review

Role of cholesterol in regulating apolipoprotein B secretion by the liver

Gilbert R. Thompson,[1] Rossitza P. Naoumova, and Gerald F. Watts*

MRC Lipoprotein Team, Clinical Sciences Centre, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 ONN, United Kingdom, and University Department of Medicine,* University of Western Australia, Perth, Australia

Abstract This review examines the evidence that the supply of cholesterol available for incorporation into nascent lipoprotein particles exerts a regulatory influence on apolipoprotein (apo) B secretion by the liver. Support for this hypothesis comes both from in vitro experiments and from recent observations in normal subjects and patients with dyslipidemia associated with familial hypercholesterolemia, obesity, non-insulin dependent diabetes mellitus, growth hormone deficiency and cholesteryl ester storage disease. The findings do not negate a role for triglyceride synthesis in determining apoB secretion in very low density lipoprotein, but the inhibitory effects on the latter process of pharmacological blockade of cholesterol synthesis or esterification suggest that it is conditional upon an adequate supply of cholesteryl ester.–**Thompson, G. R., R. P. Naoumova, and G. F. Watts.** Role of cholesterol in regulating apolipoprotein B secretion by the liver. *J. Lipid Res.* 1996. **37:** 439–447.

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Factors that determine the rate of secretion of very low density lipoprotein (VLDL) by the liver arguably play a key role in atherosclerosis in that the major products of VLDL metabolism are potentially atherogenic. This is exemplified by the predisposition to premature coronary heart disease (CHD) of subjects with type III hyperlipidemia and familial hypercholesterolemia (FH), whose plasma contains excessive amounts of VLDL remnants and low density lipoprotein (LDL) particles, respectively.

Plasma VLDL consists of 90% lipid, of which 56% is triglyceride, 19% is phospholipid, and 17% is cholesterol; just over half of the latter is esterified (1). The remaining 10% of VLDL consists of protein, mainly apolipoprotein (apo)B-100 and apoC-III. As apoC-III is largely acquired after entry of VLDL into plasma, it is unlikely to be involved in VLDL synthesis and secretion. Most of the data on the latter topic are derived from in vitro studies using either perfused liver preparations, freshly isolated hepatocytes, or various cell lines, including HepG2 cells. However, the suitability of HepG2 cells as a model has been questioned on account of their relative lack of smooth endoplasmic reticulum (SER), which is thought to play a crucial role in VLDL formation (2).

This review examines published data on the regulation of VLDL secretion based on in vitro experiments as well as from investigations performed in vivo, with special emphasis on the pathophysiological and pharmacological insights gained from recent studies involving simultaneous measurement of cholesterol synthesis and VLDL-apoB secretion in humans. The findings provide a new perspective of the role of cholesterol, supplied either by synthesis or recycling, in regulating VLDL secretion by the liver.

In vitro models of apoB secretion

Almost 20 years ago Goh and Heimberg (3) showed in the perfused rat liver that stimulation of hydroxymethylglutarylcoenzyme A (HMG-CoA) reductase activity and output of cholesterol were both proportional to the increase in triglyceride secretion engendered by adding free fatty acid (FFA) to the perfusion medium. In contrast, prior feeding with the HMG-CoA reductase inhibitor lovastatin markedly reduced the secretion of VLDL triglyceride into the perfusate (4). This inhibition could be reversed by inclusion of 0.1% cholesterol in the diet of the lovastatin-fed rats. Sub-

Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; apo, apolipoprotein; CHD, coronary heart disease; ER, endoplasmic reticulum; FFA, free fatty acid; FH, familial hypercholesterolemia; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; MTP, microsomal triglyceride transfer protein; MVA, mevalonic acid; NIDDM, non-insulin dependent diabetes mellitus; o,p'DDD, ortho, para, dichlorodiphenyl dichloroethane; SER, smooth endoplasmic reticulum; VLDL, very low density lipoprotein.

[1]To whom correspondence should be addressed.

sequently it was shown that reduction of VLDL secretion by lovastatin in the absence of supplementary cholesterol was not due to inhibition of triglyceride synthesis (5). More recent studies by the same group showed that prior feeding of rats with cholesterol stimulated VLDL triglyceride secretion by their perfused livers (6). These authors concluded that the synthesis and transport of VLDL triglyceride and cholesteryl ester were interdependent.

