRNA level for this enzyme

was not significantly different from control values incubated in 10% LPDS only (83 ± 28%). HDL caused a significant decrease in cholesterol 7α-hydroxylase mRNA expression, parallel to the effect on activity of the enzyme (-46 ± 4%). Sterol 27-hydroxylase mRNA expression was not affected by addition of βVLDL (93 ± 33%) or LDL (102 ± 16), but did show a significant decrease upon incubation with HDL (-50 ± 20%). Identical results were obtained with lipoproteins isolated from rat serum (data not shown), i.e. selective strong up-regulation of cholesterol 7α-hydroxylase mRNA by βVLDL (2.9-fold), while sterol 27-hydroxylase was not affected (data not shown).

As also shown in Fig. 3b, simultaneous addition of both 50 μM of CI-976 and 40 μg/ml βVLDL, did not result in further increase in cholesterol 7α-hydroxylase mRNA level (278 ± 89%), as compared with the level obtained after incubation with βVLDL only. Additional increase of bile acid synthesis, as found under these conditions, is apparently solely the result of increase in substrate-supply, having no further regulatory potential. Similarly, CI-976 alone had no effect on cholesterol 7α-hydroxylase mRNA levels (94 ± 11%), while stimulating bile acid synthesis (Fig. 1). Simultaneous addition of both 40 μg/ml βVLDL and 10 mM NH_4Cl prevented the increase in cholesterol 7α-hydroxylase mRNA, indicating that lysosomal routing of βVLDL is a prerequisite for stimulation of cholesterol 7α-hydroxylase mRNA levels. No adverse effects of NH_4Cl were found on mRNA expression of either enzyme (83 ± 1% and 75 ± 3% for cholesterol 7α-hydroxylase mRNA and sterol 27-hydroxylase mRNA, respectively).

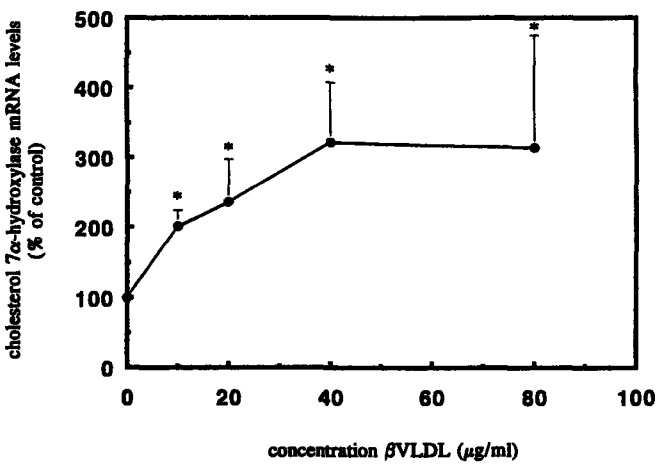


Figure 4. Dose-dependency of stimulation of cholesterol 7α-hydroxylase mRNA by βVLDL. Rat hepatocytes were incubated for 24 hours, from 18 to 42 h of culture, with various amounts of βVLDL (10-80 μg protein/ml). Total RNA was isolated and cholesterol 7α-hydroxylase mRNA amounts were determined, relative to β-actin mRNA expression, as described in "Materials and Methods". Data are expressed as a percentage of control (cells incubated in 10% LPDS) and are a means ± S.D. of independent experiments using hepatocytes from 3-4 rats. * indicates a significant difference ($p < 0.05$) compared with the control value.

A clear dose-dependency of cholesterol 7α-hydroxylase mRNA levels was shown when cells were incubated with different concentrations of βVLDL (10-80 μg/ml) (Fig. 4). With 10 μg/ml βVLDL there was already a 2-fold increase of cholesterol 7α-hydroxylase mRNA as compared with cells incubated in 10 % LPDS only. This stimulation was increased further in a dose-dependent way, with a maximal stimulation of 322 ± 85% at 40 μg/ml βVLDL.

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

To further assess the molecular level at which the effect of βVLDL is exerted, transcriptional activity of the cholesterol 7α-hydroxylase gene was determined by means of nuclear run-off assays. Hepatocytes were incubated with 40 μg/ml of βVLDL for different periods of time, between 18 and 42 h of culture, and harvested simultaneously after 42 h of culture time for the isolation of nuclei, as described in "Materials and Methods". Figure 5 shows a rapid up-regulation of cholesterol 7α-hydroxylase transcriptional activity: within 1.5 h of incubation

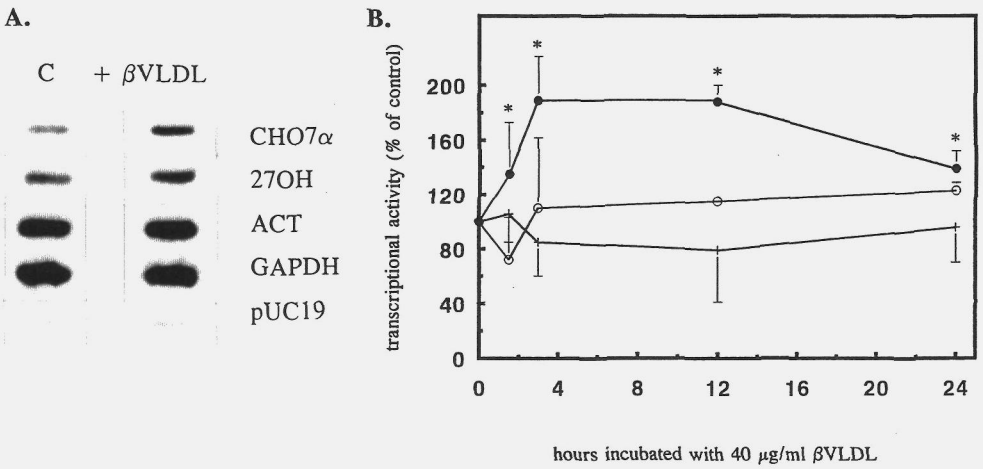


Figure 5. Time course of cholesterol 7α-hydroxylase and sterol 27-hydroxylase transcriptional activity in response to βVLDL. Rat hepatocytes were incubated with 40 μg/ml βVLDL for different lenghts of time between 18 and 42 h of culturing, and hepatocytes were harvested simultanously after 42 h of culture time for the preparation of nuclei. Transcriptional activity of the different genes was determined by nuclear run-off assay, as described in "Materials and Methods", 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.

(A) Typical autoradiograph of a hybridization experiment. Probes used for detection of specific mRNA's consisted of: CHO7α (cholesterol 7α-hydroxylase), 27OH (sterol 27-hydroxylase), GAPDH, and ACT (β-actin).

(B) Relative transcriptional activities of cholesterol 7α-hydroxylase (-●-), sterol 27-hydroxylase (-○-), and GAPDH (-+-) are expressed as % of control value (hepatocytes cultured in 10% LPDS only) and are means ± S.D. of independent experiments using hepatocytes from 3-4 rats. * indicates a significant difference ($p < 0.05$) compared with control values.

with βVLDL, an increased activity was observed (135 ± 38%), while the maximal stimulation was reached in the period from 3 to 12 h (190 ± 11%), as compared with culturing in 10% LPDS only.

The transcriptional activity of the sterol 27-hydroxylase gene was not affected by βVLDL in accordance with the absence of 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 response upon addition of βVLDL.

Effect of βVLDL on CAT-activity in cells transfected with -348R*cat*

Hoekman et al (15) recently described a region of 348 nt proximal to the transcription initiation site of the cholesterol 7α-hydroxylase gene, harboring a major transcription-activating element for promoter activity of the enzyme. To asses whether a region responsive to βVLDL might be located within this proximal part of the cholesterol 7α-hydroxylase promoter, transient expression studies were performed. The -348R*cat*-construct, consisting of the first 348 basepairs of the cholesterol 7α-hydroxylase promoter fused proximally to the CAT-reporter gene, as described in detail in ref. 15, was used in these experiments. Transfected rat hepatocytes were cultured in 10% LPDS-containing medium in the absence or presence of 40 μg/ml βVLDL. As shown in Table 2, promoter activity of the -348R*cat*-contruct was increased in the presence of βVLDL (2-fold), in accordance with the approximately 2-fold stimulation of the transcriptional activity of the cholesterol 7α-hydroxylase gene as determined by nuclear run-off assays.

Table 2. Effect of βVLDL on CAT activity of cholesterol 7α-hydroxylase promoter-reporter construct -348R*cat* in transfected cultured rat hepatocytes.

	CAT-activity (% of control)
WE/10% FCS	100
WE/10% LPDS (controls)	42 ± 7 (100%)
" + 40 μg/ml βVLDL	82 ± 6 (195%)

Transient expression experiments were performed as described under "Material and Methods", using the -348R*cat* construct described in ref. 15. Transfected cells were subsequently cultured in medium (WE) containing 10% LPDS only, or supplemented with 40 μg/ml βVLDL, for 48 hours. CAT activity was assessed after this culture period in homogenates of cells, as described. Values expressed are a means ± S.D., and as percentage of CAT-activity in FCS-containing medium, of 4 independent experiments, and in parentheses as a percentage of CAT activity in LPDS-containing medium.

DISCUSSION

This study shows that in rat hepatocytes, exogenous cholesterol supply in the form of lipoprotein has a stimulatory effect on the rate-limiting enzyme in the neutral pathway to formation of bile acids, cholesterol 7α-hydroxylase, in addition to supplying substrate for bile acid synthesis. Specifically βVLDL was demonstrated to have a direct effect on the transcriptional activity of cholesterol 7α-hydroxylase, while the LDL and HDL fractions did not have such regulatory capacity. Previous reports have suggested that cholesterol derived from *de novo* synthesis is the preferred substrate for bile acid synthesis (47,48). Increase in bile acid synthesis and cholesterol 7α-hydroxylase activity, as a result of chronic- or short-term biliary diversion, has been shown to be dependent on *de novo* cholesterol for supply of sufficient substrate to meet demands as a result of an enlarged bile acid synthetic capacity, indicating that this particular cholesterol pool is regulatory under such circumstances (23-25). The latter was also taken to indicate that circulating lipoprotein-cholesterol could not provide substrate, or act as a regulator, for bile acid formation under such conditions (25).

Our present results, however, show that both sources of cholesterol, be they exogenously derived via receptor mediated uptake of serum lipoproteins, or endogenously synthesized from precursors, are equally efficiently directed to bile acid synthesis (Fig. 1). An explanation for the apparant discrepancy between *in vivo* and *in vitro* data may lie in the heterogeneity of cholesterol synthetic- and catabolic pathways. As reported by Singer (49) and Li (50), cholesterol synthetic enzymes HMG-CoA reductase and HMG-CoA synthase are located exclusively in the periportal region of the liver acinus, whereas bile acid biosynthesis, and key-enzymes involved therein, are perivenously localized (51,52). Under normal circumstances, therefore, cholesterol synthesis and bile acid biosynthesis are strictly separated (51,52), suggesting that in this case bile acid formation utilizes preferably preformed-cholesterol, as reported by Scheibner and coworkers (53). Distribution patterns may change, however, upon dietary manipulation of bile acid synthesis, e.g. by administration of bile acid sequestrants. A reduced flux of bile acids to the liver, as a result of this treatment, leads to expansion of expression of bile acid synthetic capacity to a larger section of the liver acinus (51), as a result of enhanced transcriptional activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes (52). Such stimulated circumstances thus provide the possibility of a direct linkage between cholesterol synthetic- and catabolic pathways (50-52), and result in increased utilization of *de novo* cholesterol as a substrate for bile acid formation (53,54). Taken together, use of a certain substrate pool for bile acid synthesis appears to be more a result of bile acid synthetic capacity and related distribution patterns of key-enzymes under certain physiological- or feeding conditions, rather than preference of these enzymes for a particular cholesterol pool.

Our data show that not all lipoprotein fractions contribute equally to bile acid synthesis.

βVLDL-derived cholesterol was the more potent substrate, showing a 1.8-fold increase in the formation of bile acids, while LDL, added in equimolar amounts of cholesterol, led to a 1.5-fold increase. There were no major differences in uptake of βVLDL or LDL, as both lipoproteins led to accumulation of large amounts of intracellular cholesteryl ester (Table 1). The latter was due to the activation of ACAT, and not simply a consequence of uptake of core CE from these lipoproteins, as the rise in intracellular CE could be prevented by simultaneous incubation with CI-976. On the other hand, addition of HDL did not stimulate bile acid synthesis, allthough it should be noted that the HDL-fraction (50% of serum concentrations) contained significantly less cholesterol, as compared with either βVLDL or LDL (approximately 15% thereof). Consequently, no rise in intracellular level of CE was observed upon addition of HDL. Previous reports have shown that higher concentrations (500 μg protein/ml) HDL did stimulate bile acid synthesis in cultured rat hepatocytes (55-57). In a similar study by Whiting and coworkers (58), a dose-dependent stimulation of bile acid synthesis was found for βVLDL, LDL, and HDL in cultured rabbit hepatocytes, incubated with lipoproteins isolated from 1% cholesterol-fed rabbits. In this case, use of HDL in a concentration equivalent to 50% serum HDL-levels was sufficient to observe an effect. In contrast, others have shown that addition of HDL in equimolar cholesterol amounts, as compared with VLDL+IDL, to medium of cultured rat hepatocytes failed to stimulate bile acid synthesis, indicating that some species related differences may exist (19).

