review

Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells

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INTRODUCTION

ApoB is required for the assembly and secretion of apoB-containing lipoproteins (LPs) which transport hydrophobic lipids, cholesteryl ester, and triglycerides in their cores. Human apolipoprotein (apo) B-100 is a large hydrophobic protein of 4536 amino acids and a molecular mass of approximately 520 kD that is synthesized in the liver (1–4). ApoB comprises 30–40% of the protein content of plasma very low density lipoprotein (VLDL) and greater than 95% of the protein in plasma low density lipoprotein (LDL). There is also a truncated form of apoB called apoB-48 that is synthesized in human small intestine. ApoB-48 is also synthesized in livers of several lower species, but not in human liver. Except where noted, the term apoB will refer to apoB-100 throughout the course of this review. ApoB contains both hydrophobic lipid binding regions, which probably participate in the assembly of nascent lipoprotein complexes, as well as hydrophilic sequences, which interact with the polar aqueous environment (5). ApoB also contains an LDL receptor binding domain, located in the region between amino acids 3100–3600 (2, 3), that is involved in the uptake of plasma intermediate density lipoprotein and LDL, and possibly some VLDL, by tissues.

ApoB-containing LPs are assembled in the endoplasmic reticulum (ER), with maturation occurring in the Golgi apparatus of hepatocytes prior to secretion (5). Regulation of the assembly and secretion of apoB-containing LPs has become an active area of investigation as it is recognized that overproduction of apoB-containing LPs may be responsible for hyperlipidemia in a large percentage of patients (6–10). The purpose of this review is to summarize the current information available concerning regulation of the hepatic assembly and secretion of apoB-containing LPs, with a special emphasis on studies utilizing the continuous human hepatoma cell line, HepG2, as a model for the human hepatocyte.

HEPG2 CELLS AS A MODEL FOR HEPATIC LIPOPROTEIN METABOLISM

Many studies concerning hepatic LP metabolism have used HepG2 cells. These cells were derived from a human hepatoma and exhibit many differentiated functions of human parenchymal cells, including expression of secretory proteins (11). Apos A-I, A-II, A-IV, B, C-II, C-III, and E have all been identified in association with various secreted LPs in HepG2 medium (12, 13). HepG2 cells have also been shown to synthesize bile acids and many liver specific proteins (14). Although HepG2 cells have been shown to secrete α-fetoprotein, they appear to be a mature epithelial cell line that can form structures resembling bile canaliculi in tissue culture (15). The use of HepG2 cells as a model to study cholesterol and bile acid metabolism in hepatocytes was recently reviewed by Javitt (14).

The lipid composition of HepG2 cells grown to confluence in serum-containing medium has recently been compared to that of human liver by Wang et al. (16). HepG2 total lipid content (255 μg/mg protein) was much higher than that of normal liver tissue (143 μg/mg protein). The increased total lipid content of HepG2 cells was the result of an elevated triglyceride and phospholipid content, whereas the cholesterol content was comparable to that of human liver. Although these comparisons did not take into account the fact that human liver contains four major cell types (parenchymal, endothelial, Kupffer, and stellate cells), they do indicate that HepG2 cells, although a rapidly growing cell line, are not triglyceride- or phospholipid-poor.

Abbreviations: apo, apolipoprotein; ER, endoplasmic reticulum; LDL, low density lipoprotein; LP, lipoprotein; VLDL, very low density lipoprotein.

REGULATION OF APOB SECRETION: A POST-TRANSLATIONAL PHENOMENON

The apoB gene has been cloned and studied, and genetic variations in the apoB gene have been identified (for reviews see (17–21). However, as the apoB mRNA level has been found not to change in situations where apoB secretion is altered over a wide range, it appears that apoB secretion is not regulated at the transcriptional or mRNA level. Pullinger et al. (22) found that the level of apoB mRNA in HepG2 cells was refractory to treatment with oleate or insulin, although the former stimulated and the latter inhibited secretion of apoB. Similar discordant effects of oleate on apoB message and secretion were obtained by Moberly et al. (23). Dashti, Williams, and Alaupovic (24) also showed that apoB mRNA levels were unaffected by oleate or insulin treatment. However, in their studies, while insulin inhibited apoB secretion, treatment with 0.8 mM oleate/3% BSA failed to stimulate apoB secretion compared to controls (24). These observations on the refractory nature of apoB mRNA levels in HepG2 cells are in agreement with several studies of the effects of diet on the hepatic levels of apoB mRNA in African green monkeys (25), rabbits (26), and baboons (27). In the study by Sorci-Thomas et al. (25), monkeys consuming diets high in cholesterol for 5 years had higher levels of plasma apoB and LDL cholesterol compared to controls, but showed no change in the abundance of apoB mRNA in liver. Additionally, Leighton et al. (28) reported that total apoB secretion was decreased in hepatocytes isolated from fasted rats without any change in apoB mRNA level.

However, in a recent study, cebus monkeys fed diets containing coconut oil and cholesterol for 3 years or longer had hepatic levels of apoB mRNA that were 87% greater than those of animals fed corn oil without cholesterol (29). Additionally, Srivastava et al. (30) have recently reported that the effects of lipogenic diets on apoB transcription and mRNA stability in the mouse are complex and require further study. Very recently, Dashti (31) reported an increase in apoB mRNA after treating HepG2 cells with 25-hydroxycholesterol. The addition of free cholesterol to HepG2 cells did not, in the same study, alter apoB mRNA.

