The Late Addition of Core Lipids to Nascent Apolipoprotein B100, Resulting in the Assembly and Secretion of Triglyceride-rich Lipoproteins, Is Independent of Both Microsomal Triglyceride Transfer Protein Activity and New Triglyceride Synthesis*

Received for publication, August 3, 2001, and in revised form, November 2, 2001 Published, JBC Papers in Press, November 9, 2001, DOI 10.1074/jbc.M107460200

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Although microsomal triglyceride transfer protein (MTP) and newly synthesized triglyceride (TG) are critical for co-translational targeting of apolipoprotein B (apoB100) to lipoprotein assembly in hepatoma cell lines, their roles in the latter stages of lipoprotein assembly remain unclear. Using N-acetyl-Leu-Leu-norleucinal to prevent proteasomal degradation, HepG2 cells were radiolabeled and chased for 0–90 min (chase I). The medium was changed and cells chased for another 150 min (chase II) in the absence (control) or presence of Pfizer MTP inhibitor CP-10447 (CP). As chase I was extended, inhibition of apoB100 secretion by CP during chase II decreased from 75.9% to only 15% of control (no CP during chase II). Additional studies were conducted in which chase I was either 0 or 90 min, and chase II was in the presence of [3H]glycerol and either BSA (control), CP (inhibits both MTP activity and TG synthesis), BMS-1976360-1 (BMS) (inhibits only MTP activity), or triacsin C (TC) (inhibits only TG synthesis). When chase I was 0 min, CP, BMS, and TC reduced apoB100 secretion during chase II by 75.3, 73.9, and 53.9%. However, when chase I was 90 min, those agents reduced apoB100 secretion during chase II by only 16.0, 19.2, and 13.9%. Of note, all three inhibited secretion of newly synthesized TG during chase II by 80, 80, and 40%, whether chase I was 0 or 90 min. In both HepG2 cells and McA-RH7777 cells, if chase I was at least 60 min, inhibition of TG synthesis and/or MTP activity did not affect the density of secreted apoB100 lipoproteins under basal conditions. Oleic acid increased secretion of TG-enriched apoB100 lipoproteins similarly in the absence or presence of either of CP, BMS, or TC. We conclude that neither MTP nor newly synthesized TG is necessary for the later stages of apoB100-lipoprotein assembly and secretion in either HepG2 or McA-RH7777 cells.

The assembly of apolipoprotein B100 (apoB100) is a complex process that requires the coordinated assembly of core lipids (triglyceride (TG) and cholesteryl esters), and surface components (phospholipid, free cholesterol, and apoB100). Significant progress has been made in recent years concerning the hepatic formation and secretion of apoB100-containing very low density lipoproteins (VLDL) (1–3). Studies with primary rat hepatocytes and the rat hepatoma cell line McA-RH7777 have allowed investigators to characterize the assembly of lipoproteins with either full-length apoB100 or with apoB48, which in humans is only expressed in the intestine. Studies of B48-VLDL have indicated that it is assembled via two discontinuous lipidation steps in the endoplasmic reticulum (ER) (4–6). In the first step, apoB48 is associated with a small amount of lipid to form a primordial particle with high buoyant density. These high density lipoprotein (HDL) particles (designated B48-HDL) may be secreted from the cells if further lipid recruitment does not occur. Alternatively, the B48-HDL particle can undergo a second lipidation step, expanding its lipid content, primarily triglyceride, to form apoB48-VLDL. This conversion of B48-HDL into B48-VLDL is associated with increased synthesis of cellular lipid. The assembly of apoB100-VLDL also appears to involve a two-step pathway in McA-RH7777 cells (4, 7). ApoB100-VLDL, however, are mostly degraded intracellularly in McA-RH7777 cells in the absence of adequate lipid to stimulate their conversion to VLDL. Furthermore, characterization of each discrete stage of apoB100-VLDL formation in McA-RH7777 cells has been much less well developed than it has been for apoB48-VLDL formation (6, 8). It is also unclear if, in the presence of oleic acid, the assembly of apoB100-VLDL in HepG2 cells occurs in a single or a two-step process.