While both βVLDL and LDL stimulated bile acid synthesis, only βVLDL stimulated expression of key-enzyme cholesterol 7α-hydroxylase. Stimulation was exerted at the level of cholesterol 7α-hydroxylase mRNA, showing a dose-dependent and maximal stimulation (3.2-fold) at 40 μg/ml βVLDL (Fig. 4). Similar uptake and activation of ACAT by βVLDL and LDL, yet yielding such regulatory differences, suggests that both particles may undergo different intracellular processing and routing to different cholesterol pools. Evidence for the latter has been obtained in various studies (59-62). Mouse peritoneal macrofages incubated with labelled βVLDL or LDL, displayed a clear-cut difference in targeting of these lipoproteins within the cell (59). βVLDL was localized within distinct vesicles throughout the cytoplasm, while LDL was directed to more diffuse perinuclear vesicles. It was concluded that βVLDL and LDL were taken up by similar receptor-mediated mechanisms, but that βVLDL was processed more slowly, in a different cellular compartment, and was a more potent stimulator of ACAT. It was further postulated that large, cholesterol-loaded βVLDL particles, rich in multivalent apoEs, act by binding to various LDL-receptor molecules simultaneously, resulting in a slower release from the receptor within the acidic sorting endosome. This was further demonstrated by neutralization of apoE by immunotitration, leading to an intracellular distribution pattern similar to that of LDL (60). In a subsequent study by Lombardi et al. (61), using HepG2 cells, a similar difference in processing of these lipoproteins was observed. VLDL and LPL-treated VLDL (presumed similar to βVLDL) showed a slower transport rate from early endosomes to lysosomes, as

compared with LDL. However, in contrast to findings of Tabas (59), the slower transport rate from the endosomal compartment did not result in activation of ACAT in these cells, but did result in enhanced retroendocytosis. Taken together, different intracellular processing of βVLDL and LDL may explain the observed different effects of these lipoproteins on bile acid synthesis in cultured rat hepatocytes, allthough the latter cells appear to differ from HepG2 with respect to routing of lipoproteins to a ACAT-accessable pool.

Determining the lipoprotein entity responsible for directing βVLDL-cholesterol to the regulatory pool is currently under research. As stated, immunotitration of apoE leads to intracellular processing of βVLDL in a mannner similar to LDL (60), a lipoprotein which does not stimulate cholesterol 7α-hydroxylase (Figs. 2 and 3). Furthermore, E2-βVLDL showed a pattern of endocytosis similar to LDL (59), implicating apoE and apoE-phenotype as an important determinant in this respect.

We have demonstrated that lysosomal degradation is a prerequisite for observed up-regulation by βVLDL: inhibition of lysosomal degradation using NH_4CL prevented stimulation of cholesterol 7α-hydroxylase mRNA expression (Fig. 3*b*), in agreement with De Water et al. (63), showing prevention of routing of βVLDL to bile acid synthesis *in vivo* in rats after administration of chloroquine. In this respect, HDL may also be degraded differently from βVLDL, as it has been shown that stimulation of bile acid synthesis by HDL is not affected by chloroquine, indication of a lysosome-independent processing (64,65). It has also been shown that CE from HDL is selectively taken up by the hepatocyte, with no parallel uptake of the apoA1-moiety (65,66). HDL-mediated stimulation was prevented after simultaneous inhibition of ACAT (57), indicating that HDL-cholesterol first mixes with an ACAT accessable cholesterol pool, prior to serving as substrate for bile acid synthesis. We have demonstrated that addition of ACAT inhibitor CI-976 together with βVLDL leads to a further increase of bile acid synthesis (Fig. 1), indicating that βVLDL-cholesterol and CE is efficiently routed to a regulatory pool of cholesterol prior to a pool accessable to ACAT. We have also shown that mevalonate is an efficient precursor for the formation of bile acids (Fig. 1), and leads to strong activation of ACAT in cultured rat hepatocytes (Table 1), as also reported by others, both in cultured rat (19) and rabbit hepatocytes (58). Stimulation of bile acid synthesis by mevalonate was further enhanced upon addition of ACAT inhibitor 58-035 (67,68). It has also been shown that administration of mevalonate to rats induces cholesterol 7α-hydroxylase (27,69). Taken together, the results indicate similarities between mevalonate (and/or derivatives thereof), and βVLDL-derived cholesterol with respect to routing to a pool regulating bile acid synthesis.

Up-regulation of cholesterol 7α-hydroxylase mRNA by βVLDL was exerted at the level of transcription of the gene, which was shown to be rapidly stimulated (2-fold) within 3-12 hours. This result indicated the presence of a positive sterol responsive site within the cholesterol 7α-hydroxylase promoter, which was further corroberated with transient

expression experiments. A βVLDL-cholesterol responsive element was localized within the proximal 348 nucleotides of the cholesterol 7α-hydroxylase promoter (Table 2), showing a similar 2-fold induction of expression upon addition of the particular lipoprotein to the medium of transfected cells. Similarly, responsiveness to exogenous cholesterol has recently been shown in H2.35 cells transfected with a construct containing the proximal 342 nucleotides of the rat cholesterol 7α-hydroxylase promoter, in addition to an enhancer thereof (70). As previously reported by Hoekman and coworkers (15), this same proximal region also harbored a bile acid responsive element (BARE), indicating the presence of a composite sequence responsive to various physiological signals. In an elegant review by Russell and Setchell (71), it was envisioned that both positive and negative effects, exerted by cholesterol and bile acids, respectively, are mediated via positive SRE's and negative BARE's, together titrating expression of the cholesterol 7α-hydroxylase gene. Such a scheme of events, within a genetic background of closely situated sequences, may suggest that competition for anchoring sequences between different ligand-transcription factor complexes can occur. In line with this thought, simultaneous addition of bile acid taurocholate (50 μM) and βVLDL (40 μg/ml) prevented increase or suppression of cholesterol 7α-hydroxylase by either of the two effectors (Twisk, Van der Fits, Hoekman, Princen, unpublished observation).

Analogous differential effects of lipoproteins have been reported with respect to other genes involved in cholesterol homeostasis. Kamps and coworkers (72) have shown a clear difference in capacity of βVLDL and LDL to down-regulate LDL-receptor activity. In accordance, it was shown that only βVLDL, and not LDL or HDL, was capable of down-regulating conversion of acetate to cholesterol in cultured rat hepatocytes (19). Both the LDL-receptor gene, and the gene coding for the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase, contain sequences (SRE's) sensitive to sterols and sterol-derivatives. It seems possible that the relatively slow processing of βVLDL within the cell enables direction of the cholesterol entity towards a regulatory pool within the hepatocyte responsible for regulation of expression of multiple genes. Judging from our results, a similar pool may be involved in stimulation of the cholesterol 7α-hydroxylase gene by βVLDL-derived cholesterol, just as it is responsible for down-regulation of the LDL-receptor and HMG-CoA reductase gene expression. In contrast, sterol 27-hydroxylase expression was not affected by exogenous cholesterol of any source. Previous reports (16,17) have shown regulation of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase by similar mediators, indicating that both enzymes are regulated coordinately to efficiently attain cholesterol homeostasis. Both enzymes diverge, however, with respect to regulation by exogenous cholesterol, which is in contrast to a recent report by Hasan et al. (73), describing induction of sterol 27-hydroxylase by dietary cholesterol in low responding baboons. It is unclear, however, whether determinations of enzyme activity were carried out under conditions of saturating cholesterol concentrations, and differences in enzyme activity may well reflect hepatic-cholesterol concentrations, i.e. low reponders having higher hepatic-cholesterol levels in reponse to the

cholesterol diet.

Not all available data, however, are in agreement with a direct role of cholesterol in regulation of bile acid synthesis. While feeding studies in rats with either mevalonate or cholesterol have shown an up-regulated bile acid synthesis and cholesterol 7α-hydroxylase (12,27,28,69), Björkhem and coworkers concluded from a sequence of different studies (28,30-32) that dietary cholesterol caused stimulation of bile acid synthesis and cholesterol 7α-hydroxylase activity via intestinal malabsorption of bile acids. Infusion of cholesterol-enriched Intralipid failed to stimulate the key-enzyme, and dietary stimulation of bile acid synthesis by cholesterol persisted in lymph-fistulated rats, blocking transport of cholesterol to the liver. In the present study, however, we clearly show that cholesterol *per se* is not sufficient to stimulate cholesterol 7α-hydroxylase, but that lipoprotein composition and specific intracellular processing determines regulatory potency. In this respect, infusion of rats with Intralipid may not yield conclusive results, as this source for cholesterol lacks specific additional entities important for stimulation of cholesterol 7α-hydroxylase. Furthermore, the authors showed that administration of cholesterol caused a decrease in half-life of labelled cholic acid, based on which it was concluded that cholesterol stimulated bile acid synthesis indirectly, and that bile acids remained the major regulators of cholesterol 7α-hydroxylase. In a later paper by Duane (74), however, feeding rats a diet rich in cholesterol did decrease the half-life of cholic acid, but had no effect on intestinal resorption and half-life of chenodeoxycholic acid. The latter was taken to indicate that cholesterol may indeed affect the intestinal resorption of bile acids, but that more hydrophobic bile acids are still taken up by enterocytes. The latter are potent regulators of bile acid synthesis (9,13,14), in which case a scenario of the ratio of cholesterol to bile acids determining the transcriptional activity of the cholesterol 7α-hydroxylase gene, seems more likely (29,71).

In conclusion, we have shown that circulating cholesterol-laden lipoproteins are not only substrate for bile acid synthesis, but that, given a specific lipoprotein composition, they are directly regulatory at the level of cholesterol 7α-hydroxylase gene transcription. We suggest that specific intracellular processing determines regulatory potency of lipoprotein-derived cholesterol, possibly directing cholesterol to a common regulatory pool responsible for the regulation of a number of genes involved in maintainance of cholesterol homeostasis.

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CHAPTER 7

INSULIN SUPPRESSES BILE ACID SYNTHESIS IN CULTURED RAT HEPATOCYTES BY DOWN-REGULATION OF CHOLESTEROL 7α-HYDROXYLASE AND STEROL 27-HYDROXYLASE GENE TRANSCRIPTION

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ABSTRACT

Evidence from in vivo studies indicates that the bile acid pool and bile acid excretion is increased during diabetes mellitus in man and experimental diabetic animals, and that both parameters return to normal levels after administration of insulin. To investigate the biochemical background of these changes, the effects of insulin on bile acid synthesis and cholesterol 7α-hydroxylase and sterol 27-hydroxylase, two key-enzymes in routing of cholesterol towards bile acids, were studied in cultured rat hepatocytes. Mass production of bile acids was dose-dependently diminished, showing significant reduction (-33 to -53%) at physiological concentrations of the hormone (1.4 to 14 nM) and a maximal decrease at 140 nM (-65%). The decrease of bile acid synthesis correlated well with the suppression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity. The enzyme activity for cholesterol 7α-hydroxylase, examined in more detail, was dose-dependently diminished upon incubation of hepatocytes with various concentrations of insulin, reaching maximal reduction at 14 nM insulin. Maximal decrease of the enzyme activity was seen after 8 h of incubation (-70%). Insulin strongly reduced the rise in cholesterol 7α-hydroxylase activity induced by incubation with dexamethasone. Sterol 27-hydroxylase activity was inhibited up to -58% after 24 hours of incubation with 140 nM insulin.

To study the mechanism of suppression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, the effects of insulin on their respective levels of mRNA and gene transcription were assessed. The decrease in enzyme activities could be explained by a concomitant reduction in the cholesterol 7α-hydroxylase (-76%) and sterol 27-hydroxylase (-62%) mRNA level. Transcriptional activity, as assessed by nuclear run-off assays, was decreased to the same extent, i.e. -60% for cholesterol 7α-hydroxylase and -75% for sterol 27-hydroxylase.

Transient-expression experiments using a construct containing the proximal 348 basepairs of the cholesterol 7α-hydroxylase promoter fused to the CAT-gene (-348R*cat*) showed a significant reduction of transcriptional activity (-64%) with insulin, indicating that a sequence important for an insulin induced transcriptional response is located within the first 348 basepairs, preceding the transcription start of the cholesterol 7α-hydroxylase promoter.

We conclude that physiological concentrations of insulin suppress bile acid synthesis by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene transcription, and that this effect is mediated through a direct action of the hormone on the hepatocyte. These results may provide an explanation for the increased bile acid pool and excretion as found during untreated diabetes mellitus in humans and with insulin deficiency in experimental animals.

INTRODUCTION

The liver plays an important role in the synthesis and catabolism of cholesterol (1). Conversion of cholesterol into bile acids takes place exclusively in the liver and excretion of cholesterol and bile acids via the bile represents the major pathway for elimination of cholesterol from the body (1,2). According to current concepts, the primary pathway of bile acid biosynthesis in rats and humans is initiated with 7α-hydroxylation of cholesterol. This reaction is catalyzed by the microsomal cholesterol 7α-hydroxylase, a cytochrome P-450-dependent enzyme (3-5). Recently the mRNAs for rat and human 7α-hydroxylase have been isolated and cloned (6-9), presenting a valuable tool for regulation studies. Cholesterol 7α-hydroxylase displays a response to various physiological signals, the most important of which is considered to be exerted through the enterohepatic circulation of bile acids. Cholesterol 7α-hydroxylase activity (3,4,10) and mRNA (8,9,11) in animals and enzyme activity in humans (12,13) is subject to end-product suppression by bile acids. Regulation takes place through a direct effect of bile acids on the hepatocyte, at concentrations commonly observed in portal blood (14,15), both at a transcriptional and posttranscriptional level (11,15,16).