In summary, the results of many, but not all previous in vitro and in vivo studies have indicated that the hepatic concentration of apoB mRNA and most likely the transcriptional rate of the apoB gene is not, in most circumstances, responsive to changes in lipid metabolism. These observations are in line with the observation that the half-life of apoB mRNA in HepG2 cells is relatively long, approximately 16 h (22). Thus, the regulation of hepatic apoB secretion by major substrates and hormones does not appear to be modulated transcriptionally, but rather translationally or post-translationally (22).

POST-TRANSLATIONAL REGULATION OF APOB SECRETION

Location and translocation of nascent apoB in the rough endoplasmic reticulum

Secretory proteins are synthesized in the rough endoplasmic reticulum (RER) and then directed to the lumen of the RER after translocation through the membrane (32). During the early phase of translation, a signal recognition particle binds to the elongating secretory polypeptide containing an amino terminal signal sequence, stopping translation. The signal recognition particle then binds to its receptor on the surface of the ER, establishing a ribosome-ER membrane junction. The signal recognition particle and its receptor are then released, and translation resumes. The polypeptide chain elongates through a putative aqueous channel in the membrane (33) and the signal sequence is cleaved on the luminal side of the ER membrane (34). In contrast, integral membrane proteins are not fully translocated through the ER membrane, but rather their translocation is halted by a stop-transfer sequence when they have only partially passed through the membrane (35). Proteins that span the membrane several times are thought to contain multiple alternating signal and stop-transfer sequences (36). ApoB contains an amino terminal cleavable signal sequence which should direct the emerging peptide chain through the membrane (37). As no classical stop transfer signal, or complete hydrophobic membrane spanning region, has been identified in the sequence of apoB, its translocation through the ER membrane should occur without interference.

There are now numerous reports, however, that apoB becomes associated with the ER membrane, either cotranslationally or very early in the post-translational period. Thus Boström et al. (38) reported that 60–70% of apoB isolated in the ER of HepG2 cells was membrane-associated. In a later study (39), this group observed that after a 3-min pulse, apoB remained associated with the ER membrane for approximately 15–17 min before being transferred to the ER lumen. In more recent kinetic experiments, Borén et al. (40) showed that following a 5-min pulse with [^{35}S]methionine, >90%, 75%, and >50% of labeled apoB was recovered in the ER membrane in cells after 15, 20, and 30 min of chase, respectively. Furthermore, Borén et al. (40) showed that after leaving the ER membrane, very little apoB could be recovered in the ER lumen or Golgi membrane, whereas a moderate amount could be recovered in the Golgi lumen. These observations indicated that once released from the ER membrane, apoB is rapidly transported from the ER lumen to the Golgi lumen. Bamberger and Lane (41) confirmed that apoB was located in the ER membrane as they observed that approximately 40% of apoB remained in the membrane fraction after alkaline treatment of both heavy and light ER isolated from chick hepatocytes.

Davis et al. (42) reported that apoB was not only associated with the ER membrane, but was, in fact, located on the outer surface of the ER membrane. In RER isolated from rat liver (42), 56% of apoB*L* (apoB-100) and 70% of apoB*S* (apoB-48) were sensitive to exogenous trypsin, indicating that a significant amount of apoB remained on the cytosolic face of the ER membrane. On the other hand, only a small amount of apoB was accessible to trypsin in smooth ER and none was accessible in Golgi. Dixon et al. (43, 44), using immunoelectron microscopy, observed that 96% of a gold-labeled antibody to chick apoB was localized in close association with the membrane of isolated RER vesicles. Furthermore, apoB could not be dislodged from the ER membrane by treatment at pH 9.1 (which causes the formation of small pores in the membrane (45)), but could be completely removed from the membrane upon carbonate (pH 11) treatment. These data indicate that although apoB is tightly associated with the ER membrane, it is not an integral membrane protein in the classical sense. Similar to the results of Davis et al. (42), 60–75% of apoB in chick RER was degraded when isolated vesicles were treated with exogenous protease K (44).

The mechanism whereby nascent apoB becomes associated with the ER membrane was investigated by Chuck et al. (46) in studies of the translocation of a truncated form of apoB (apoB-15) across membranes of dog pancreas microsomes. After apoB-15 was translated in reticulocyte lysates and allowed to translocate for 20–90 min, the microsomes were treated with protease K. At early time points, apoB-15 was almost completely susceptible, except for certain small regions, to protease K treatment, indicating that a large portion of apoB-15 was located on the cytosolic side of the microsomes. At later time points, apoB-15 was almost completely protected from exogenous protease K. These data suggested that apoB-15 moved slowly across the microsomal membrane into the lumenal environment, and not rapidly through a channel in the membrane as observed for other secretory proteins. In later studies, Chuck and Lingappa (47) identified a 33 amino acid pause sequence in apoB-15 which stops translocation, and a second sequence more than 200 amino acids downstream, which appears to restart translocation across the membrane.