The influence of these non-polar or neutral lipids on apoB secretion has been the subject of numerous studies in cultured cells. Stimulation of apoB secretion by addition of oleic acid to the incubation medium has been demonstrated both in HepG2 cells (7) and in rat hepatoma cells (8). In both instances this effect was mediated by decreased intracellular degradation of apoB, not by increased apoB synthesis. Other studies in HepG2 cells suggested that oleate acts by promoting triglyceride synthesis and that this action is independent of the rate of intracellular synthesis of cholesterol and its subsequent esterification by acyl coenzyme A cholesterol acyltransferase (ACAT). Thus, neither an HMG-CoA reductase inhibitor (9) nor an ACAT inhibitor (10) prevented oleate-induced apoB secretion whereas this effect was blocked by an inhibitor of fatty acyl coenzyme A synthase (10).

Contrasting results were obtained, however, by Cianflone et al. (11) who showed marked decreases in oleate-stimulated apoB secretion in HepG2 cells in the presence of inhibitors of either HMG-CoA reductase or ACAT. These authors concluded that synthesis of cholesteryl ester rather than triglyceride was the prime regulator of apoB secretion. Independent confirmation of this conclusion has come from experiments in which fenofibrate was incubated with HepG2 cells; the resultant decreases in cholesteryl ester synthesis and apoB secretion occurred long before there was any reduction in triglyceride synthesis (12). However, recent studies from the Montreal group suggest that apoB secretion correlates better with the total mass of cholesteryl ester present within HepG2 cells than with the rate of cholesteryl ester synthesis (13). These data are in keeping with an earlier study which showed that incubating HepG2 cells with 25-hydroxycholesterol both increased their cholesteryl ester content and enhanced their secretion of apoB (14).

Although there may be conflicting evidence in HepG2 cells that cholesterol plays an active role in regulating apoB secretion, in vitro data using other models provide support for this concept. Addition of cholesterol to cultured human hepatocytes increased their rate of secretion of apoB, mainly in the form of VLDL (15). Similarly, addition of LDL to rabbit hepatocytes resulted in closely correlated increases in cholesteryl ester content and apoB secretion, the latter again reflecting decreased intracellular degradation of apoB (16). The opposite effect was achieved by the addition of the HMG-CoA reductase inhibitor pravastatin, which inhibited apoB secretion in hepatocytes cultured from both normal and Watanabe rabbits, which lack LDL receptors.

Non-rodent models of apoB secretion

Additional evidence regarding the role of cholesterol, specifically cholesteryl ester, in regulating apoB secretion has come from studies of apoB turnover in minipigs (17), in which treatment with an ACAT inhibitor decreased apoB secretion by 65%. This effect was attributed to inhibition of cholesteryl ester synthesis as the ACAT inhibitor used had no effect on triglyceride metabolism.

In another, more recent study, livers from cholesterol-fed African green monkeys were perfused with various ACAT inhibitors (18). The results showed a close correlation between the consequent decreases in secretion of cholesteryl ester and apoB in the perfusate, effects that were most marked in particles of $d < 1.006$ g/ml. In the studies with the most selective of the ACAT inhibitors (CI-976) there was no change in triglyceride secretion, despite addition of FFA to the perfusion medium. These results suggest that an adequate supply of cholesteryl ester is a necessary prerequisite for VLDL secretion, presumably by playing an essential role in the formation or stabilization of the lipid core of nascent particles.

Mechanisms whereby neutral lipids regulate apoB secretion

The relevance of the nature and availability of neutral lipids in influencing apoB secretion has been the subject of two contrasting reviews as to the relative importance of triglyceride (19) and cholesteryl ester (20) in this respect. However, recent data on the role played by microsomal triglyceride transfer protein (MTP) in the assembly of VLDL particles may help to reconcile these opposing views.

The discovery that MTP was absent from the liver of individuals with abetalipoproteinemia, due to a mutation of the gene encoding this protein, has shed new light on the mechanisms involved in VLDL formation (21). The absence of VLDL secretion in such individuals suggests that MTP normally plays a key role in this process, which takes place in the endoplasmic reticulum (ER) of the hepatocyte. As reviewed recently, translocation of nascent apoB across the ER membrane is a crucial step in the post-translational regulation of apoB production (22). Because it is so hydrophobic, apoB must associate with lipids either in the ER or in nascent lipoprotein particles adjacent to the ER. Failure to asso-

ciate with lipid results in degradation of apoB by proteolysis, a process which possibly occurs on the cytoplasmic side of the ER (23).