The assembly of apoB100-lipoproteins requires the initial translocation of apoB100 across the ER membrane, during which apoB100 interacts with a number of molecular chaperones, including Hsp70 in the cytosol, calnexin in the ER membrane, and microsomal triglyceride transfer protein (MTP) in the ER lumen (9–13). In the course of translocation, the hydrophobic apoB100 polypeptide is lipidated via an MTP-dependent process. Mutations that inactivate or abolish MTP activity are associated with the syndrome of abetalipoproteinemia, an almost total absence of apoB100-containing lipoproteins in the plasma (14, 15). Additionally, in liver-derived cells, inactiva-
tion of MTP with MTP-specific inhibitors abolishes the initial translocation of apoB100 across the ER, resulting in degradation of the nascent protein and absent secretion (16, 17). The role of MTP in the later, second-step lipidation of apoB100 is, however, much less certain. Thus, despite general agreement that MTP activity is required for efficient, initial translocation of apoB100 across the ER membrane in cultured cells (18, 19), its role in the addition of the majority of core lipid to nascent apoB100, and in particular to apoB100 in liver cells, during the later stages of lipoprotein assembly is much less clear (20, 21).

We have demonstrated previously that, in HepG2 cells, assembly and secretion of nascent (radiolabeled) apoB100-li-poproteins were not impaired by an MTP inhibitor if the inhibitor was added 60 min after the cells were pulse-labeled and then chased in the presence of a proteasome inhibitor (22). In contrast, if MTP was inhibited immediately after radiolabeling, assembly, and secretion of apoB100-lipoproteins were almost completely abolished. On the basis of these data, we speculated that there probably exists a window of time after the initiation of translation during which the dependence of apoB100 upon MTP for secretion diminishes. A similar model had been proposed for the formation and secretion of apoB48-VLDL in McA-RH7777 cells (8). The goal of this study was to extend our previous observation and explore, in detail, the role of MTP, as well as that of newly synthesized TG, in the assembly and subsequent secretion of apoB100-VLDL in both HepG2 and McA-RH7777 cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**N-Acetyl-Leu-Leu-norleucinal (ALLN), dimethyl sulfoxide, leupeptin, pepstatin A, and bovine serum albumin (fatty acid-free) were purchased from Sigma. MTP inhibitors (CP-10447, BMS-19567, and other compounds) were generous gifts of Pfizer Inc. (CP-10447) and Bristol-Squibb (BMS-19567), respectively. Sheep anti-human apoB100 polyclonal antibodies were from Roche. [3H]Glycerol and [35S]methionine/cysteine were from PerkinElmer Life Sciences. Protein A-Sepha-

**Growth of Cells—**HepG2 cells and McA-RH7777 cells, obtained from ATCC, were grown in six-well plates coated with collagen. HepG2 cells were maintained in MEM containing 0.1 mM nonessential amino acids, 10 mM Hepes, pH 7.5; and finally re-suspended and homogenized 10 times with a pre-chilled Dounce homogenizer in 3 ml of cold buffer containing 250 mM sucrose, 10 mM Hepes, pH 7.5, 0.5 mM dithiothreitol, 5 mg/ml trypsin inhibitor, 40 mM EDTA, 30 μM ALLN, 70 μl/ml PMSF, and 150 mM NaCl freshly added PMSF. The homogenate was spun at 1,900 g for 10 min at 4 °C. The supernatant was further spun at 50,000 rpm (Beckman TL-100) for 60 min at 4 °C to precipitate the microsomes. Microsome pellets were resuspended in 1 ml of 250 mM sucrose, 10 mM Hepes, pH 7.4, 2 ml of 0.0625% deoxycholate in PBS, and 0.5 ml of 0.1 sodium carbonate, with final pH 10.5. The mixture was incubated for 25 min at room temperature. To improve recovery of radiolabeled apoB100, albumin was added to get a final concentration of 0.75%, and the incubation was extended for 5 min. The mixture was then adjusted to contain 12.5% sucrose, 1× PBS, and 150 mM NaCl for sucrose gradient ultracentrifugation.