Several caveats should be noted about the studies of Chuck et al. (46) and Chuck and Lingappa (47). First, they were performed with only very small portions of the apoB-100 protein. Second, dog pancreas microsomes may be quite physiologically different than human hepatic microsomes. And third, translocation of proteins in in vitro systems occurs very slowly. However, the observations of these investigators are conceptually important because they indicate that the mechanism of apoB translocation may be quite different from that of most other secretory proteins.

Pease, Harrison, and Scott (48) have recently reported similar translation/translocation experiments, but with quite different results. In their studies, also utilizing dog pancreas microsomes, truncated apoBs (B-15 and B-17) were not sensitive to exogenous protease treatment at any time during translocation. However, B-15 and B-17 were shown to become associated with the ER membrane as only approximately 50% of each could be extracted with carbonate pH 11 buffer and none could be displaced after treatment with 0.05% saponin. Therefore, Pease et al. (48) concluded that apoB was co-translationally translocated across the cytosolic leaflet, but then became inserted into the inner leaflet of the ER membrane.

The reason for the discordant findings in the studies of Chuck et al. (46, 47) and Pease et al. (48) is not apparent. However, the findings of Davis et al. (42) and Dixon et al. (44) in studies on the location of full-length apoB in isolated hepatic ER vesicles support the findings of Chuck et al. (46, 47).

Assembly of apoB-containing LPs

The studies of Borchardt and Davis (49) and Bostrom et al. (39) indicated that nascent apoB, during secretion, resides in the ER much longer than in the Golgi, and that the ER, therefore, is probably the site of the initial assembly of apoB-containing LPs. Glaumann, Bergstrand, and Ericsson (50), using electronmicroscopy, observed immature LPs in the rough ER of rat liver. These particles, although rare, were 200–400 Å in diameter and floated at a density of $d < 1.03$ g/ml, probably due to a low lipid content at this step in the secretory pathway. Particles observed in the smooth ER were more abundant and of larger size (200–600 Å). Stein and Stein (51) observed autoradiographic grains resulting from the synthesis of tritiated triglyceride in both rough and smooth ER of rat hepatocytes 2 min after injection of [^{3}H]glycerol. After 10 min, grains were seen in Golgi. Alexander, Hamilton, and Havel (52) reported that lipid particles observed in regions of smooth ER did not contain antigenic sites to apoB, indicating that nascent VLDL lipid particles are assembled in the smooth ER and pick up apoLPs at the junction of the smooth and rough ER. Finally, both mature and immature apoB-containing LPs have been observed by electronmicroscopy in Golgi isolated from rat liver (53), indicating that some further assembly of apoB-containing LPs may occur in this organelle. However, a later study has suggested that the immature LPs that were seen were derived from multivesicular bodies (54).

Biochemical studies in HepG2 cells have complemented the electron microscopic observations made in rat liver. Boström et al. (38) reported that the incorporation of lipids into an initial apoB-lipid complex occurred in the ER membrane, where apoB resided for approximately 15 min. Dense apoB-containing lipoprotein particles ($1.065 < d < 1.17$ g/ml) were initially found in the

microsomal lumen and subsequently converted to less dense particles. In more recent studies (40), these workers demonstrated that the transfer of apoB from the membrane bound "preassembly pool" to the lumen of the secretory pathway occurred in a smooth membrane fraction, apparently a pre-Golgi compartment analogous to the smooth endoplasmic reticulum. ApoB was not found to be present in the Golgi membrane at any time during the course of the pulse-chase experiment, and only an extremely small fraction of the total labeled apoB could be isolated from the lumen of ER. However, a larger fraction of apoB could be isolated from the Golgi lumen, indicating that once released into the ER lumen, apoB-containing LPs were rapidly transferred to the Golgi and transported out of the cell.

Several studies have indicated that nascent phospholipids are incorporated into LPs in the Golgi (53, 55, 56). This might involve the active exchange of polar lipids between Golgi membranes and the LP surface (53). Whether complexing of core lipids with apoB also occurs in the Golgi is more controversial, although several reports indicate that this, indeed, does occur (41, 57, 58). In kinetic studies in estrogen-induced cultured chick hepatocytes, Bamberger and Lane (41, 57) reported that apoB, without lipid, was rapidly transported from the ER to the Golgi, where most of the complexing of lipid with apoB occurred. However, it must be noted that the regulation of apoB secretion in estrogen-stimulated chick hepatocytes, which secrete massive amounts of apoB, may be different from that in human hepatocytes or HepG2 cells.

In summary, kinetic studies in HepG2 cells (38–40) and in rat hepatocytes (49) indicate that the rate-limiting step in apoB-containing LP secretion was the assembly of apoB-lipid complexes in the ER membrane. On the other hand, electron microscopic studies suggest that primordial apoB-lipid complexes, most likely formed in the rough ER, can assemble into more mature LPs utilizing core lipids generated in the smooth ER (52).