Additionally, several hormones have been implicated to play a physiological role in the regulation of cholesterol 7α-hydroxylase (5). Several observations suggest the involvement of glucocorticoids in modulation of the enzyme. Enzyme activity, protein and mRNA, in parallel with plasma corticosterone levels in rat, show a diurnal variation (17-19). Adrenalectomy subsequently abolishes the diurnal rhythm of cholesterol 7α-hydroxylase activity (20,21). Using primary monolayer cultures of rat hepatocytes our group has shown that glucocorticoids and no other steroid hormones induce bile acid synthesis by stimulation of cholesterol 7α-hydroxylase activity (22,23). Other investigators recently showed that regulation by dexamethasone takes place at the level of mRNA (24,25) and transcription (25) in a rat hepatoma cell line and cultured rat hepatocytes. Furthermore, thyroid hormone was found to increase cholesterol 7α-hydroxylase activity (26) and mRNA (27) in vivo. It has recently been reported that thyroxine stimulates cholesterol 7α-hydroxylase gene transcription in cultured rat hepatocytes (16,25).

Insulin has also been implicated to play an important role in cholesterol metabolism. However, the effects of this hormone on bile acid synthesis and cholesterol 7α-hydroxylase are less clear. Experimental diabetes in the rat (28-31) and uncontrolled diabetes mellitus in humans (32) have been shown to lead to a marked increase in bile acid pool and biliary lipid and bile acid excretion. These parameters returned to normal levels upon insulin administration (28,31,32). The higher bile acid excretion in diabetes was ascribed to an increased bile acid synthesis (28,32), however other investigators found no change in bile acid production (29). Furthermore, divergent results have been reported with regard to the effect of experimental diabetes on cholesterol 7α-hydroxylase activity in rats. Both an

increased (33), unchanged (29,34) and decreased (35) activity of cholesterol 7α-hydroxylase were found. In none of the latter studies the site of regulatory control was investigated.

There is accumulating evidence that in normal human subjects an alternative pathway towards bile acids exists, involving initial 27-hydroxylation of cholesterol (36). It has also been shown that the alternative route contributes substantially to bile acid synthesis in vitro, accounting for approximately 50% of total synthesis in cultured human and rat hepatocytes (37). Sterol 27-hydroxylase, responsible for catalysis of the initial step in alternative routing of cholesterol, is a member of the cytochrome P-450 superfamily, and located in the inner mitochondrial membrane. The enzymes from rabbit (38,39) as well as rat (40) and pig (41) liver have been characterized, as well as corresponding cDNA sequences for this enzyme from rabbit (42), rat (43,44) and human (45). Little is known about possible regulatory processes affecting sterol 27-hydroxylase. It has been suggested that the enzyme is of minor importance for the regulation of bile acid synthesis and composition of bile acids formed in vivo in rat (46). However, it is conceivable that in view of the reported significant contribution of the 27-hydroxylase pathway to total bile acid synthesis, any mediators of the latter may regulate the enzyme, as in the case of well-documented regulatory processes affecting cholesterol 7α-hydroxylase.

In this study we have assessed the effects of insulin on bile acid synthesis in primary monolayer cultures of rat hepatocytes and we have investigated the mechanism of regulation. Our data indicate that physiological concentrations of insulin inhibit bile acid synthesis by decreasing cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity. Regulation takes place by down-regulation of gene transcription for both enzymes.

MATERIALS AND METHODS

Materials used for isolation and culturing of rat hepatocytes, determination of bile acid synthesis from radiolabelled cholesterol and of mass production of bile acids, and assaying cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity were obtained from sources described previously (22,47,48). Insulin (Actrapid, 100 IE/ml) was from Novo Industri (Copenhagen, Denmark). [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (400 Ci/mmol) and [4-^{14}C]-cholesterol (60 mCi/mol) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, UK.

Male Wistar rats weighing 250-350 g were used throughout and were maintained on standard chow and water ad libitum. For preparation of hepatocytes, animals were sacrificed between 9 and 10 a.m. Institutional guidelines for animal care were observed in all experiments.

Rat hepatocyte isolation and culture

Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously (22,47,48). Viability, as determined by trypan blue exclusion, was higher than 90%. The cells were seeded on 60-mm diameter plastic tissue culture dishes or 6-well cluster plates (Costar, Cambridge, MA, USA) at a density of $1.5*10^5$ cells/cm^2 in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37°C in a 5% CO_2/95% air atmosphere (22,47,48). After a 4-hour attachment period, and 20 h thereafter, medium was refreshed with 1.0 ml (6-well plates) or 2.5 ml (dishes) of culture medium with the appropriate insulin concentrations, as indicated. Hepatocytes were cultured for 2 days under these conditions. After a 48-hour culture period, cells were harvested for determination of bile acid synthesis, cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and assessment of mRNA and transcriptional activity levels. In time course experiments insulin was added to the hepatocytes at various times between 24 and 48 hours of culture age as indicated in the results section.

Quantification of bile acid synthesis

Synthesis of bile acids by rat hepatocytes was measured by determination of mass production of bile acids with gas liquid chromatography during the second 24-hour culture period from 24 to 48 hours, as described before (22). Bile acid synthesis was also determined by measuring conversion of pre-existing radiolabelled cholesterol (0.15 μCi of [4-^{14}C]-cholesterol per 10 cm^2 of cells) into bile acids during the same period, i.e. between 24 to 48 hours, as reported previously (22,47).

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

Cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity were measured in homogenates of hepatocytes, or in microsomes and mitochondria, respectively, as reported previously (47-49). [^{14}C]-labelled products were analysed by thin layer chromatography, and the amount of [^{14}C]-7α-hydroxycholesterol and [^{14}C]-27-hydroxycholesterol was quantitated by scraping off and counting of the spots containing these products, using the [^{14}C]-cholesterol input as a recovery standard. Blank values, determined by running parallel incubations without a NADPH-generating system, were subtracted before calculating enzyme activity. Protein and cholesterol were assayed as previously described (48).

RNA isolation, blotting and hybridization procedures

Total RNA was isolated from cultured rat hepatocytes as previously described (15). Equal amounts of total RNA from different incubations were fractionated by electrophoresis on a 0.8% agarose gel containing 1 M formaldehyde, and transferred to Hybond-N$^+$ filter

(Amersham). For slotblotting of total RNA, samples were diluted to appropriate concentrations and applied onto a filter using the Minifold II slotblotting apparatus (Schleicher and Schuell). After both procedures, filters were crosslinked with UV-light for 5 min and then hybridized with different probes as described previously (15). Each blot was hybridized with 25 ng of probe, labelled by the random-primer method (Mega-prime, Amersham) to approximately 6 x 10^8 cpm/μg DNA. After hybridization and washing, the filters were exposed to Hyperfilm MP (Amersham) together with an intensifying screen (Eastman-Kodak Co.) for 48-120 h at -80°C. For quantitation of the relative amounts of mRNA, the autoradiographs were scanned using a Shimadzu CS 910 chromatograph scanner, and areas under the curves were integrated using a data processor (Shimadzu Corp. Kyoto, Japan). The mRNA levels were quantitated by using three different amounts of total RNA, between 2 and 8 μg, giving a linear relation between the specific mRNA signal and the amount of RNA applied.

The following DNA fragments were used as probes in hybridization experiments: a 1.6 kb PCR-synthesized fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region as described in detail in ref. 15; a 1.6 kb HindIII/XbaI fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. Jerome Strauss (44), and isolated from a rat liver cDNA library using the rabbit sterol 27-hydroxylase cDNA, previously isolated by Russell and coworkers (42), as a probe; a 773 bp HindIII fragment of hamster HMG-CoA reductase cDNA (50); a 1.2 kb PstI fragment of hamster actin cDNA; and a 1.1 kb PstI fragment of rat GAPDH. The latter two served as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter.

Nuclear run-off studies

Nuclear run-off studies were conducted essentially as described by Groudine et al. (51), with minor modifications (15).

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

RNA labelling and isolation- An aliquot of frozen nuclei was spinned down and resuspended in 200 μl of transcription buffer (10 mM Tris-HCl pH 7.9, 140 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM of dGTP, dATP, dCTP, 0.1 mM s-adenosyl-1-methionine, 14 mM

β-mercaptoethanol, 1 mg/ml heparin sulfate, 1.7 mM spermidine, 10 mM creatine phosphate, 40 μg/ml creatine kinase, 25% glycerol and 100 μCi of [α^{32}P]UTP), and incubated while shaking at 30°C for 30 min. 600 μl of a buffer containing 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris-HCl pH 7.4 and 200 U/ml DNAseI (Bethesda Research Laboratories) was added, and the mixture was incubated for an additional 5 min. at 30°C. 200 μl of SDS/Tris (5% w/v SDS, 0.5 M Tris-HCl pH 7.4, 0.125 M EDTA) with 200 μg/ml Proteinase K (Boehringer Mannheim) was added, and the mixture was incubated for 30 min at 42°C. RNA was extracted with 1 volume of phenol/chloroform/isoamylalcohol (50:49:1), precipitated with 2.5 volumes of ethanol and 10 μg/ml tRNA, washed, and taken up in 50 μl of Tris/EDTA (10 mM Tris-HCl pH 7.4, 1 mM EDTA). Labelled RNA was separated from free nucleotides by passage over a Sephadex G50(fine)-column (Boehringer Mannheim). The RNA was mildly degraded by a 10 min incubation on ice in 0.25 M NaOH, and the mixture was neutralized by addition of a half volume of 1 M HEPES (free acid) and precipitated with 2 volumes of ethanol and 0.1 volume of 3 M NaAc. Incorporation of label was measured by liquid scintillation counting, and equal amounts of labelled RNA were added to the filters.

Hybridization- Target DNA, being 5 μg of plasmid material containing cDNA sequences of rat cholesterol 7α-hydroxylase, rat sterol 27-hydroxylase, hamster HMG-CoA reductase, hamster actin and rat GAPDH were slotblotted onto strips of Hybond-N^+ filter (Amersham) and crosslinked. The filters were hybridized with the labelled RNA for 36 hours, washed and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantitation of relative amounts of mRNA synthesized was conducted as described above.

Transfection experiments and CAT-assays

At 22 hours after isolation cells were subjected to transfection using recombinant plasmid -348R*cat*, and CAT-assays were performed, as described previously (16). -348R*cat* contains the proximal 348 nucleotides of the cholesterol 7α-hydroxylase promoter fused to the bacterial chloramphenicol acetyltransferase gene, used as a reporter. The amounts of acetylated product as represented by autoradiography were quantitated using a Phosphor-imager 400B (Molecular Dynamics). Data were corrected for protein and transfection efficiency.

Statistical analysis

Data were analyzed statistically using Student's paired t-test. Values are expressed as means ± SD.

RESULTS

Effect of insulin on bile acid synthesis in cultured rat hepatocytes

To determine the influence of insulin on bile acid synthesis, hepatocytes were incubated with [4-^{14}C]-cholesterol as a substrate. Addition of 140 nM insulin to the culture medium resulted in a significant ($P < 0.005$) 58 ± 19% decrease in total bile acid synthesis, from 8760 ± 3370 dpm/24 h per mg of cell protein to 3680 ± 1320 dpm/24 h per mg of cell protein (means ± S.D., n = 4) in the period from 24-48 h. In these experiments, bile acid synthesis was determined by measuring conversion of pre-existent radiolabelled cholesterol into bile acids. To exclude the possibility that insulin decreased bile acid synthesis from exogenous cholesterol by changing the amount of total cholesterol available for bile acid formation, consequently leading to changes in the specific activity of the precursor pool of cholesterol, mass production of bile acids was determined. As can be seen in Fig. 1, addition of insulin to the cells caused a dose-dependent decrease in total bile acid synthesis. Significant inhibition (-33 to -53%) was achieved at physiological concentrations of insulin (1.4 to 14

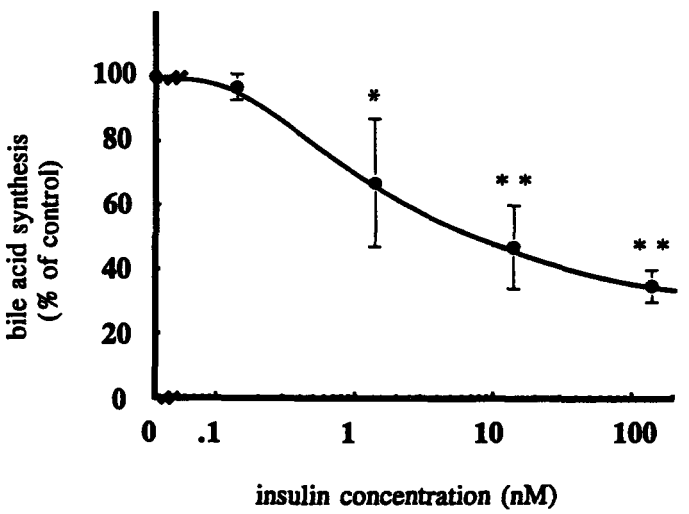


Figure 1. Effect of insulin concentration on mass production of bile acids in cultured rat hepatocytes. Hepatocytes were cultured as described in the Materials and methods section. After a 4-h attachment period and 20 h thereafter, cells were refreshed with medium without (control) or with the indicated insulin concentrations. Bile acid synthesis was measured in the period from 24-48 h in cells and media. Values shown are expressed as percentage of bile acid synthesis in control incubations and are means (± S.D.) of duplicate incubations of hepatocytes from six rats. Absolute synthesis rate in the absence of insulin was 1.29 ± 0.80 μg/24 h per mg cell protein. Cholic acid was 23 ± 7% of total bile acid synthesis, β-muricholic and cheno-deoxycholic acids 77 ± 7%. A significant difference between control and insulin-treated cells is indicated by an asterix (*, $P < 0.05$; **, $P < 0.005$).

nM), whereas maximal inhibition was reached at 140 nM (-65 ± 5%). The latter value compared well with the data obtained from the [^{14}C]-cholesterol conversion measure-ment. No change was observed in the proportion of individual bile acids synthesized in the presence or absence of insulin. The contribution of cholic acid to total bile acid synthesis was 23 ± 7% and of β-murichолic acid and chenodeoxycholic acid 77 ± 7%.