MTP catalyzes the movement of triglyceride and cholesteryl ester between phospholipid surfaces and is thus an important determinant of lipid transport within the lumen of the ER. In vitro, the relative rates of MTP-catalyzed transfer of triglyceride and cholesteryl ester are approximately 1:0.6 (24). As MTP-mediated association with neutral lipids protects apoB from intracellular degradation, increased availability of either triglyceride or cholesteryl ester for incorporation into lipoprotein particles would be expected to increase apoB secretion by the liver. As illustrated in **Fig. 1** the rate of transfer of neutral lipid may also determine whether the secreted particles exhibit the characteristics of VLDL or LDL (23).

Methods for measuring cholesterol synthesis and VLDL-apoB secretion

In a series of papers published during the last 2 years, we and our co-workers reported the results of studies based on the analysis of cholesterol synthesis and apoB secretion rates in both normal and hyperlipidemic subjects (25–31). Synthesis of cholesterol was assessed by measurement of the plasma concentration of two of its precursors, mevalonic acid (MVA) which is the product of an early step on the synthetic pathway, and lathosterol which marks a much later step.

A close correlation exists between the 9 A.M. plasma level of MVA after an overnight fast and the rate of cholesterol synthesis as determined by the sterol balance method (32). Plasma MVA levels also correlate closely with the rate of incorporation of deuterium into plasma free cholesterol (33) and with HMG-CoA reductase activity in human liver biopsies (34). Administration of the HMG-CoA reductase inhibitor lovastatin to hypercholesterolemic patients induced a 30% decrease in their 24-h urinary excretion of MVA (35). Urinary measurement of MVA provides an overall index of plasma levels, which fluctuate in a circadian manner, but is less sensitive in detecting acute changes in cholesterol synthesis. Current methods for quantifying MVA include radioenzymatic assay (35) and gas chromatography–electron capture mass spectrometry (36).

In an manner analogous to MVA, plasma lathosterol levels measured by gas chromatography correlate both with whole body cholesterol synthesis (37) and with HMG-CoA reductase activity in human liver (38). Furthermore, plasma lathosterol levels increase during