Post-translational regulation of apoB secretion through intracellular degradation

The first indication that regulation of apoB secretion occurs post-translationally came in the studies of Borchardt and Davis (49), who reported that only 36 and 60% of newly synthesized apoB-100 and apoB-48, respectively, were secreted by primary rat hepatocytes in culture. These results indicated that a large percentage of both forms of nascent apoB was being degraded intracellularly. Boström et al. (39) noted that the inclusion of oleate in the medium of HepG2 cells increased the proportion of the intracellular pool of apoB that was secreted. Recently, Sato et al. (59) and Dixon, Furukawa, and Ginsberg (60) reported that apoB in HepG2 cells was degraded in the secretory pathway, indicating that post-translational regulation is involved in modulating apoB secretion in these cells. Furthermore, Dixon et al. (60) observed that the extent of intracellular apoB degradation could be modulated by treatment of the cells with oleate, such that in the presence of oleate, apoB degradation was decreased and its secretion increased. These results were very recently confirmed in McArdle-RH7777 cells by White et al. (61). After transfecting these cells with apoB cDNA constructs of varying lengths, these authors demonstrated that oleate treatment only protected apoB that was of sufficient size to form buoyant lipoproteins.

Whereas oleate protected apoB, treatment of cultured rat hepatocytes with 10 nM insulin has been shown by Sparks and Sparks (62) to stimulate the intracellular degradation of newly synthesized apoB-100 and apoB-48, 45 and 27%, respectively, compared to hepatocytes treated with a much lower level of insulin (0.1 nM). There were additional effects of insulin, however, as it was reported that insulin also inhibited the synthesis of total apoB by 48% (62).

The location of early degradation of nascent apoB in hepatocytes has been investigated by several groups. Davis et al. (63) reported that proteolytic fragments derived from apoB were detected in rough and smooth ER of rat hepatocytes, but not in Golgi fractions. In a later study (42) these investigators studied the metabolism of apoB leading to degradation in the ER. As noted earlier, protease protection assays and Western blot analysis indicated that significant amounts of rat hepatic nascent apoB remained on the cytosolic surface. In subsequent pulse-chase experiments using cultured rat hepatocytes, accessibility of both forms of apoB (B-48 and B-100) to exogenous trypsin in microsomes increased from about 25% accessible after 0 min of chase, to about 45% accessible after 40 min of chase. From these observations Davis et al. (42) concluded that there are two populations of apoB in the ER: a lumenal pool comprised of apoB that was translocated across the ER membrane and assembled into LPs, and a pool of apoB that remained accessible to trypsin and thus was not translocated across the membrane. These authors proposed that the latter pool was subsequently diverted for intracellular degradation. This hypothesis provides an attractive model for post-translational control of apoB secretion. However, it still requires rigorous testing. For example, the pool of apoB found on the cytosolic side of the ER membrane after 40 min of chase (approximately 20% of the total apoB synthesized during the pulse) may represent apoB that escaped both assembly into LPs and early intracellular degradation.

Sato et al. (59) also presented data indicating that apoB degradation occurred in the ER. They observed that when HepG2 cells were pretreated with brefeldin A, a compound which blocks ER to Golgi transport (64), degradation of newly labeled apoB continued as in unblocked cells, whereas another apoLP, apoA-I, remained stable. As there was no decline in labeled apoA-I, which

must also be trapped in the ER of brefeldin A-treated cells, degradation appeared to be specific for apoB. These results strongly suggested that intracellular degradation of newly synthesized apoB occurs in the ER compartment. However, brefeldin A also causes the backflow of Golgi resident proteins to the ER (65–67), leaving the possibility that degradation of apoB in brefeldin A-treated cells may be actually due to a protease originating from the Golgi. Recent studies in our laboratory (68) with isolated ER and Golgi fractions and studies utilizing nocodazole, an inhibitor of retrograde Golgi to ER transport (69), have indicated that the ER is, indeed, the site of early apoB degradation.

Other mechanisms which might be involved in the regulation of apoB secretion

ApoB is known to undergo post-translational glycosylation, but it appears not to be crucial for regulation as treatment of rat and chicken hepatocytes with tunicamycin did not interfere with the secretion of apoB (70, 71). HepG2 apoB also has been reported to undergo fatty acid acylation with either stearic or palmitic acid (72). This post-translational modification could be involved in the LP assembly process, by altering the hydrophobicity of regions of the apoB polypeptide chain. Another post-translational modification of apoB that has been reported is phosphorylation of serine residues (73, 74). These studies were performed in cultured rat hepatocytes, and it is not known whether phosphorylation of apoB-100 occurs in human cells. Further investigations are needed to determine whether any of the above post-translational modifications are indeed involved in the regulation of apoB secretion.

APOB-CONTAINING LPS SECRETED BY HEPG2 CELLS

Thrift et al. (75) demonstrated that HepG2 cells, when grown under serum-free conditions, secrete a majority of their apoB in LP particles having a buoyant density in the LDL range, as only 1% of the protein found in the $d < 1.063$ g/ml fraction floated in the $d < 1.006$ range. These particles were also observed to be similar in size to LDL (25 nm) (76). Whereas the composition of human plasma VLDL is approximately 5–10% protein, 55–80% triglyceride, 10–20% phospholipid, and 10–17% cholesterol (including both free and esterified), particles isolated in the $d < 1.063$ g/ml fraction of HepG2 medium contained a higher content of total protein (27%) and phospholipid (28%), but a lower content of triglyceride (25%). The cholesterol content (20%), however, was similar to that found in plasma VLDL, but with a majority of the cholesterol in unesterified form. The major apolipoprotein in HepG2 LDL was apoB, with very little apoE or other apolipoproteins present (75). Similar apoB-containing LPs were described by Ellsworth, Erickson, and Cooper (77), who observed that when HepG2 were incubated with 0.8 mM oleate, secreted apoB and triglyceride were both redistributed from the LDL to the VLDL density range. ApoE, while absent from HepG2 LDL, was present in VLDL secreted by both control and oleate stimulated cells (77). With oleate present, apoE accounted for 7% of the labeled protein in VLDL. Recently, Wang et al. (16) reported that 47% of the triglyceride secreted by their HepG2 cells was present in the IDL density range.