Effect of insulin on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity in cultured rat hepatocytes

The effect of incubation of hepatocytes with 140 nM insulin on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities is shown in Fig. 2. The suppression of the activities of both enzymes (-57% for cholesterol 7α-hydroxylase and -58% for sterol 27-hydroxylase) paralleled the decrease in bile acid synthesis (-65%). The dose-dependency and time course of the suppression of cholesterol 7α-hydroxylase activity by insulin were studied in more detail.

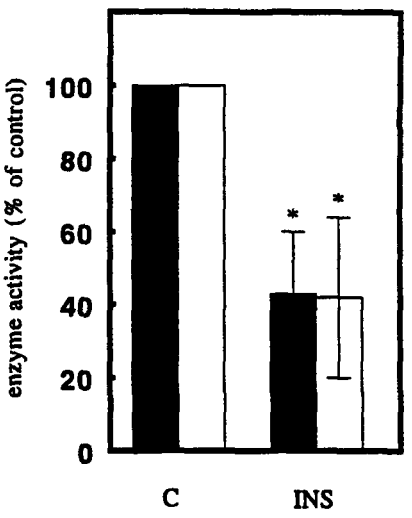


Figure 2. Effect of insulin on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity in cultured rat hepatocytes. Experiments were performed as described in the legend to Fig. 1. Hepatocytes were harvested at 48 hours of culture, after a 24-hour incubation with insulin, or solute (controls), and enzyme activities were determined. Values shown are expressed as a percentage of enzyme activity in control incubations and are a means (± S.D.) of duplicate incubations, using hepatocytes from 5-6 rats. Cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity in control hepatocytes amounted to 203 ± 154 and 70 ± 24 pmol/h per mg of cell protein, respectively. A significant difference between control and insulin-treated cells is indicated by an asterix ($P < 0.005$).

Addition of various concentrations of insulin to the culture medium resulted in a dose-dependent suppression of cholesterol 7α-hydroxylase activity between 24-48 h of culture (Fig. 3). Maximal reduction (-60% ± 20%) was reached at 14 nM insulin whereas 1.4 nM was already sufficient to obtain significant inhibition.

To assess the time course of inhibition, cells were exposed to 140 nM insulin for different lengths of time between 24 and 48 h of culture and hepatocytes were harvested

simultaneously after 48 h of culture time. Figure 4 shows that maximal inhibition was achieved after an incubation period of 8 hours. The rat hepatocytes were refractory to induction of cholesterol 7α-hydroxylase activity during the first 24 h of culture. This finding is in line with previous reports on poor hormonal induction of enzymes, including cholesterol 7α-hydroxylase (22) and other mono-oxygenases (52) shortly after hepatocyte isolation (53,54).

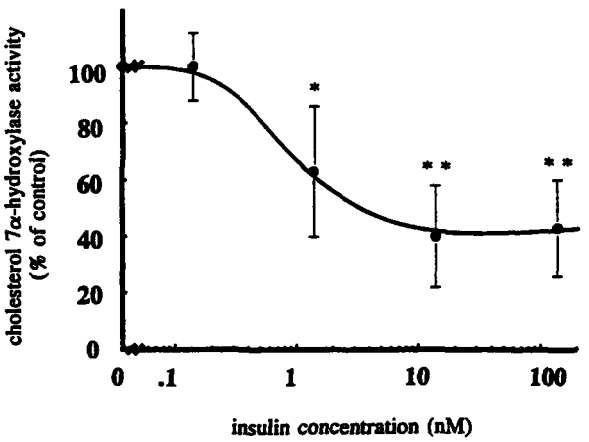


Figure 3. Effect of insulin concentration on cholesterol 7α-hydroxylase activity in cultured rat hepatocytes. Experiments were performed as described in the legend to Fig. 1. Hepatocytes were harvested at 48 h of culture and enzyme activity was determined. Values shown are expressed as percentage of enzyme activity in control incubations and are means (± S.D.) of duplicate incubations, using hepatocytes from 3-9 rats. Absolute values for cholesterol 7α-hydroxylase activity were 203 ± 154 pmol/h per mg of cell protein. A significant difference between control and insulin-treated cells is indicated by an asterix (*, $P < 0.05$; **, $P < 0.005$).

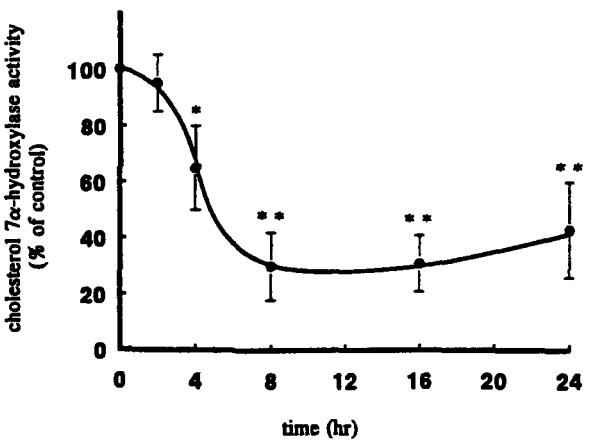


Figure 4. Time course of inhibition of cholesterol 7α-hydroxylase activity in cultured rat hepatocytes by insulin. At the indicated times before harvesting 140 nM insulin was added to the culture medium. Hepatocytes of all incubations were harvested simultaneously, at 48 h of culture time. Values shown are expressed as percentage of enzyme activity in control incubations and are means ± S.D. of duplicate incubations, using hepatocytes from 3-9 rats. Cholesterol 7α-hydroxylase activity in control hepatocytes amounted to 203 ± 154 pmol/h per mg of cell protein. A significant difference between control and insulin-treated cells is indicated by an asterix (*, $P < 0.05$; **, $P < 0.005$).

We have previously shown that glucocorticoids stimulate bile acid synthesis in cultured rat hepatocytes by inducing cholesterol 7α-hydroxylase (22,23). To investigate whether insulin had an effect on the stimulation of the enzyme activity by dexamethasone, hepatocytes were incubated for 24 h without hormones, with 50 nM dexamethasone or in the presence of 50 nM dexamethasone and 140 nM insulin. The induction of 7α-hydroxylase activity by 50 nM dexamethasone (5.7-fold (S.D. = 2.5, n = 6) with respect to control cultures without hormones) was strongly blocked by simultaneous addition of 140 nM insulin. Stimulation was found to be only 2.0-fold (S.D. = 1.4, n = 6) in the presence of both hormones.

The suppression of activity of either enzyme cannot be attributed to a decrease in substrate availability, since the cellular free cholesterol content was increased significantly ($P < 0.05$) from 16.4 ± 1.5 μg/mg of cell protein (n = 5) in control cells to 20.5 ± 1.9 μg/mg of cell protein (n = 5) in hepatocytes maintained in the presence of 140 nM insulin. Furthermore, free cholesterol from cells comprises only 33% of total free cholesterol in the enzyme assay (48), and the slight dilution of specific radioactivity of substrate as a result of the increase of cholesterol content of cells was corrected for in the enzyme assay. To determine whether insulin exerted its effect by direct inhibition of cholesterol 7α-hydroxylase, as suggested previously (33), or of sterol 27-hydroxylase, the hormone was added directly to the assay mixture. Both in incubations with isolated liver microsomes or mitochondria, and homogenates of hepatocytes, 140 nM insulin had no effect on enzyme activity (data not shown).

Effect of insulin on cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA and transcriptional activity

To assess the level of regulation of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity by insulin, steady-state mRNA levels and transcriptional activity of both genes were determined in hepatocytes which had been incubated with 140 nM insulin.

Northern-blot analysis of total RNA isolated from cultured hepatocytes shows a strong down-regulation of cholesterol 7α-hydroxylase as well as sterol 27-hydroxylase mRNA in response to 140 nM insulin, as opposed to the actin and GAPDH mRNA, used as internal standards (Fig. 5a). The three distinct mRNAs for cholesterol 7α-hydroxylase, as reported before (15), all show equal down-regulation by insulin. For reasons of comparison, mRNA levels for HMG-CoA reductase were also assessed. Figure 5a clearly shows that this particular messenger is strongly upregulated in response to an 8-hour incubation with insulin (6.5-fold), indicative for the fact that not all mRNAs for enzymes involved in maintainance of cholesterol homeostasis behave similarly in response to insulin. Stimulation was transient, and declined to normal levels after prolonged incubation with the compound (results not shown). Results described herein are in good agreement with in vitro studies using cultured rat hepatocytes (55) and recent in vivo studies with streptozotocin-treated diabetic rats (56),

which show rapid stimulation of HMG-CoA reductase activity and mRNA upon administration of insulin. Figure 5b shows a rapid decline of mRNA-levels for both cholesterol 7α-hydroxylase and sterol 27-hydroxylase in response to insulin. After 8 hours of incubation with the compound, the messenger level had decreased up to -76 $\pm$ 15% and -62 $\pm$ 19% for cholesterol 7α-hydroxylase and sterol 27-hydroxylase, respectively.

Nuclear run-off assays were conducted to assess whether insulin had an effect on transcriptional activity of the genes for the two key-enzymes. Nuclei were isolated from hepatocytes incubated in the presence or absence of 140 nM insulin for 8 hours. [α^{32}P]-labelled total RNA was hybridized to rat cholesterol 7α-hydroxylase cDNA, rat sterol 27-hydroxylase cDNA, hamster HMG-CoA reductase cDNA, rat GAPDH and hamster actin cDNA. The latter two served as transcriptional activity controls between the different samples and specific transcriptional activity is hence expressed relative to that of actin.

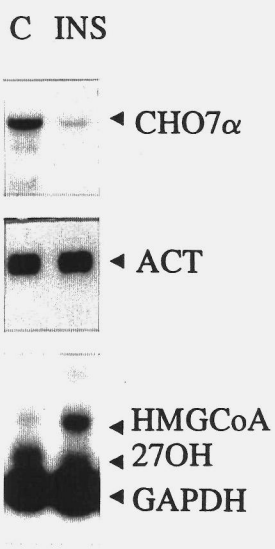
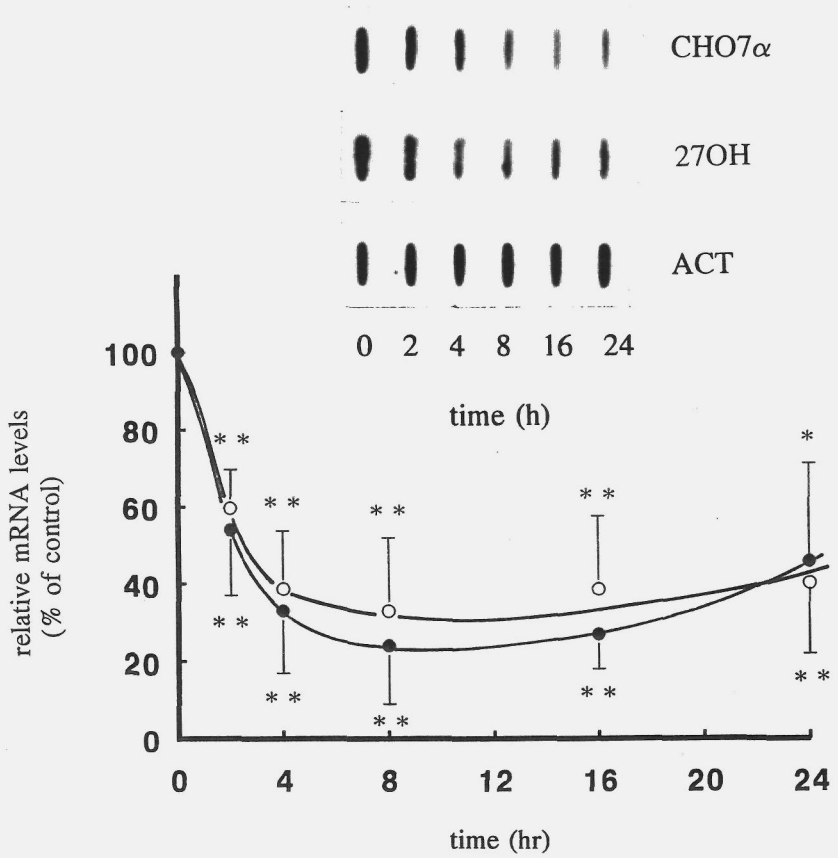


Figure 5. Time course of inhibition of cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA by insulin in cultured rat hepatocytes.
A. Northern-blot hybridization of total RNA, isolated from hepatocytes incubated in the presence (INS) or absence (C) of 140 nM insulin for 8 h. 10 μg of total RNA was separated on a 1% agarose gel containing formaldehyde, transferred to a Hybond-N$^+$ filter, and hybridized with [^{32}P]-labelled cholesterol 7α-hydroxylase (CHO7α) cDNA, sterol 27-hydroxylase (27OH), HMG-CoA reductase (HMG-CoA) cDNA, GAPDH cDNA, or actin (ACT) cDNA as described under "Materials and Methods".