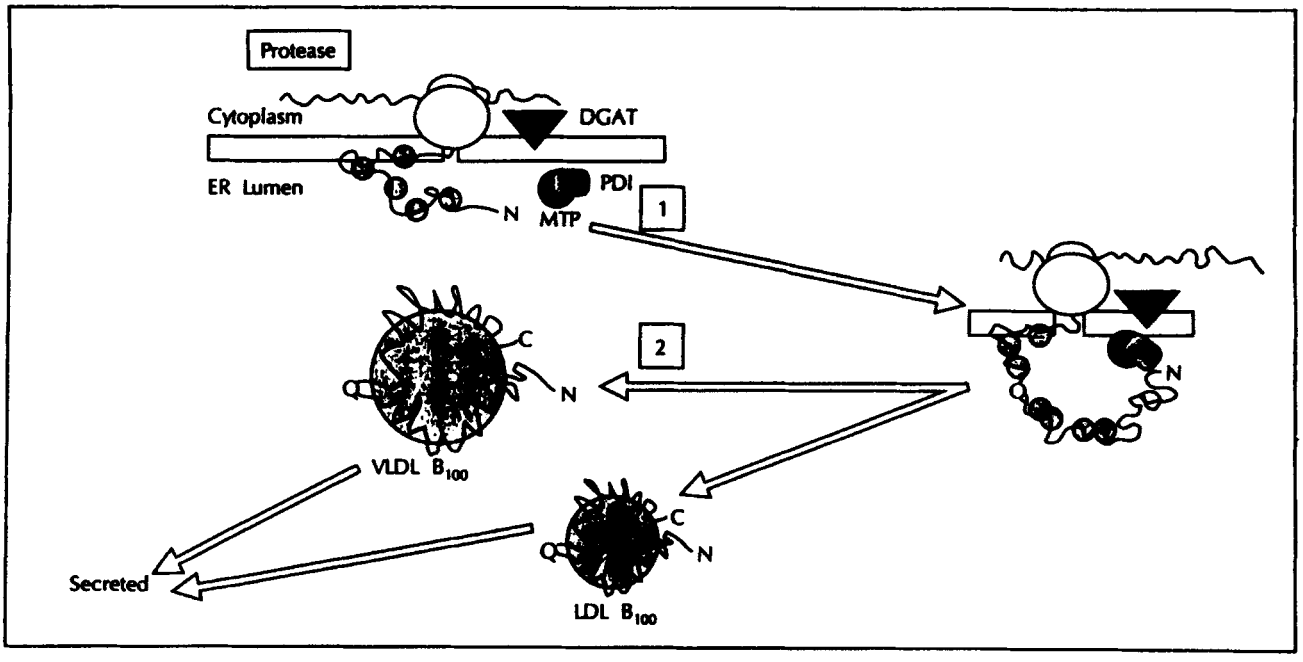

Fig. 1. Schematic diagram of steps involved in the hepatic formation of apoB-100-containing lipoproteins (reproduced with permission from Sparks, J. D., and C. E. Sparks. 1993. *Curr. Opin. Lipidol.* **4:** 177–186). 1. Co-translational association of sterol and newly formed triglyceride with apoB. 2. When the lipid core is large enough to bind the entire length of B-100, a lipoprotein particle forms. Low rates of neutral lipid transfer produce LDL particles (23–27 nm) whereas high rates favor production of VLDL particles (50–90 nm). DGAT, diglyceride acyl transferase; PDI, protein disulphide isomerase; MTP, microsomal triglyceride transfer protein.

treatment with cholestyramine, which is known to stimulate cholesterol synthesis, and decrease during treatment with an HMG-CoA reductase inhibitor (39). Disadvantages of plasma lathosterol compared with MVA are that it is transported mainly in LDL and is therefore subject to dietary (40) and therapeutic influences (25); this has led to the use of the lathosterol:cholesterol ratio rather than the plasma level of lathosterol as an index of cholesterol synthesis (41).

In comparison with traditional methods of estimating cholesterol synthesis, such as sterol balance, measurement of plasma or urinary MVA and the plasma lathosterol:cholesterol ratio have the advantages of convenience and of enabling rapid changes in synthesis to be monitored in relative terms. Their main disadvantage is that they provide only a semi-quantitative estimate of the absolute rate of cholesterol synthesis in the individual concerned.

Modern methods of measuring VLDL apoB secretion depend upon the use of stable isotopes and therefore require a mass spectrometer (42). This involves administering an intravenous bolus or primed constant infusion over 8 h of a stable isotope-labeled amino acid, such as [^{13}C]leucine, and determining isotopic enrichment of apoB in VLDL. With a primed constant infusion, isotopic enrichment of α-ketoisocaproic acid in plasma may be used as an index of the level of tracer in the precursor (43). In view of the heterogeneous nature of VLDL particles, multicompartmental analysis of the data to determine the fractional secretion rate is preferable to monoexponential analysis, although the error introduced by using the latter method can be minimized by modifying it to take account of the delay before labeled apoB appears in VLDL (44).

Studies of cholesterol synthesis and VLDL apoB secretion in humans

The acute effect on cholesterol synthesis of reducing LDL levels by LDL apheresis was studied by Pfohl et al. (25) in patients with familial hypercholesterolemia, both off and on HMG-CoA reductase inhibitors. There was a strong inverse correlation between the post-apheresis level of LDL cholesterol and the magnitude of the increase in cholesterol synthesis observed on the next day. This effect was only transient but occurred despite concomitant therapy with an HMG-CoA reductase inhibitor; the latter accentuated the reduction in LDL induced by apheresis. These findings, together with subsequent studies in normal subjects (45), suggest that the threshold below which LDL cholesterol must be reduced for cholesterol synthesis to be up-regulated is approximately 1.5 mmol/l.

Using similar methods to measure changes in cholesterol synthesis, Russell-Jones et al. (26) showed that growth hormone replacement therapy in adults with growth hormone deficiency resulted in significant decreases in both LDL cholesterol and plasma MVA. Data from other sources suggest that growth hormone up-regulates LDL receptors and, on the basis of the present findings, this could be secondary to hormonal down-regulation of HMG-CoA reductase. However, apoB turnover studies are necessary in order to determine whether growth hormone acts by increasing LDL catabolism or by decreasing VLDL secretion.