Therefore, HepG2 cells secrete apoB in triglyceride-rich particles that have a buoyant density mainly in the LDL range. In a sense, these particles appear to be a smaller version of VLDL. When a source of fatty acid or a fatty acid precursor is provided, the secreted particles become larger (due to an increase in triglyceride content), pick up apoE, and begin to more closely resemble plasma VLDL. The fact that apoE and possibly some apoCs are primarily found in HepG2 VLDL, and not in HepG2 LDL, reinforces the concept that core lipids are important in determining the apolipoprotein content of secreted particles. Secretion of HepG2 apoB in a small, dense particle may be primarily related to the low rates of triglyceride secretion in these cells (1.29 and 3.38 μg/mg cell protein per 24 h for control and oleate-treated HepG2 cells, respectively (22)), compared to 47 and 77 μg/mg cell protein per 24 h for control and oleate-treated primary rat hepatocytes, respectively (78). In addition to oleate, other energy sources can influence the rate of triglyceride secretion and the size and density of the LP particle observed in the medium. For example, when HepG2 cells were cultured in medium containing a high concentration of glucose, triglyceride secretion increased greatly, from 8.9 μg/mg protein per 24 h (5.5 mM glucose) to 29.4 μg/mg protein per 24 h for cells treated with 25 mM glucose (16).

APOB-CONTAINING LPS SECRETED BY RAT HEPATOCYTES

In contrast to the heterogeneous particles secreted by HepG2 cells, the characteristic apoB-containing LP particle secreted by rat liver (79) and rat hepatocytes in culture (80) is VLDL. However, there are conditions under which smaller particles are secreted by rat liver. In both nephrosis (81) and cholestasis (82), rat liver secretes a more heterogeneous mixture of apoB-containing particles, including those which float in the IDL and LDL fractions. In cultured rat hepatocytes, too, it also appears that the density of the nascent apoB-containing LP secreted is dependent upon the dietary regimen of the donor rat and/or the cul-

ture conditions employed. Belle-Quint and Forte (83) showed that hepatocytes obtained from fasted rats secreted lower amounts of triglyceride and VLDL compared to controls, whereas hepatocytes from sucrose-fed rats secreted 10-fold greater VLDL than those from fasted rats. In medium of control rat hepatocytes after short-term incubations (<6.5 hr), 56% of the total LP mass was found in VLDL, 20% in LDL, and 24% in HDL (83). Patsch, Franz, and Schonfeld (84) reported a similar distribution for rat hepatocytes cultured on a fibronectin matrix for 16 h, with 43% of apoB in VLDL, 27% in LDL, and 29% in HDL (determined by chromatography on Sepharose 6B). However, when Belle-Quint and Forte (83) cultured rat hepatocytes for longer periods (17 and 48 h), the VLDL fraction contained only approximately 20% of the total LP mass, possibly the result of a lower rate of triglyceride synthesis due to lower availability of precursor substrates, or because the hepatocytes were becoming dedifferentiated (85, 86).

Recently Duerden, Bartlett, and Gibbons (87) reported that rat hepatocyte triglyceride synthesis and VLDL triglyceride output could be maintained at high rates for 3 days (approximately 100 μg/mg protein per 24 h) when 1 μM dexamethasone, 10 mM lactate, 1 mM pyruvate, and 0.75 mM oleate were added to the medium. Whether continued high rates of triglyceride secretion were due to the inclusion of precursor substrates (lactate, pyruvate, oleate) or due to the glucocorticoid (dexamethasone), which would help keep the cells in a differentiated state, was not addressed. The inclusion of substrates such as pyruvate and lactate may be extremely important in hepatocyte studies because glucose, due to the high K_m of the glucose transporter in these cells (88), is not a good energy source for hepatocytes except at extremely high concentrations.

In summary, it appears that the basal rate of triglyceride secretion is low in HepG2 cells compared to cultured rat hepatocytes. It is possible that HepG2 cells, which are rapidly growing cells and require substantial amounts of energy for cell division, although not deficient in lipid stores (16), resemble fasted rat hepatocytes more than fed rat hepatocytes. Hence, they exhibit a low rate of triglyceride secretion and secrete apoB mainly in a particle similar in size and density, but not in composition, to LDL.

LIPID METABOLISM AS A REGULATOR OF APOB SECRETION

Nascent apoB-containing LPs contain triglyceride, free and esterified cholesterol, and phospholipid. The syntheses of triglyceride, cholesterol, or cholesteryl ester have been proposed as regulators of apoB secretion in various model systems. It is possible that the availability of these lipids influences the post-translational metabolism of apoB, and therefore its secretion rate.