B. At the indicated times before harvesting, 140 nM insulin was added to the culture medium. Hepatocytes of all incubations were harvested simultaneously at 48 h of culture time, and mRNA was isolated as described under "Materials and Methods". The amount of cholesterol 7α-hydroxylase (CHO7α, closed symbols) and sterol 27-hydroxylase (27OH, open symbols) mRNA was assessed by slot-blotting and densitometric scanning of resulting autoradiographs (inset), using the actin (ACT) mRNA as an internal standard to correct for differences in the amount of total RNA applied to the filter. Values shown are expressed as percentages of mRNA levels in control incubations and are a means ± S.D. of duplicate incubations, using hepatocytes from 3-6 rats. A significant difference between control and insulin-treated cells is indicated by an asterix (*, $P < 0.05$; **, $P < 0.005$).

Figure 6 shows that addition of 140 nM insulin lowers the transcriptional rate of cholesterol 7α-hydroxylase by 60 ± 1%, and in the case of sterol 27-hydroxylase: by 75 ± 4%. There was no effect on transcriptional activity of the GAPDH gene. In contrast, insulin displays a strong stimulatory effect on HMG-CoA reductase gene expression, which is induced 4.0 ± 1.1-fold.

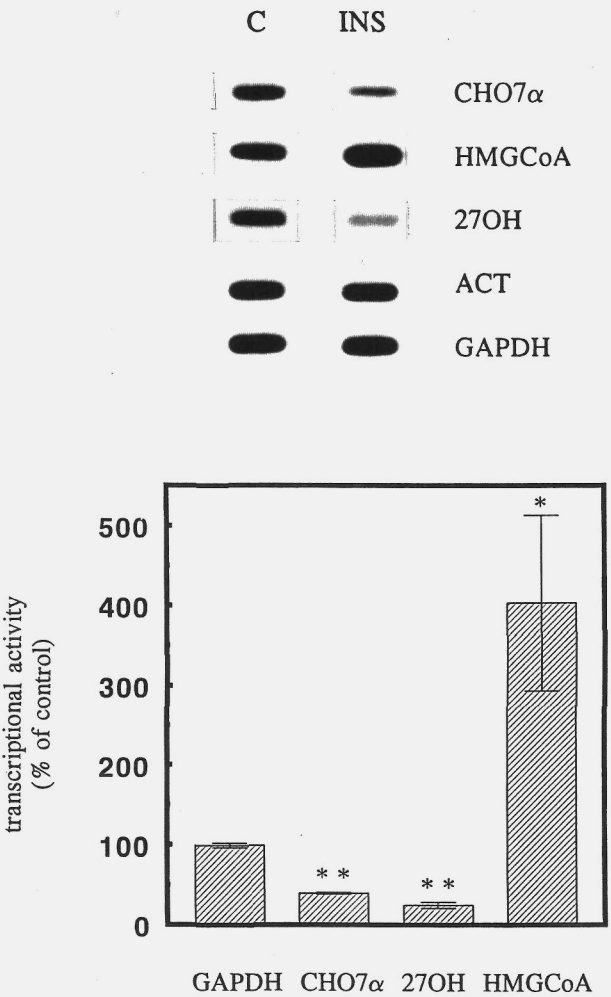


Figure 6. Effect of insulin on transcriptional activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase.

A. Transcriptional activity of several genes in nuclei isolated from hepatocytes incubated with 140 nM insulin. Cells were exposed to the hormone (INS) for 8 h, between 40 and 48 h of culture, and were harvested simultaneously with untreated cells (C) after this period. [^{32}P]-labelled total RNA was synthesized and isolated from nuclei, and hybridized to 5 μg of cholesterol 7α-hydroxylase (CHO7α) cDNA, sterol 27-hydroxylase (27OH) cDNA, HMG-CoA reductase (HMG-CoA) cDNA, actin (ACT) cDNA and GAPDH cDNA, as described under "Materials and Methods".

B. The amount of specific mRNA was assessed by densitometric scanning of resulting autoradiographs, using the actin mRNA signal as a transcriptional control. Data are expressed as transcriptional activity relative to that of actin, and as a percentage of control cells (no insulin added). Each value represents a mean ± SD of three independent experiments. A significant difference between control and insulin-treated cells is indicated by an asterix (*, $P < 0.05$; **, $P < 0.005$).

Effect of insulin on CAT-activity in cells transfected with a cholesterol 7α-hydroxylase promoter-CAT-reporter construct

Recently, Hoekman et al (16), by performing transient-expression experiments in primary cultures of rat hepatocytes, obtained evidence that a major transcription activating element of the cholesterol 7α-hydroxylase gene is located in the proximal region up to -348 nucleotides. We wanted to assess whether an insulin-responsive sequence might be localised within this proximal part of the cholesterol 7α-hydroxylase promoter. A -348R*cat*-construct,

consisting of the first 348 basepairs of the cholesterol 7α-hydroxylase promoter fused to the CAT-reporter gene (16), was used in transient-expression experiments. In accordance with down-regulation of transcriptional activity of the cholesterol 7α-hydroxylase gene as determined by nuclear run-off assays, insulin displayed a strong inhibitory effect (-64 ± 7%) on the promoter activity of the -348R*cat*-construct (Fig. 7).

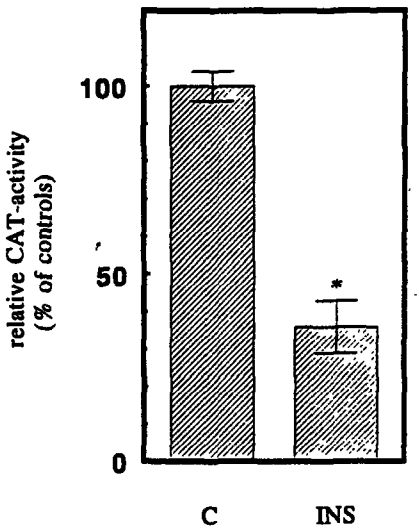


Figure 7. Effect of insulin on CAT-activity of cholesterol 7α-hydroxylase promoter-reporter construct -348R*cat* in transfected cultured rat hepatocytes. Transient-expression experiments were performed, using the -348R*cat* promoter-reporter construct described in ref. 16. After transfection, cells were cultured in standard medium in the presence (INS) or absence (C) of 140 nM insulin, and CAT-activity was assessed. Values expressed are a means ± SD of three independent experiments. *Indicates a significant difference ($P < 0.05$) between controls and insulin-treated cells.

DISCUSSION

The present study shows that bile acid synthesis in cultured rat hepatocytes is subject to down-regulation by physiological concentrations of insulin through a direct effect on the hepatocyte and that regulation takes place by suppression of transcription of the cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene. Insulin caused a maximal inhibition (-58%) of the conversion of [^{14}C]-labelled cholesterol, which is in good agreement with the 65% reduction of mass production of bile acids, i.e. cholic acid and β-muricholic acid. The latter observation excludes the possibility that insulin might affect the routing of cholesterol from an intracellular precursor pool towards bile acids, thereby causing a shift from exogenous to endogenous cholesterol as a substrate. This would ultimately result in a decrease in utilization of exogenous [^{14}C]-cholesterol. Addition of physiologically relevant concentrations of insulin (1.4 - 14 nM) already leads to a marked decrease in bile acid synthesis (-33% to -53%,

respectively), with a maximum (-65%) at 140 nM. The magnitude of suppression agrees well with results obtained in vivo in rat by other investigators, who showed a two-fold increase in bile acid pool size and biliary bile acid secretion in diabetic, insulin-deficient rats, which were restored to normal values after daily injection with insulin (28,31). Treatment of insulin resistance with insulin in patients having maturity-onset diabetes mellitus also reduced bile acid pool size and fecal bile acid excretion (32).

With regard to the mechanism of the increased bile acid synthesis in diabetic animals contradictory reports have appeared, showing unchanged (29,34), increased (33) and decreased (35) activity of cholesterol 7α-hydroxylase. Our results show that addition of insulin to the culture medium of hepatocytes results in a time- and dose-dependent decline of cholesterol 7α-hydroxylase activity. In addition, insulin displayed a concomitant suppressive effect on sterol 27-hydroxylase, involved in alternative routing of cholesterol to bile acids. The decrease of these enzyme activities correlated well with the suppression of bile acid synthesis.

It has been postulated that cholesterol 7α-hydroxylase activity, as that of other major enzymes involved in cholesterol homeostasis, are coordinately regulated by phosphorylation/dephosphorylation processes (57). Insulin does affect a number of enzyme activities through modulation of their phosphorylation state (58). However, we and others have not found indications for such a type of regulation (48,59). Down-regulation of cholesterol 7α-hydroxylase activity by insulin did not differ upon addition or absence of 50 mM fluoride (data not shown). Additionally, our results indicate that regulation by insulin takes place at the level of cholesterol 7α-hydroxylase mRNA, resulting in a 76% decrease of the steady-state mRNA levels after 8 h, similar to the suppression of enzyme activity.

In addition to normal routing of cholesterol towards bile acids via cholesterol 7α-hydroxylase as first and rate-limiting step, a substantial contribution to bile acids is made via an 'alternative' or '27-hydroxylase' pathway (36,37). Synthesis via initial 27-hydroxylation has been estimated to amount up to 50% in cultured human and rat hepatocytes (37). Down-regulation of cholesterol 7α-hydroxylase alone may thus not be sufficient to explain inhibitory effects of insulin on bile acid synthesis, since this would leave a major portion of synthesis unaffected. The results clearly show that, in addition to cholesterol 7α-hydroxylase, sterol 27-hydroxylase is affected to a similar extent. Insulin (140 nM) caused suppression of sterol 27-hydroxylase activity (-58%) and mRNA (-62%), comparable to the effects on cholesterol 7α-hydroxylase. Measurement of transcriptional activity for both genes via nuclear run-off assays and via transient-expression assays for cholesterol 7α-hydroxylase shows that the inhibitory effect of insulin thereon can fully explain the decline in activity and mRNA for these key-enzymes.

Insulin increased the intracellular amount of cholesterol by 25% (from 16.4 to 20.5 μg/mg cell protein). This is, however, not a mechanism by way of which the hormone can exert its suppressive effect on the key-enzymes described. As has been shown by other

investigators (8,60-63) dietary cholesterol has stimulatory effects on cholesterol 7α-hydroxylase activity, mRNA and gene expression, possibly by a reduced feedback inhibition due to cholesterol-induced malabsorption of bile acids (60). Sterol 27-hydroxylase is not affected by this mediator (J. Twisk, L. v.d. Fits, H.M.G. Princen, unpublished observation). In this light, the negative effect of insulin on cholesterol 7α-hydroxylase might well be underestimated due to simultaneous positive effects through enhanced intracellular cholesterol levels. The increase of cellular cholesterol may have multiple causes, since insulin has multiple effects on hepatic lipid and lipoprotein metabolism. Insulin has a stimulatory effect on the key enzyme in cholesterol synthesis, HMG-CoA reductase, both in vivo and in cultured rat hepatocytes (55,56,64-66). Streptozotocin-treated rats infused with insulin showed a rapid recovery of activity and mRNA levels for this enzyme (56). Similarly, treatment of primary monolayer cultures of rat hepatocytes with insulin resulted in an increase of both HMG-CoA reductase activity and mRNA (55). These effects can be explained by induction of gene expression (this study). Additionally, the hormone has been shown to increase the receptor-mediated uptake of lipoproteins (67,68), to decrease the synthesis and secretion of apoB-containing lipoproteins (69-71) and to inhibit bile acid synthesis (this study).

We conclude that physiological concentrations of insulin suppress bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene transcription. These findings may provide an explanation for the increased bile acid pool size and excretion as found in association with insulin resistance in untreated non-insulin-dependent diabetes mellitus in humans or insulin deficiency in experimental diabetic animals, and normalization thereof upon insulin administration.

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CHAPTER 8

GENERAL DISCUSSION & SUMMARY

The liver-specific process of bile acid biosynthesis is the most quantitive route to bodily excretion of cholesterol. However, mechanisms involved in regulation of the pathway, and importance of various enzymes therein, have been subject for dispute.