Simultaneously measured VLDL apoB secretion and cholesterol synthesis rates were both found to be raised in studies by Cummings et al. on obese subjects (27) and patients with non-insulin dependent diabetes mellitus (NIDDM) (28). In each situation VLDL apoB secretion and plasma MVA levels were significantly correlated. A unifying explanation for these findings is that in both conditions increased cholesterol synthesis was secondary to insulin resistance and resulted in an increased rate of secretion of VLDL apoB. The inhibitory action of insulin on VLDL apoB secretion and blunting of this effect in obese subjects have been shown previously by Lewis et al. (46).

In a recent study in normal subjects, we showed a strong and highly significant, positive correlation between fasting plasma MVA levels and VLDL apoB secretion rates (29). This correlation, shown in **Fig. 2,** was independent of other influences, including the apoE phenotype, and provides evidence that under normal circumstances cholesterol synthesis and apoB secretion are causally linked. Studies of the effect of simvastatin

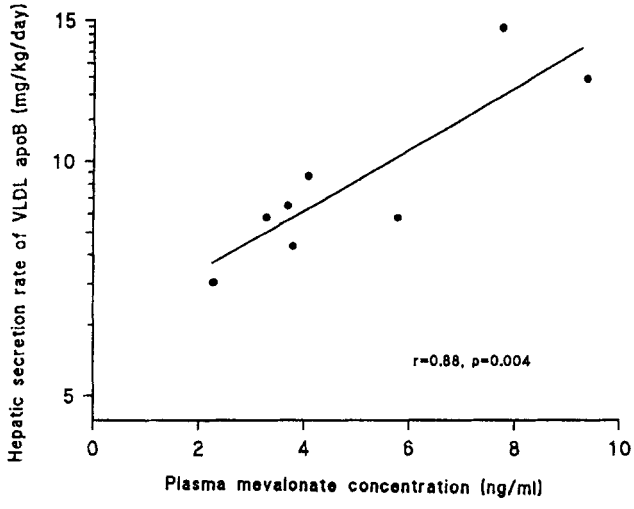

Fig. 2. Correlation between the rate of secretion of VLDL apoB and plasma mevalonate level in eight normolipidemic subjects (reproduced with permission from Watts, G. F. et al. 1995. *Metabolism.* **44:** 1052–1057).

on both these processes are now in progress.

Paired studies in patients with heterozygous FH showed that treatment with simvastatin reduced both cholesterol synthesis and VLDL apoB secretion rates but these decreases were not correlated (30). The effect of simvastatin in decreasing VLDL secretion in that study was not directly attributable to inhibition of cholesterol synthesis but may have been mediated via up-regulation of LDL receptors. One consequence of this effect in this group of patients would be to restore catabolism of LDL via the receptor pathway and thereby reduce non-receptor-mediated uptake of LDL. The latter source of cholesterol would lead in the untreated state to an increased availability of cholesterol for lipoprotein synthesis and thereby promote VLDL apoB secretion in FH, as has been demonstrated (47). Turnover studies by Fisher et al. (48, 49) support the notion that hepatic cholesterol overload is the prime determinant of apoB secretion in FH (48) and suggest that in this condition one-third of the apoB is secreted as intermediate density lipoprotein (IDL) and LDL, rather than VLDL (49). This suggests that not only the mass but also the nature of the neutral lipid available for association with apoB can influence the size of particle secreted (see Fig. 1).

In contrast with FH, where MVA levels are often normal, increased cholesterol synthesis appears to be the main reason for the increase in VLDL apoB secretion documented in a patient with cholesteryl ester storage disease (31). The increase in cholesterol synthesis reflects absence of the enzyme normally responsible for hydrolyzing cholesteryl ester to free cholesterol, thereby precluding down-regulation of HMG-CoA reductase. Treatment of another such patient with lovastatin has been shown to decrease both cholesterol synthesis and VLDL apoB secretion (50).