Regulation through triglyceride synthesis

A major role for apoB-containing LPs is the transport of triglyceride from the liver to peripheral tissues, especially adipose and muscle. Hepatic triglycerides are synthesized from glycerol and fatty acids, either taken up from the plasma or newly synthesized in the cytosol of the hepatocyte. Availability of triglyceride for lipoprotein assembly greatly influences the size of the VLDL particle secreted by the liver (89). Glaumann et al. (50) reported that maximal incorporation of [^{3}H]glycerol into triglyceride was attained simultaneously (5 min after injection) in rough and smooth ER isolated from rat liver, supporting other work (90, 91) indicating that triglyceride synthesis occurs in both compartments. Glaumann et al. (50) also showed that there are three pools of triglyceride in the rat hepatocyte: *1)* a cytosolic pool which has a long half-life, presumably triglyceride located in storage lipid droplets; *2)* an ER pool which has a very short half-life; and *3)* a lumenal/lipoprotein pool which also has a short half-life. Identification of the rough and smooth ER as sites of triglyceride synthesis gives support to the concept that triglyceride is available in these fractions for assembly with apoB in the ER.

It is well established that increased availability of free fatty acids stimulates hepatic triglyceride synthesis and secretion (92–94). Part of this stimulation is the result of fatty acid-induced activation of phosphatidate phosphohydrolase (95). Furthermore, in the postabsorptive condition, the concentration of plasma triglycerides is regulated by the metabolism of nonesterified fatty acids delivered to the liver from the periphery (96). An important question concerning apoB metabolism is whether availability of triglyceride derived from exogenous fatty acids modulates apoB secretion. Initial studies with HepG2 cells did not give consistent answers to this question. In one series of reports, oleic acid treatment had only minimal effects on the rate of apoB secretion (24, 77, 97–99). For example, Ellsworth et al. (77), in an elegant series of experiments, demonstrated that adding oleate to HepG2 cells caused a major redistribution of both secreted apoB and triglyceride from HepG2 LDL into VLDL, but did not stimulate apoB secretion. In another group of papers, the rate of apoB secretion was reported to be stimulated by 2- to 5-fold when HepG2 cells were treated with oleic acid or linoleate (12, 22, 23, 39, 40, 100, 101).

The basis of these conflicting reports may be found in the study by Dixon et al. (60), who observed that not only did oleate stimulate apoB secretion, but it did so with a lag time of only 40 min, a time period corresponding to the transit time for apoB in the secretory pathway. When oleate was removed from the medium, the apoB secretion rate returned to the control rate, also with a lag time of

40 min. These responses of apoB secretion to exogenous oleate indicated that newly synthesized lipid may be very rapidly associated with apoB early in the secretory pathway, leading to increased secretion. As the ER pool of triglyceride has a very short half-life (50), a reasonable hypothesis is that triglyceride is one of the lipids responsible for modulation of apoB secretion by oleate. Therefore, upon removal of oleate, triglyceride availability rapidly returns to the control level, leading to decreased complexing of triglyceride with apoB in the ER and increased degradation of apoB. In contrast to what occurs in rat hepatocytes (102), it appears that in HepG2 cells triglyceride from cytosolic stores cannot be provided to apoB in sufficient quantities to protect it from degradation. Thus, ongoing oleate transport into the cell from the medium may be required to observe a fatty acid-induced stimulation in the rate of apoB secretion.

Besides the ongoing requirement for oleate during the experiment, there may be several other reasons why there is such a wide discrepancy among reports studying the effects of fatty acids on apoB metabolism. First, the concentrations of oleate and BSA used in experiments are extremely important, as high concentrations of oleate are toxic to cells, and albumin alone is inhibitory for apoB secretion (22). Second, if the length of the incubation period is too long, remodeling or uptake of secreted LPs may occur (103). Third, apoB and triglyceride should be measured in whole medium or whole concentrated medium before extensive manipulation, so that methodologic artifacts may be avoided. Finally, different batches of HepG2 cells may respond differently to a fatty acid challenge. It may be important for investigators to use a uniform and well-characterized batch of HepG2 cells in their studies.

In contrast to the observations in HepG2 cells, it is now well established that whereas exogenous oleate stimulates triglyceride secretion, it does not stimulate total apoB secretion in control rat hepatocytes in culture (78, 104) or in the control perfused rat liver (105). The reason for this difference may be related to a difference in the basal rates of triglyceride synthesis and secretion in these cells, and the fact that rat hepatocytes synthesize and secrete both apoB-100 and apoB-48 and can mobilize excess triglyceride on apoB-48-containing particles. However, addition of oleate to the perfusate has been reported to stimulate apoB secretion in perfused livers from rats fasted for 24 h (105) and in livers from rats fed a high carbohydrate diet (106).