Bile acid-induced biofeedback

Bile acid synthesis may be regulated in a number of ways, most important of which is considered to be exerted by the flux of bile acids that undergo enterohepatic circulation and suppress bile acid synthesis upon return to the liver via portal blood.

Using monolayer cultures of primary rat hepatocytes, the molecular mechanism underlying this form of regulation was investigated. A dose-dependent suppression of bile acid synthesis was demonstrated, exerted at the level of cholesterol 7α-hydroxylase, upon addition of taurocholic acid (TCA) to the medium of cultured rat hepatocytes (chapter 2). Clearly, the effects observed are resultant from a direct effect of bile acids on the hepatocyte. A putative intestinal factor, dependent on bile flow for uptake and transport to the site of regulation, does not appear to play a role in bile acid-induced biofeedback.

Regulation of cholesterol 7α-hydroxylase is exerted both at a transcriptional and post-transcriptional level. In addition to possible interaction with an as yet unidentified bile acid receptor, resulting in a transcriptional event, bile acids may act by affecting cholesterol 7α-hydroxylase mRNA stability. From incubation studies using TCA, an apparent half-life of 4 hours was calculated for both enzyme and mRNA, possibly resulting from multiple AU-rich sequences within the 3'-untranslated region (UTR) of the cholesterol 7α-hydroxylase mRNA. These sites have been associated with increased endonuclease sensitivity, and rapid mRNA degradation. Indirect evidence for a post-transcriptional regulatory mechanism was provided by the large gap in potency of TCA to affect gene transcription (-40%), and enzyme activity and steady-state mRNA levels (-90%) for cholesterol 7α-hydroxylase. *In vivo* experiments with rats treated with cholestyramine also exhibited a much stronger stimulation of cholesterol 7α-hydroxylase activity and mRNA ($>$ 10-fold), as compared with stimulation of transcription (4-fold).

Molecular biology could provide a further tool for enhancement of bile acid synthesis, as a strategy for lowering of serum cholesterol levels, and the risk of CHD. Serum cholesterol levels increase with age, concomitant with a decline in bile acid formation and cholesterol 7α-hydroxylase activity. Furthermore, a low bile acid synthetic capacity has been shown to be an independent, additional risk factor for incidence of atherosclerosis, in heterozygotes suffering from FH. Conversely, the high bile acid-synthetic capacity of rats, may partly explain the resistance of these animals to atherosclerotic diets. Based on the data discussed in the above, enhancement of bile acid synthesis could be provoked by increasing expression and stability of cholesterol 7α-hydroxylase mRNA. Introduction of a cDNA-construct into the liver, harboring the coding sequence for cholesterol 7α-hydroxylase, whereby the original promoter has been replaced by a strong liver-specific one that does not harbor bile acid responsive sequences described, would render high and constitutive expression of the cholesterol 7α-hydroxylase mRNA. In addition, the 3'-UTR of cholesterol 7α-hydroxylase mRNA can be replaced by a UTR that does not harbor the AU-rich sequences described, resulting in a much more stable mRNA. Such an approach could ultimately provide an attractive basis for gene targeting of the liver, whereby the recipient is expected to show a constant higher rate of bile acid synthesis.

It should be stressed that any attempt to stimulate bile acid synthesis will result in a larger bile acid pool, and, allthough spill-over in the intestine will result in an increase in faecal excretion, enlargement of the pool will also increase intestinal absorption of dietary cholesterol and fats. In addition, enlargement of intestinal bile acid load, unaccompanied by dietary intake, will increase the free bile acid concentration, resulting in detergent activity and injury to the intestinal tract. Efficient and quantitative excretion of cholesterol therefore will remain dependent on administration of bile acid binders within combination therapy.

Involvement of sterol 27-hydroxylase in regulation of bile acid synthesis

While research has mainly focussed on cholesterol 7α-hydroxylase as main regulatory point of various processes affecting bile acid synthesis, other potentially important enzymes have largely escaped attention. The importance of contribution of the alternative pathway to total bile acid synthesis, had been reported *in vivo* in humans, and in cultured human and rat hepatocytes, but regulation of this route had hitherto not been reported. From the strong suppressive effects of TCA on bile acid synthesis, existence of the 27-hydroxylase pathway gave theoretical grounds for assuming that the pathway may be subject to regulation. In chapter 3, it is shown that indeed bile acids affect alternative routing of cholesterol to bile acids at the level of sterol 27-hydroxylase activity, mRNA, and transcription, in parallel to their effect on cholesterol 7α-hydroxylase. Effects at the transcriptional level may show similarities between the two enzymes, but the 3'-UTR of the sterol 27-hydroxylase mRNA is not rich in AU-sequences, and consequently displayed an apparent half-life of 13 hours in

incubation studies using TCA, indicative of a much more stable mRNA, while the extent of transcriptional suppression was similar (-40%).

Apart from the liver, 27-hydroxycholesterol is synthesized in many different tissues and organs. Its potential for down-regulation of both LDL-receptor expression, as well as that of HMG-CoA reductase, makes 27-hydroxycholesterol and the enzyme involved in its synthesis, a liable target for future research. Regulation of the 27-hydroxylase pathway may serve a dual purpose: regulation of extrahepatic cholesterol uptake and storage, and endogenous cholesterol synthesis, as well as determining the flux of cholesterol that undergoes the first and dedicated step towards (alternative) formation of bile acids, outside the liver. Thus stimulation of extrahepatic bile acid biosynthetic enzymes, followed by rapid hepatic clearance of resulting oxysterols, may serve to direct cholesterol away from possible storage pools. Subsequent fueling into degradational pathways can thus contribute to lowering of serum cholesterol levels. Stimulation of sterol 27-hydroxylase in the liver will probably not affect LDL-receptor expression, nor that of HMG-CoA reductase, as efficient coupling of the oxysterol to further bile acid formation can take place in this organ, increasing the demand for, and uptake of substrate cholesterol.

Be it that sterol 27-hydroxylase is a possible target for therapy, stimulation of expression the hepatic enzyme only, will result in increased synthesis of chenodeoxycholic acid, a more hydrophobic and potentially more hepatotoxic bile acid than is cholic acid.

Structural aspects of bile acids involved in biofeedback

In chapter 4, attention is focussed on possible structural aspects of bile acids important for eliciting an effect on bile acid synthesis. While bile acids were shown to down-regulate both cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, mRNA, and gene transcription, results discussed in chapters 2 and 3 indicated that not all bile acids were equally potent in this respect. Analysis of effects of 27 bile acids, differing in hydrophibicity index (HI_x), and number, position, and orientation (α/β) of hydroxyl groups present on the steroid nucleus, on cholesterol 7α-hydroxylase mRNA revealed a weak correlation between hydrophobicity *per se*, and capacity to suppress mRNA expression for the enzyme ($r = 0.61$), indicating that only some 37% of effects observed could be explained by hydrophobicity alone. Apparently, other structural aspects are important as well. Analysis of the three-dimensional structure of cholic acid (3α,7α,12α), being a potent suppressor of cholesterol 7α-hydroxylase (and sterol 27-hydroxylase), showed the OH-groups to be in close proximity of each other, forming a hydrophilic microenvironment separate from a relatively hydrophobic backbone. Such a structure could provide a hydrophilic anchor for binding to a cellular component, such as the putative bile acid receptor described, subsequently followed by a transcriptional event, either directly or indirectly, within the promoter region of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes. Deviation from such bile acid

structure, thereby disrupting the hydrophilic component and possible binding site, led to intermediate ability, or no ability at all to suppress cholesterol 7α-hydroxylase. In line with this scheme of events, use of a cholesterol 7α-hydroxylase-promoter-CAT-reporter construct harboring the putative BARE, for transient expression experiments, exhibited specificity with respect to the bile acid added to transfected cells. Addition of cholic acid (3α,7α,12α) suppressed CAT-activity in these cells (-70%), while ursocholic acid (3α,7β,12α) did not.

Knowledge of specific structural elements involved in biofeedback by bile acids, could prove useful for the development of certain bile acid analogues. Acting as a possible receptor antagonists, such compounds could render the resulting complex inactive towards promoter regions of cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes, resulting in enhanced bile acid synthesis. In this respect, it is interesting to note that some bile acids tested appeared to elicit a stimulatory effect on expression of cholestesterol 7α-hydroxylase.

Other prospects may lie in the use of bile acids as targeting molecules for drugs. Cholic acid has been used as a carrier-molecule to specifically deliver 'vastatins' to the liver, as a means to limit side-effects of these cholesterol synthesis inhibitors in extrahepatic tissue. Cholic acid is a potent suppressor of bile acid synthesis, however, and thus the carrier-molecule may result in unwanted side-effects. An interesting alternative would be to use allo-cholate, which has a comparable HI_z, and presumably equal uptake characteristics, but shows no effect on cholesterol 7α-hydroxylase.

Heterogeneity within the liver acinus and implications for regulation of bile acid synthesis

As outlined in chapter 5, the key-enzymes in both the neutral- and alternative pathways to formation of bile acids, are heterogeneously distributed within the liver lobulus, being most abundant in the pericentral zone under normal conditions (pericentral (PC):periportal (PP)-ratio's of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and mRNA expression of 7.9 and 9.9, and 2.9 and 2.5, respectively), parallel to bile acid synthetic capacity (4-fold higher in the PC zone). Opposite localization of cholesterol synthetic enzyme, HMG-CoA reductase, indicates that direct linkage between both synthetic and catabolic pathways is most probably limited to only a few cells within the lobulus. From these results, it may be concluded that under normal circumstances, the liver utilizes preformed cholesterol for bile acid synthesis. Both cholesterol 7α-hydroxylase and sterol 27-hydroxylase metabolize lipoprotein-cholesterol, not because of an intrinsic preference for serum-derived cholesterol, but simply because *de novo* cholesterol is synthesized elsewhere, enabling direct adaptation of bile acid synthetic rate to fluctuation in serum cholesterol levels. It was further shown that such heterogeneity results from a higher transcriptional activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes, and possibly of enhanced mRNA stability (cholesterol 7α-hydroxylase), within the pericentral hepatocytes.

Localization of both key-enzymes in the pericentral zone of the rat liver lobulus, inversely correlated to a strong lobular bile acid gradient, provides *in vivo* validation for the dose-dependent down-regulation of both enzymes by bile acids in cultured rat hepatocytes, as described in chapters 2,3 and 4. The lobular architecture of the liver thus provides the means for a dynamic regulation of bile acid synthesis. Variation in the portal bile acid load will determine the extent of the bile acid gradient, and the number of hepatocytes over the portocentral axis involved in uptake of bile acids, thereby ultimately suppressing bile acid synthesis within a large or small part of the liver lobulus. As demonstrated by administration of the bile acid resin colestid to rats, the enhanced bile acid synthetic capacity and need for precursor cholesterol as substrate for the pathway, resulted in extension of expression of the bile acid synthetic enzymes up to the portal area. Thus, a direct link between cholesterol synthetic and bile acid synthetic processes is provided. The increase resulted from enhanced transcriptional activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes along the entire portocentral axis, and in expression of activity and mRNA for these enzymes within a large lobular section. This phenomenon was further demonstrated by *in situ* hybridization techniques, showing predominant pericentral expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNAs in control rats, which were augmented and extended towards the periportal area upon administration of colestid.

Such a mechanism limits the success of bile acid sequestrants in lowering serum cholesterol, and explains the greater potential of combining resins with inhibitors of cholesterol synthesis (e.g. vastatins), in which case the possibility of a direct link between *de novo* cholesterol synthesis and (enhanced) bile acid synthesis is prevented.

Another finding was the periportal localization of mRNA and transcriptional activity of the lithocholic acid 6β-hydroxylase gene. From a physiological point of view, such distribution may be explained by the fact that periportal hepatocytes experience the highest bile acid load, and involvement of the latter enzyme in metabolism of lithocholic acid, suggests that periportal localization of lithocholic acid 6β-hydroxylase may serve to protect the liver from hepatotoxicity of certain incoming bile acids. The mRNA and transcriptional activity is decreased upon treatment of rats with colestid, suggesting regulation of the enzyme by bile acid load in an opposite fashion, as compared with either cholesterol 7α-hydroxylase or sterol 27-hydroxylase.

Regulation of bile acid synthesis by substrate cholesterol

As discussed in chapter 6, supply of either endogenous or exogenous cholesterol to cultured rat hepatocytes, led to stimulation of bile acid synthesis. From the lipoproteins used in the study, both βVLDL and LDL stimulated bile acid synthesis, but only βVLDL enhanced cholesterol 7α-hydroxylase activity and mRNA expression dose-dependently, at the level of gene transcription (2-fold within 3 hours of incubation). No effect of lipoprotein-derived

cholesterol was found on expression of sterol 27-hydroxylase, indicating that both enzymes diverge in this respect. Responsiveness to βVLDL was found to reside within the 5'-proximal part of the cholesterol 7α-hydroxylase promoter. Therefore, the ultimate effects on cholesterol 7α-hydroxylase, exerted by cholesterol and bile acids, respectively, are determined at the transcriptional level by the relative amounts of these compounds reaching the liver from the flux of nutrients absorbed in the ileum.