Raised plasma MVA levels have also been observed in patients with Cushing's disease and secondary hypercholesterolemia due to treatment with ortho, para, dichlorodiphenyl dichloroethane (o,p'DDD). It has been suggested that this reflects a failure of down-regulation of HMG-CoA reductase, due to impaired formation of oxysterols caused by iatrogenic inhibition of cytochrome P450 (51). The regulatory role of 27-hydroxycholesterol has recently been demonstrated in fibroblasts (52), but it remains to be shown whether a decreased level of this or any other oxysterol occurs in o,p'DDD-treated patients and, if so, whether their raised cholesterol synthesis results in an increased rate of apoB secretion. The elevated levels of MVA and LDL cholesterol in these patients when on o,p'DDD and subsequent decreases in both variables achieved by simvastatin are in accord with such an explanation.

HMG-CoA reductase inhibitors have a similarly striking effect in reversing the secondary hypercholesterolemia of patients with the nephrotic syndrome, in some of whom an ensuing decrease in apoB secretion has been documented; this was attributed to a reduction in the synthesis and availability of cholesterol in the liver (53). However, both here and in familial combined hyperlipidemia (54) it is probable that increased secretion of apoB is primarily driven by increased hepatic uptake of free fatty acids (55), any change in cholesterol synthesis presumably being a secondary phenomenon. The analogous syndrome of hyperapobetalipoproteinemia is purportedly due to dysfunction of the adipsin-acylation stimulating protein pathway (56); this causes impaired synthesis of triglyceride in adipose tissue and a consequent increased availability of free fatty acid for hepatic uptake, which results in increased secretion of apoB (57).

Conclusions

The hypothetical pools of free and esterified cholesterol and the main metabolic pathways involved in the synthesis of bile acids and secretion of apoB-containing lipoprotein particles by the liver are illustrated in **Fig. 3.** The supply of cholesteryl ester for incorporation into VLDL originates from esterification by ACAT of both newly synthesized and recycled LDL cholesterol. In normal subjects the latter is derived mainly from receptor-mediated uptake, but in FH much of the excess LDL in plasma is taken up by the liver via non-receptor pathways. In both instances cholesteryl ester predominates, which presumably must first undergo hydrolysis and partial re-esterification before incorporation into VLDL. In FH, treatment with HMG-CoA reductase inhibitors reduces VLDL secretion indirectly, by increasing the receptor-mediated component of LDL catabolism and thereby reducing recycling of LDL, rather than by decreasing cholesterol synthesis which is usually already down-regulated. The opposite occurs in cholesteryl ester storage disease where the inability to hydrolyze cholesteryl ester results in failure of down-regulation of HMG-CoA reductase and leads to increased synthesis of cholesterol and secretion of apoB. Treatment with an HMG-CoA reductase inhibitor reduces cholesterol synthesis, and thereby VLDL secretion, and normalizes serum lipids.

It seems reasonable to speculate that the interrelationship between apoB secretion and the availability of cholesteryl ester may explain why plasma triglyceride levels increase during treatment with anion exchange resins, which stimulate cholesterol synthesis, and decrease during treatment with HMG-CoA reductase inhibitors, which depress cholesterol synthesis. In this context it is noteworthy that administration of atorvastatin, a new HMG-CoA reductase inhibitor (58), resulted in dose-related decreases in both LDL cholesterol and plasma triglyceride in hypercholesterolemic subjects