The role of triglyceride synthesis in regulating apoB secretion was studied recently by Arbeeny et al. (107). They observed that treatment of primary cultures of hamster hepatocytes with oleate stimulated the secretion of triglyceride and the synthesis of cholesteryl ester without affecting apoB secretion. When the cells were treated with TOFA (5-(tetradecyloxy)-2-furancarboxylic acid), an inhibitor of acetyl CoA carboxylase, the synthesis of fatty acids and triglyceride and the secretion of triglyceride were inhibited 98, 76, and 90%, respectively. TOFA also inhibited apoB secretion (50%) and cholesteryl ester synthesis (38%). Treatment of TOFA-inhibited cells with oleate partially reversed many of the effects of TOFA on lipid metabolism. These data indicate that whereas stimulation of triglyceride synthesis does not increase apoB secretion, significant inhibition of triglyceride synthesis decreases apoB secretion. Therefore, it appears that triglyceride availability in hamster hepatocytes under basal conditions is adequate to support maximal apoB secretion, and effects on apoB secretion are only observed when triglyceride availability falls below some threshold level.

Regulation through cholesterol or cholesteryl ester synthesis

There have been recent reports that cholesterol or cholesteryl ester availability, which can also be influenced by fatty acids (108), may be important in regulating hepatic apoB secretion (9, 109–112). Cholesterol metabolism in the liver is complex, as there are several functional pools of cholesterol in the hepatocyte, including a metabolically active pool, an ACAT substrate pool, and a cholesteryl ester pool (113). The various pools of cholesterol in the cell and the various pathways that connect them are coordinated such that the free cholesterol pool in the cell is usually maintained at a precise level. When HepG2 cells were incubated with LDL for 20 h, although the total concentration of cellular cholesterol increased (despite down-regulation of the LDL receptor), the concentration of free cholesterol was maintained at a constant level (114). This occurred because the esterification of cholesterol increased to buffer the cell from increased free cholesterol. In fact, the concentration of free cholesterol did not change even when HepG2 cells were incubated with both LDL and Sandoz 58-035, an inhibitor of ACAT. In this case, free cholesterol content probably remained constant through reductions in LDL receptor activity and cholesterol synthesis, and adaptations in other pathways of cholesterol metabolism such as the 7-α hydroxylase and bile secretion pathways (114).

There have been reports that the direct addition of cholesterol to the medium of human primary hepatocytes (115) or HepG2 cells (112) increased the secretion of apoB. However, this has not been observed by other investigators. Thus, in a recent study by Dashti (31), the addition of free cholesterol to the medium of HepG2 cells had no effect on apoB secretion. In contrast, the addition of 25-hydroxycholesterol (20 μg/ml medium), a form of cholesterol that is preferentially esterified by the cell, increased the secretion of triglyceride, cholesteryl ester, and apoB by 1.4-, 3.2-, and 2.5-fold, respectively. Furthermore, the apoB mRNA concentration and the synthesis of apoB after a short pulse period were both significantly in-

creased, indicating transcriptional or post-transcriptional regulation. The physiologic importance of this finding remains to be determined.

It is possible that cholesterol must be presented to the cell in a more physiological form in order to observe a stimulation. Thus Craig, Nutik, and Cooper (98) observed that treatment of HepG2 cells with rat chylomicron remnants (cholesterol/triglyceride = 0.12) or rat βVLDL (cholesterol/triglyceride = 4.65) for 24 h stimulated total apoB secretion during a subsequent 24-h period, 2.6- and 4.2-fold, respectively. There have been conflicting reports on the effects of LDL on apoB secretion. Whereas Sato et al. (59) reported that treatment of HepG2 cells with LDL for 24 h decreased the rate of apoB secretion, Dashti (31) recently observed that addition of 100 μg LDL protein/ml to HepG2 medium increased apoB secretion by 50%. More studies on the effects of LPs on the secretion of apoB from hepatocytes are needed.

As the enzyme that esterifies cholesterol with fatty acid, acyl-CoA:cholesterol acyltransferase (ACAT), is located predominately in the RER of rat liver (116–118), and may be in close approximation to the location of apoB synthesis, cholesteryl ester synthesis may be especially important in the regulation of apoB secretion. In a recent study on the regulation of apoB secretion in HepG2 cells, Cianflone et al. (111) performed experiments in order to determine whether the stimulation of apoB secretion observed with exogenous free fatty acid occurred through effects on triglyceride synthesis or cholesteryl ester synthesis. Over a 24-h period addition of oleate (1 mM) stimulated the secretion of apoB (approximately 150%) and the synthesis of cholesteryl ester (200%) and triglyceride (400%). When either lovastatin or the ACAT inhibitor, 58-035, were added to oleate-stimulated cells, both the accumulation of apoB in medium (−25% with either agent) and the synthesis of cholesteryl ester (−45% with lovastatin and −60% with 58-035) decreased, whereas the synthesis of triglyceride remained unchanged or increased (111). In another recent study (119), these authors reported that treatment of HepG2 cells with 25 mM glucose increased the synthesis of triglyceride without stimulating either the synthesis of cholesteryl ester or the secretion of apoB. These authors concluded that cholesteryl ester synthesis was the primary drive that influences the rate of apoB secretion. However, other interpretations of these data are possible. First, although both lovastatin and 58-035 decreased the rate of apoB secretion in oleate-treated cells, the rate of apoB secretion in these cells remained greatly enhanced compared to that observed in control cells (without oleate). Thus, oleate stimulation of triglyceride synthesis may have been quantitatively more important than the oleate-induced increase in cholesteryl ester synthesis. Second, although incubation of cells with glucose increased triglyceride but not apoB secretion, it is possible that triglycerides that are synthesized endogenously from glucose (through fatty acids synthesis) might affect apoB secretion differently than triglycerides that are synthesized from exogenous fatty acids. Therefore, although these two studies (111, 119) suggest that increased cholesteryl ester synthesis can influence apoB secretion from HepG2 cells, more evidence is required to support the hypothesis that cholesteryl ester synthesis is the primary drive.