The results indicate that an increased supply of potentially atherogenic particles, is efficiently routed to bile acid synthesis within the hepatocyte. Such a mechanism is important to prevent prolonged circulation of these lipoproteins in blood, and storage of cholesterol in peripheral tissue and vessel walls. An entirely new aspect is formed by the contention that βVLDL, and possibly other remnant-like particles (CR, VLDL-remnants, IDL), have a potency to stimulate expression of cholesterol 7α-hydroxylase, in addition to supplying substrate for the bile acid synthetic pathway.

Effect of insulin on bile acid synthesis

Bile acid pool size, and biliary bile acid excretion is increased during uncontrolled maturity-onset diabetes mellitus in man, and in experimental diabetic animals. In chapter 7, the biochemical background of normalization of these parameters by insulin therapy, was investigated. Cultured rat hepatocytes treated with insulin showed a dose-dependent down-regulation of bile acid synthesis, as a result of suppression of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, mRNA, and gene transcription (-50%). In addition, administration of insulin was shown to increase HMG-CoA reductase gene expression and mRNA levels (5-fold).

These results may seem puzzling, while, allthough the underlying molecular mechanism provides an explanation for lowering of bile acid synthesis by insulin in diabetic subjects, the results appear paradoxical to reported lowering of serum cholesterol levels by the hormone. Insuline therapy elicits multiple effects, however, and decrease in bile acid formation by the hormone, is apparently overruled by a concomitant decrease of VLDL production, and restored peripheral levels of lipoprotein lipase, as well as stimulated receptor-mediated uptake of resulting remnants by the liver. Possible adverse effects of insulin-treatment remain, however, as decrease in bile acid synthesis combined with increased liver-cholesterol levels will cause supersaturation of bile, leading possibly to enhanced gallstone formation.

SAMENVATTING

Quantitatief de belangrijkste route voor uitscheiding van cholesterol, vindt plaats via galzuur-afhankelijke biliaire excretie, of via de synthese van galzuren, een lever-specifiek proces. Een lage synthese capaciteit voor galzuren wordt gezien als een onafhankelijke risico-factor voor het optreden van coronaire hartziekten. Bovendien is progressie van atherosclerose en coronaire sterfte, in patienten die lijden aan een heterozygote vorm van Familiaire Hypercholesterolemie (FH), negatief gecorreleerd aan het niveau van galzuursynthese. Anderzijds neemt galzuursynthese capaciteit af met de leeftijd, terwijl serum LDL-waarden toenemen.

Vorming van galzuren, en vooral mechanismen betrokken bij de regulatie van deze metabole route, zijn daarom een belangrijk aandachtsgebied binnen het hart- en vaatziekten onderzoek, en een mogelijk instrument voor cholesterol-verlagende therapie.

Negatieve terugkoppeling door galzuren

Galzuren worden gevormd in de lever parenchymcellen (hepatocyten), via een reeks van verschillende metabole reakties. Galzuursynthese wordt gereguleerd via een aantal mechanismen, waarvan de meest belangrijke wordt bewerkstelligd door galzuren zelf. Na excretie in de darm wordt het grootste gedeelte van de galzuren, tesamen met andere voedingsfactoren, weer geabsorbeerd, en keert terug naar de lever via portaal bloed, resulterend in onderdrukking van galzuursynthese in dit orgaan.

In hoofdstuk 2 is het moleculair mechanisme, dat ten grondslag ligt aan deze vorm van regulatie, nader onderzocht. Gebruik makend van monolaag-culturen van primaire hepatocyten van de rat, werd een dosis-afhankelijke onderdrukking van galzuursynthese capaciteit waargenomen, na toevoeging van taurocholaat (TCA) aan het medium. Maximale onderdrukking vondt plaats bij een concentratie van 50 μM, wat binnen de grenzen van galzuur concentraties in portaal bloed ligt. Onderdrukking *in vitro* liet bovendien duidelijk zien dat remming door galzuren het gevolg is van een direct effect op de hepatocyt. Het idee dat een mogelijke darm-factor, afhankelijk van galzuren voor opname en transport naar de lever, verantwoordelijk zou zijn voor onderdrukking van galzuursynthese, lijkt daarom niet aannemelijk.

Regulatie vondt plaats op het niveau van het snelheids-bepalend enzym, cholesterol 7α-hydroxylase, via een effect op zowel het transcriptionele- als ook het post-transcriptionele niveau. Afgezien van een nog-te-identificeren galzuur-receptor, verantwoordelijk voor een

transcriptionele gebeurtenis, kunnen galzuren ook een effect hebben op stabiliteit van de cholesterol 7α-hydroxylase boodschapper-RNA (mRNA). Een schijnbare half-waarde tijd voor zowel het enzym, als ook de mRNA, kon worden berekend uit incubatie studies met TCA. Deze relatief korte half-waarde tijd (4 uur) hangt mogelijk samen met de aanwezigheid van meerdere AU-rijke sequenties binnen het 3'-niet-coderende-gebied (UTR) van de cholesterol 7α-hydroxylase mRNA. Dergelijke codes zijn gecorreleerd aan gevoeligheid voor endonucleases, en een snelle degradatie van mRNA. Indirecte bewijzen voor een post-transcriptioneel effect van galzuren, zoals hierboven beschreven, zijn o.a. het verschil in effect van TCA op transcriptie (40% onderdrukking), en mRNA niveau (-90%). Behandeling van ratten met het galzuur-bindende cholestyramine liet bovendien een veel sterkere inductie van cholesterol 7α-hydroxylase enzym aktiviteit en mRNA-niveau zien (> 10-voudig), vergeleken bij stimulatie van de transcriptie-snelheid van het gen (4-voudig).

De moleculaire biologie zou een middel kunnen vormen voor een serum cholesterol-verlagende strategie. Een constante, en hogere galzuursynthese capaciteit kan worden gerealiseerd door verhoging van expressie en stabiliteit van de cholesterol 7α-hydroxylase mRNA. Introductie in de lever van een cholesterol 7α-hydroxylase cDNA construct, waarbij de galzuur-gevoelige sequenties ontbreken, en vervangen zijn door een andere, sterke en lever-specifieke promoter, resulteert in constante en hoge expressie van de mRNA. Bovendien kan de stabiliteit ervan worden bevorderd door de AU-rijke UTR te vervangen door een stabielere 3'-staart. Een dergelijke benadering kan een goede basis zijn voor gen-therapie, met als doel-orgaan de lever, waarbij de hogere galzuursynthese capaciteit naar verwachting zou resulteren in een toegenomen vraag naar (exogeen) cholesterol.

Het moet echter worden benadrukt dat verhoging van de hoeveelheid aan galzuren, ook al geeft dit een verhoogde faecale uitscheiding van galzuren en cholesterol, ook tot gevolg heeft dat er meer dietair cholesterol en vetten kunnen worden opgenomen. Bovendien treedt verhoging op van de vrije galzuur-concentratie in de darm. Dit laatste heeft consequenties, daar de detergerende aktiviteit van galzuren zo het darm-epitheel kan beschadigen. Quantitatieve en efficiente uitscheiding van cholesterol via galzuursynthese blijft daarom afhankelijk van voldoende faecale excretie van galzuren, mogelijk door middel van combinatie therapie met galzuur-binders.

Betrokkenheid van sterol 27-hydroxylase bij regulatie van galzuur synthese

Terwijl onderzoek in het verleden veelal gericht geweest is op cholesterol 7α-hydroxylase als belangrijk punt van regulatie van galzuursynthese door diverse mediatoren, zijn andere enzymen grotendeels aan de aandacht ontsnapt. Een belangrijke rol voor een alternatieve route van galzuur-vorming was al gerapporteerd bij de mens *in vivo*, en in gecultiveerde humane- en rat-hepatocyten, maar over regulatie van deze route was tot nu toe niets bekend. De sterke onderdrukking van galzuursynthese door TCA, en het bestaan van de alternatieve

of '27-hydroxylase' route in het onderzochte cel-systeem, deed vermoeden dat de route tevens reguleerbaar moest zijn. In hoofdstuk 3 wordt aangetoond dat galzuren (zoals ook insuline (hoofdstuk 7)) ook aangrijpen op het sterol 27-hydroxylase, het enzym dat verantwoordelijk is voor de eerste stap in alternatieve omzetting van cholesterol. Regulatie vindt plaats op het niveau van enzym aktiviteit, mRNA, en transcriptie, parallel met een effect op cholesterol 7α-hydroxylase. De UTR van sterol 27-hydroxylase mRNA bevat echter geen AU-rijke sequenties, en laatstgenoemde mRNA vertoonde een schijnbare half-waarde tijd van 13 uur bij incubatie studies met 50 μM TCA. Het effect van TCA op transcriptie was echter vergelijkbaar (40% onderdrukking)

Behalve in de lever, wordt 27-hydroxycholesterol ook gesynthetiseerd in andere organen, en in sommige weefsels. Als regulator van zowel LDL-receptor expressie, als ook die van HMG-CoA reductase aktiviteit, is dit intermediair in galzuur-vorming, en het enzym betrokken bij de synthese ervan, een mogelijk aangrijpingspunt voor toekomstig onderzoek. Regulatie van sterol 27-hydroxylase zou een twee-ledig doel dienen: regulatie van extrahepatische cholesterol opname en opslag, alsmede endogene cholesterol synthese, als ook regulatie van de flux van cholesterol dat de eerste en toegewijde stap naar (alternatieve) galzuurvorming ondergaat, buiten de lever. Stimulatie van extrahepatische enzymen, betrokken bij galzuursynthese, gevolgd door een snelle klaring door de lever van resulterende oxysterolen, kan zo het potentieel atherogene cholesterol weg-dirigeren van opslag (o.a. in de vaatwand), naar degradatie routes in de lever.

Ook al biedt reguleerbaarheid van sterol 27-hydroxylase perspectieven voor gen-therapie, zoals in het geval van cholesterol 7α-hydroxylase, zal stimulatie van expressie van sterol 27-hydroxylase alléén, resulteren in relatieve verhoging van chenodeoxycholaat synthese, welke een hepatotoxisch galzuur betreft.

Strukturele aspekten van galzuren betrokken bij regulatie van galzuursynthese

In hoofdstuk 4 wordt aandacht besteed aan mogelijke structurele elementen van galzuren die betrokken zijn bij galzuur-gemedieerde terugkoppeling. Hoewel galzuren zowel cholesterol 7α-hydroxylase als ook sterol 27-hydroxylase aktiviteit onderdrukken, bleek reeds uit hoofdstuk 2 en 3, dat niet alle galzuren even aktief waren in dit opzicht. Analyse van het effect van 27 verschillende galzuren op expressie van het cholesterol 7α-hydroxylase mRNA, werd daarom ondernomen. Deze varieerden in hydrofobiciteit index (HI_x), en in aantal, positie, en orientatie (α/β) van de verschillende OH-groepen op de sterole kern. Wanneer vervolgens remmende capaciteit werd uitgezet tegen de HI_x van het galzuur, bleek dit slechts een geringe correlatie coefficient op te leveren (r= 0.61). Verdere analyse van de drie-dimensionale struktuur door middel van computer-modellering, van cholaat (3α,7α,12α), een potente remmer van cholesterol 7α-hydroxylase (en van sterol 27-hydroxylase), liet zien dat de verschillende OH-groepen op de steroide kern van dit molekuul zich in elkaars nabijheid

bevinden. Zo wordt een hydrofiel compartiment gecreeerd, binnen een overigens hydrofoob molekuul. Het is mogelijk dat een dergelijke struktuur een hydrofiel anker vormt voor binding aan een cellulaire component, zoals de voorgestelde galzuur-receptor. Een dergelijke binding kan dan gevolgd worden door een transcriptioneel effect, binnen de promoter regio's van beide sleutel-enzymen. Afwijking van een dergelijke strukturering van een galzuur, door positionering van OH-groepen anders dan in elkaars nabijheid, resulteerde in een intermediair effect op cholesterol 7α-hydroxylase mRNA expressie, of zelfs in afwezigheid van remmende capaciteit. Transiente expressie experimenten werden uitgevoerd, waarbij primaire hepatocyten werden getransfecteerd met een cholesterol 7α-hydroxylase promoter-CAT-reporter-construct, bevattende de galzuur-gevoelige sequentie (of BARE). Ook in dit geval bleek er specificiteit voor galzuur-struktuur te zijn. Toevoeging van cholaat (3α,7α,12α) onderdrukte CAT-aktiviteit, terwijl additie van ursocholaat (3α,7β,12α) geen effect vertoonde.

Kennis omtrent specifieke strukturele elementen van galzuren, die betrokken zijn bij negatieve terugkoppeling op sleutel-enzymen in de galzuursynthese, kan van betekenis zijn voor de ontwikkeling van bepaalde galzuur-analoga. Als mogelijke receptor antagonisten, zouden dergelijke stoffen de negatieve interactie met de promoter-regio's van zowel cholesterol 7α-hydroxylase en sterol 27-hydroxylase kunnen verhinderen, en zo galzuursynthese capaciteit verhogen.