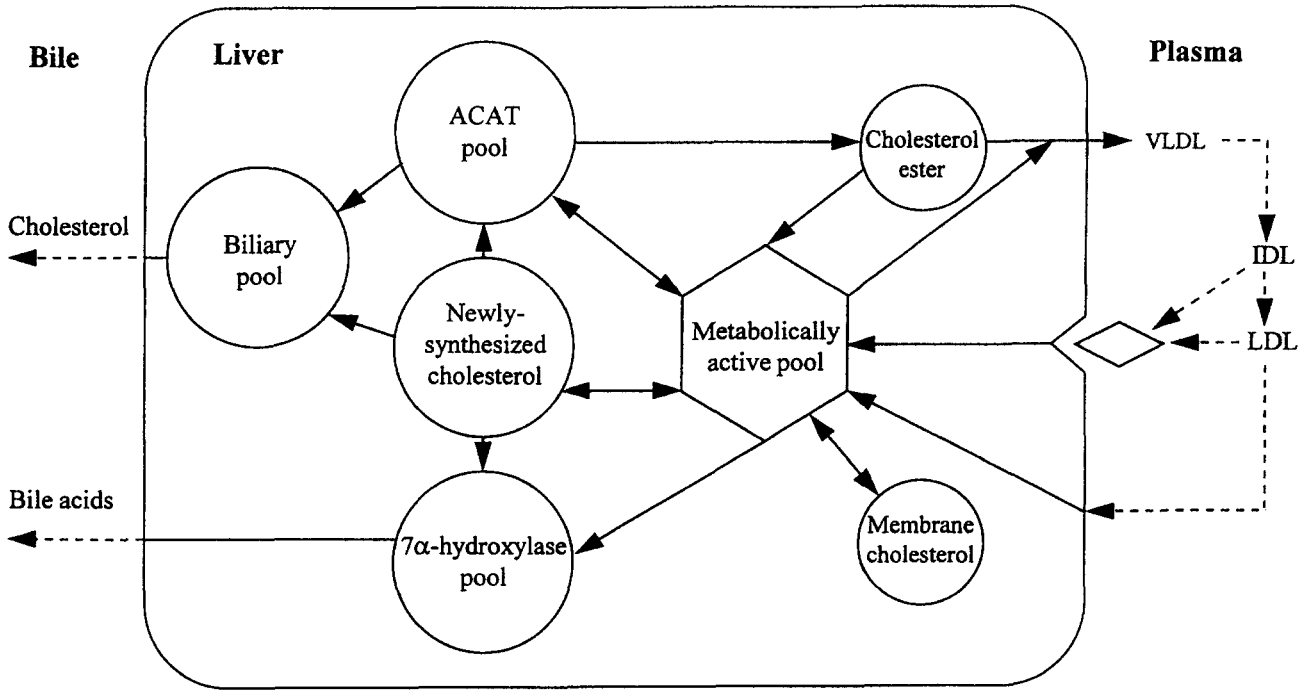

Fig. 3. Pathways of cholesterol involved in bile acid formation and biliary cholesterol excretion, and in VLDL secretion (modified with permission from Suckling, K. E., and E. F. Stange. 1985. *J. Lipid Res.* **26:** 647–671). Receptor-mediated uptake of IDL and LDL is indicated by the diamond-shaped symbol, receptor-independent uptake of LDL by the broken line leading to the surface of the liver. The metabolically active pool includes free cholesterol derived from LDL by the action of cholesteryl ester hydrolase, which influences the pool of newly synthesized cholesterol by regulating HMG-CoA reductase activity.

(59). Studies in an animal model of acquired hypercholesterolemia, the casein-fed rabbit, show that atorvastatin lowers LDL cholesterol primarily by decreasing apoB secretion; equivalent doses of lovastatin were less effective (60). Recent studies in FH patients show atorvastatin to be more effective than pravastatin and simvastatin in reducing both LDL cholesterol and plasma MVA levels (61). Evidence that the more marked inhibition of cholesterol synthesis by atorvastatin is accompanied by a greater reduction in apoB secretion than is achieved by other statins would not only support the hypothesis that the hepatic supply of cholesterol influences apoB secretion but could also explain the more marked triglyceride-lowering effect of atorvastatin.

Two fundamental issues that remain unresolved are the relative effects on hepatic apoB secretion in humans of variations in the supply of cholesteryl ester versus triglyceride and the respective contributions made to apoB secretion by the differing pools of cholesterol within the liver (see Fig. 3). The first issue could be examined by stable isotope turnover studies of the independent and additive effects of inhibitors of hepatic triglyceride synthesis, such as eicosapentaenoic acid (62), and of HMG-CoA reductase in both normal and hyperlipidemic subjects (62, 63). The second issue might be addressed by stable isotope studies of the effects of ACAT inhibitors in patients with cholesteryl ester storage disease (31) and Nieman-Pick Type C disease; the latter appear to have a genetic defect in LDL-mediated stimulation of cholesterol esterification (64). Kinetic studies using specific inhibitors of cholesterol transport within cells would also be germane (64).

As has already been discussed, increased apoB secretion is a feature of several common forms of hyperlipidemia, both inherited and acquired. Confirmation that HMG-CoA reductase inhibitors decrease apoB secretion would not only shed light on the mode of action of these compounds but would also emphasize the therapeutic potential of absorbable ACAT inhibitors. The two should make a formidable combination.

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