Uncertainty about the role of cholesterol or cholesteryl ester synthesis or availability in the regulation of apoB secretion also derives from studies on the effects of inhibitors of HMG-CoA reductase on apoB production in cells not stimulated with fatty acids. Rather than decreasing apoB secretion, studies of the effects of inhibitors of HMG-CoA reductase have reported either stimulation or no effect on apoB secretion in rat hepatocytes (120) or HepG2 cells (121), respectively.

Recent studies on the regulation of apoB secretion in the perfused rat liver have focused on the coordinate roles that triglyceride and cholesterol (or its ester) may play in the assembly and secretion of apoB-containing LPs. Studies by Salam, Wilcox, and Heimberg (105) showed that increased availability of free fatty acids increased triglyceride secretion and particle size, but not apoB secretion (and therefore particle number) in fed rats. However, in fasted rats, increased fatty acid availability did increase the secretion of apoB (105). The results in hepatocytes from fasted rats are similar to those observed after treatment of HepG2 cells with oleate. On the other hand, Fungwe et al. (122) reported that apoB and triglyceride secretion were both increased in livers from rats fed increased amounts of cholesterol in the diet. In addition, although the surface ratio of phospholipid/free cholesterol of secreted VLDL remained the same, the core lipids (with triglyceride still remaining the primary core lipid) became more enriched in cholesteryl ester. The changes in core lipids are in accord with earlier observations reported by Davis and Malone-McNeal (123) using primary rat hepatocytes. In that study, however, there was no increase in apoB secretion. In HepG2 cells, excess cholesterol appears to have less of an effect on the secretion of apoB, especially when triglyceride synthesis in these cells is adequate or increased. These studies, overall, suggest that cholesterol or cholesteryl ester are available in HepG2 cells in quantities adequate to support apoB secretion at most levels of triglyceride synthesis: the availability of cholesterol or cholesteryl ester may only be rate limiting under certain conditions. Finally, the effects of delivery of LP cholesteryl esters to HepG2 cells may depend on the status of cholesterol metabolism in the cells and/or the particular LP used.

Regulation through phospholipid synthesis

Phospholipid availability is also required for high rates of secretion of apoB-containing LPs. Phosphatidylcholine is the predominate phospholipid in plasma LPs, although phosphatidylethanolamine, sphingomyelin, lysophosphatidylcholine, phosphatidylserine, and phosphatidylinositol are present in smaller quantities. Recent studies have indicated that although most phosphatidylcholine is made through the CDP-choline pathway, phosphatidylcholine in LPs may be derived from the phosphatidylserine/phosphatidylethanolamine pathway (124). Furthermore, it appears that secreted phospholipids are predominantly derived from newly synthesized pools (125), and that phosphatidylcholine, and not other phospholipids, is absolutely essential for VLDL secretion from rat hepatocytes (126, 127). Phospholipids, like triglycerides, are made in both rough and smooth ER (91). However, unlike triglyceride, phospholipids made in the ER have a rather long half-life (50). Therefore, while some minimal quantity of phospholipids appears to be required for the normal secretion of apoB-containing LPs, it appears likely that phospholipids would always be available, except in certain extreme metabolic conditions, for assembly into LPs. Thus, it is unlikely that phospholipids would regulate apoB secretion under most conditions. However, the possibility that the rapid effects of other lipids, particularly fatty acids, are mediated through the generation of specific phospholipids cannot be ruled out.

SUMMARY

A major theme of this review is that apoB secretion is regulated post-translationally, and that apoB secretion reacts rapidly to the current state of lipid metabolism in the cell. Therefore, as discussed by Fungwe et al. (122), the metabolism of triglyceride and of cholesteryl ester, in so far as both can be used as core lipids for apoB-containing LPs, are inextricably linked, and the shortage of one or both of these lipids could, by "allowing" increased intracellular degradation in the ER, inhibit the secretion of apoB.

Another theme in this review is that the regulation of apoB secretion may be quite different in rat hepatocytes compared to cultured cells (HepG2) used as a model for human hepatocytes. Exogenous fatty acids appear to modulate the rate of apoB secretion in HepG2 cells, whereas they have only minimal effects on apoB secretion in rat hepatocytes or liver. Increased dietary cholesterol, on the other hand, appears to be an important modulator of apoB secretion in rats, but the evidence for effects of cholesterol on apoB secretion in HepG2 cells is less convincing.

Finally, because HepG2 cells are an immortalized cell line, there could be many differences between these cells and human hepatocytes in vivo. Therefore, many of the results obtained with HepG2 cells should be corroborated in primary cultures of human hepatocytes. However, investigators utilizing primary human hepatocytes should be sure that the culture conditions are adequate to maintain the continued transcription of liver specific genes and to prevent the dedifferentiation of these cells in culture (85, 86).

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