Andere mogelijkheden liggen in het gebruik van galzuren om bepaalde pharmaca gericht af te leveren. Zo wordt cholaat gebruikt als drager-molekuul om 'vastatines' (cholesterol synthese remmers) gericht naar de lever te vervoeren, zodat bijwerkingen in de rest van het lichaam worden geminimaliseerd. Cholaat is een potente remmer van galzuursynthese, en dus kan de drager in dit geval tot ongewenste neven-effecten leiden. Een alternatief zou zijn allocholaat, dat een vergelijkbare HI_x bezit, en vermoedelijk vergelijkbare opname-karakteristieken, maar dat geen effect op cholesterol 7α-hydroxylase mRNA vertoont.

Heterogeniteit in de lever lobulus en betekenis voor de regulatie van galzuursynthese

Zoals uiteen gezet in hoofdstuk 5, vertonen de sleutel-enzymen in zowel de neutrale als ook de alternatieve route een heterogene verdeling binnen de lever lobulus. Expressie van cholesterol 7α-hydroxylase en sterol 27-hydroxylase aktiviteit en mRNA bevindt zich onder normale omstandigheden vooral in het pericentrale gebied (pericentraal (PC):periportaal (PP)-ratio van 7.9 en 9.9, respectievelijk 2.9 en 2.5), parallel met de galzuursynthese capaciteit in de lobulus (PC:PP-ratio van 4). Tegenovergestelde lokalisatie werd gevonden voor HMG-CoA reductase, wat suggereert dat een directe koppeling tussen cholesterol synthese en afbraak waarschijnlijk beperkt blijft tot enkele cellen in de lobulus. Hieruit kan tevens worden afgeleid dat onder normale omstandigheden, de lever voornamelijk gebruik maakt van exogeen cholesterol voor galzuursynthese. Beide galzuursynthese routes verbruiken serum-cholesterol, niet vanwege een expliciete voorkeur voor deze vorm van substraat, maar

omdat *de novo* cholesterol elders gesynthetiseerd wordt. Zodoende kan de lever direkt inspringen op fluctuaties in circulerende cholesterol niveau's.

Verder bleek dat de heterogene verdeling van cholesterol 7α-hydroxylase en sterol 27-hydroxylase aktiviteit en mRNA expressie binnen de lever lobulus het gevolg is van een verschil in transcriptionele aktiviteit van de corresponderende genen, tussen periportale en pericentrale zone (PC:PP-ratio's van 4.4 en 1.7, respectievelijk). In het geval van cholesterol 7α-hydroxylase, bleek mRNA-stabiliteit waarschijnlijk ook een rol te spelen.

Lokalisatie van beide sleutel-enzymen is negatief gecorreleerd met een sterke galzuur-*gradient* over de lever lobulus, wat een *in vivo* validering is voor de gevonden dosis-afhankelijke onderdrukking van beide enzymen in gecultiveerde rat-hepatocyten (hoofdstukken 2,3 en 4). De lobulaire architektuur geeft daarom de mogelijkheid van een 'dynamische' regulatie van galzuursynthese. Variatie in de galzuur concentratie in portaal bloed bepaalt uiteindelijk de lengte van de galzuur gradient, en het aantal hepatocyten over de portocentrale lengte-as dat betrokken is bij opname van galzuren, en dientengevolge onderhevig is aan galzuur-gemedieerde suppressie. Zoals is aangetoond met de toediening van het galzuur-bindende colestid aan ratten, resulteerde de verhoogde galzuursynthese capaciteit (door verminderde onderdrukking) en vraag naar substraat voor de synthese route, in verhoogde expressie van zowel cholesterol 7α-hydroxylase als ook sterol 27-hydroxylase aktiviteit over een groot gedeelte van de lobulus. Hierdoor wordt tevens de mogelijkheid geschapen van een directe verbinding tussen cholesterol synthese en afbraak (galzuursynthese). Verhoogde lobulaire expressie was het gevolg van een toename in transcriptionele aktiviteit van de genen coderend voor cholesterol 7α-hydroxylase en sterol 27-hydroxylase, binnen een brede lobulaire zone. Met behulp van *in situ* hybridizatie technieken werd dit fenomeen bevestigd. Een sterke en discrete pericentrale expressie werd waargenomen voor cholesterol 7α-hydroxylase mRNA in controle levers, als ook voor sterol 27-hydroxylase mRNA, hoewel minder sterk en discreet. Beide mRNAs kwamen sterker tot expressie in levers van colestid-behandelde ratten, en expressie was zichtbaar over een breed gebied binnen de lobulus.

Dynamische expressie van galzuursynthese, en de sleutel enzymen daarbij betrokken, limiteert het succes van galzuurbinders in verlaging van serum cholesterol concentraties. Het verklaart tevens waarom kombinatie therapie met remmers van *de novo* cholesterol synthese (b.v. vastatines) wel tot verlaging leidt. De mogelijkheid om gebruik te maken van nieuw-gesynthetiseerd cholesterol, noodzakelijk om aan de vraag naar substraat te voldoen bij verhoogde galzuursynthese capaciteit, wordt zo verhinderd, waardoor verbruik van exogeen cholesterol zal stijgen.

Een andere vinding binnen deze studie betrof de periportale lokalisatie van mRNA en transcriptionele aktiviteit voor lithocholaat 6β-hydroxylase. Vanuit fysiologisch oogpunt kan een dergelijke lobulaire verdeling worden verklaard uit het feit dat juist de periportale

hepatocyten blootgesteld worden aan hoge galzuur concentraties. Betrokkenheid van het enzym bij metabolisme van lithocholaat, een zeer hydrofoob galzuur, doet vermoeden dat periportale lokalisatie van het enzym de lever moet behoeden voor hepatotoxiciteit van binnenkomende galzuren. Na behandeling van ratten met colestid nam de mRNA expressie en transcriptionele aktiviteit van het lithocholaat 6β-hydroxylase gen af, wijzend op een tegenovergestelde (positieve) regulatie door galzuren in vergelijking met de situatie beschreven voor cholesterol 7α-hydroxylase en sterol 27-hydroxylase.

Regulatie van galzuursynthese door substraat cholesterol

Aanbod van substraat, hetzij endogeen of exogeen van aard, gaf stimulatie van galzuursynthese in gecultiveerde rat-hepatocyten, zoals beschreven in hoofdstuk 6. Van de lipoproteinen die in de studie werden gebruikt, lieten zowel incubatie met βVLDL als ook LDL een verhoging van galzuursynthese zien. Echter, aanbod van βVLDL had ook tot gevolg dat cholesterol 7α-hydroxylase enzym aktiviteit en mRNA expressie dosis-afhankelijk toenam. Deze toename bleek het gevolg van stimulatie van transcriptionele aktiviteit van het gen (2-voudige toename binnen 3 uur). Sterol 27-hydroxylase werd op geen enkele wijze beinvloed door lipoproteine-cholesterol, wat aangeeft dat beide enzymen uiteenlopen in dit opzicht. Gevoeligheid voor βVLDL bleek gelokaliseerd te zijn in het 5'-proximale gedeelte van de cholesterol 7α-hydroxylase promoter. Het tegenovergestelde effect van cholesterol en galzuren op cholesterol 7α-hydroxylase, impliceert dat uiteindelijk expressie *in vivo* wordt bepaald door de relatieve hoeveelheden van beide effectoren, in de flux van nutrienten die de lever bereikt vanuit de darm.

De resultaten geven aan dat een toename in het aanbod van potentieel atherogene deeltjes in serum, kan worden opgevangen door een efficiente opname door de lever en koppeling aan afbraak middels galzuursynthese. Een dergelijk mechanisme is belangrijk om opslag van cholesterol, afkomstig van circulerende lipoproteinen, in de vaatwand te kunnen minimaliseren. Een nieuw aspekt wordt gevormd door het gegeven dat βVLDL, en mogelijk ook andere 'remnant' deeltjes (CR, VLDL-remnants, IDL), expressie van cholesterol 7α-hydroxylase kunnen verhogen. Zo dienen deze deeltjes, behalve louter als substraat voor galzuursynthese, om de capaciteit van de afbraak route te stimuleren in de lever.

Effect van insuline op galzuursynthese

De hoeveelheid van galzuren, en biliaire galzuur excretie, is verhoogd in patienten met ongecontroleerde vorm van 'maturity-onset' diabetus mellitus, en in experimentele diabetus in dieren. In hoofdstuk 7 is de biochemische achtergrond voor normalisering van deze waarden door insuline onderzocht. Gecultiveerde rat-hepatocyten werden behandeld met insuline, resulterend in een dosis-afhankelijke regulatie van galzuursynthese als gevolg van onderdrukking van enzym aktiviteit, mRNA niveau's, en transcriptionele expressie van zowel

cholesterol 7α-hydroxylase als sterol 27-hydroxylase (-50%). Bovendien gaf insuline een 5-voudige stimulatie van het HMG-CoA reductase mRNA niveau, en van de transcriptionele aktiviteit van het gen.

Deze resultaten lijken paradoxaal, want hoewel ze de verklaring geven voor verlaging van galzuursynthese capaciteit bij diabetici door het hormoon, heeft insuline tot gevolg dat serum cholesterol niveau's dalen bij deze patienten. Insuline vertoont echter meerdere effecten, die tesamen toch resulteren in een verlaagde concentratie aan circulerend cholesterol. Zo neemt de VLDL-productie af, neemt de receptor-gemedieerde opname van lipoproteinen toe, en worden perifere niveau's van lipoproteine lipase hersteld na insuline behandeling, waardoor lipoproteine-deeltjes makkelijker worden geklaard door de lever. Nadelige effecten van insuline zouden echter kunnen zijn dat verhoogde opname van exogeen cholesterol door de lever, in kombinatie met verlaging van galzuursynthese, super-verzadiging van gal met cholesterol tot gevolg heeft, uiteindelijk resulterend in een verhoogde kans op galsteen-vorming.

ABBREVIATIONS

ACAT	acyl-coenzyme A:cholesterol-acyltransferase
ACTH	adrenocorticotrophine
BARE	bile acid response element
BARP	bile acid responsive protein
BTE	basic transcription element
CAT	chloramphenicol acetyltransferase
CBD	complete biliary diverted
CE	cholesteryl ester
C/EBP	CCAAT-enhancer binding protein
CHD	coronary heart disease
CM	chylomicron
CR	chylomicron remnant
CTX	cerebrotendinous xanthomatosis
DBP	albumin promoter D-site binding protein
DMSO	dimethylsulfoxide
DTT	dithiothreitol (Cleland's reagent)
EHC	enterohepatic circulation
FH	familial hypercholesterolemia
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDL	high-density lipoprotein
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI_x	hydrophobicity index
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A reductase
HNF	hepatic nuclear factor
HRE	hormone regulatory element
IDL	intermediate-density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low-density lipoprotein
LFB-1	liver factor B-1
LPDS	lipoprotein deficient serum
MPS	macrophage-phagocyte system
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue)
NP-40	octylphenoxypolyethoxyethanol
PBS	phosphate buffered saline

PMSF	phenazine methosulfate
PC	pericentral
PP	periportal
RT-PCR	reversed transcriptase-polymerase chain reaction
SDS-PAGE	SDS-polyacrylamide-gel-electrophoresis
SRE	sterol regulatory element
TCA	taurocholic acid
TG	triglycerides
TRE	thyroid regulatory element
UTR	untranslated region
VLDL	very-low-density lipoprotein
βVLDL	β-migrating VLDL

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CURRICULUM VITAE

Jaap Twisk werd geboren op 10 December 1965 te Harlingen. Na de lagere school, en een deel van de middelbare school te hebben doorlopen aan de Rygårds Skole te Hellerup, Denemarken, werd in 1983 het diploma ongedeeld V.W.O. behaald aan de Rijksscholengemeenschap te Oud-Beijerland. In 1983 werd tevens een begin gemaakt aan de studie Biologie aan de Rijksuniversiteit te Leiden. Het propaedeutisch examen werd in 1984 afgelegd. Vervolgens werd het doctoraalexamen, met als specialisatie Biochemie, in september 1989 behaald. Het laatste deel van deze periode bestond uit: een 10 maanden onderzoeksperiode naar bacteriele zuivering van afvalwater (Prof.dr. J.G. Kuenen, Microbiologie/Enzymologie, Technische Universiteit Delft), een hoofdvak van 12 maanden moleculaire biologie, waarin naar regulatie van methylering van het 3'-uiteinde van *E. coli* 16S rRNA werd gekeken (J. Bact. 1989; 171: 4002-4008) (Prof.dr. P.H. van Knippenberg en Dr. B. van Gemen, Vakgroep Biochemie, Rijksuniversiteit Leiden), en 5 maanden Gistgenetica (Rijksuniversiteit Leiden/E.C. Slater Institute for Biochemical Research, Amsterdam).

Van 15 februari 1990 tot 15 februari 1994 was hij als Assistent-in-Opleiding werkzaam aan de Rijksuniversiteit te Leiden, Faculteit Geneeskunde, en gedetacheerd bij het Gaubius Laboratorium TNO-PG, te Leiden. Gedurende deze periode werd onder leiding van dr. H.M.G. Princen het in dit proefschrift beschreven onderzoek verricht. Per januari 1995 zal hij als postdoctoraal medewerker worden aangesteld bij de vakgroep Biochemie van de University of Madison-Wisconsin, College of Agricultural and Life Sciences, Madison, Wisconsin, USA, onder leiding van dr. A.D. Attie, hoofd van de afdeling Lipoproteine Metabolisme.

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