**Sucrose Gradient Ultracentrifugation—**Sucrose gradient ultracentrifugation of apoB-containing lipoproteins were conducted as described (8). Briefly, pulse-chased HepG2 or McA-RH7777 cells were washed and harvested with cold PBS. The cell pellets were further washed once with cold 150 mM sucrose, 10 mM Hepes, pH 7.5, and finally re-suspended and homogenized 10 times with a pre-chilled Dounce homogenizer in 3 ml of cold buffer containing 250 mM sucrose, 10 mM Hepes, pH 7.5, 0.5 mM dithiothreitol, 5 mg/ml trypsin inhibitor, 40 mM EDTA, 30 μM ALLN, 70 μl/ml PMSF, and 150 mM NaCl freshly added PMSF. The homogenate was spun at 1,900 g for 10 min at 4 °C. The supernatant was further spun at 50,000 rpm (Beckman TL-100) for 60 min at 4 °C to precipitate the microsomes. Microsome pellets were resuspended in 1 ml of 250 mM sucrose, 10 mM Hepes, pH 7.4, 2 ml of 0.0625% deoxycholate in PBS, and 0.5 ml of 0.1 sodium carbonate, with final pH 10.5. The mixture was incubated for 25 min at room temperature. To improve recovery of radiolabeled apoB100, albumin was added to get a final concentration of 0.75%, and the incubation was extended for 5 min. The mixture was then adjusted to contain 12.5% sucrose, 1× PBS, and 150 mM NaCl for sucrose gradient ultracentrifugation.

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**RESULTS AND DISCUSSION**

Previous studies in our laboratories revealed that, in HepG2 cells, if apoB100 degradation by the proteasome was prevented by inhibitors such as ALLN or lactacystin, nascent apoB100 could be found associated with the translocon for a prolonged period of time (as long as 2 h) after translation should have been completed (22). Additionally, despite being physically close to Seef1 (the major translocon protein) as determined by cross-linking studies, and being significantly ubiquitinated, apoB100 could be secreted rapidly after stimulation of cellular lipid synthesis with oleic acid. Finally, in those studies, we observed that, after a 1–2-h chase period (which was enabled by the use of proteasome inhibitors), apoB100 secretion appeared to be independent of MTP (22). On the basis of these data, in the present study, we conducted additional experiments to 1) confirm our prior observation that apoB100 secre-
Fig. 1. General protocol. This depiction of the general protocol used in the experiments presented in the following studies demonstrates the range of time from radiolabeling to the end of chase I. Chase II was constant in duration for all experiments, but differed by the type of inhibitor that was added: CP-10447, BMS 197636-01, triacsin C. In some experiments, [3H]glycerol was added at the start of chase II. The proteasome inhibitor, ALLN, was present in the medium throughout the entire experiment.

The Dependence of ApoB100 Secretion on MTP Diminishes as the Time from Translation Increases—To fully characterize the time frame during which assembly and secretion of apoB100-lipoproteins changes from an MTP-dependent to an MTP-independent process, we designed a protocol with two chase periods. Subconfluent HepG2 cells were first pre-incubated with 1.5% BSA for 60 min and then treated with both BSA and ALLN for an additional 60 min prior to radiolabeling. ALLN was present throughout the remainder of the protocol. After 20 min of radiolabeling with [35S]methionine/cysteine, there were two chase periods, I and II. Chase I varied from 0 to 90 min (see Fig. 1). No MTP inhibitor was applied in chase I. Chase I medium was replaced by new medium with or without 25 μM Pfizer MTP inhibitor CP-10447, and chase II was carried out for an additional 150 min. The chase II medium was collected to determine 35S-labeled apoB100 secretion in the presence or absence of MTP-inhibition. The data are presented as the percentage of apoB100 secreted during chase II as the fraction secreted from cells treated with CP-10447 versus nontreated cells during chase I were: chase I = 0 min, 4,935/20,512; chase I = 15 min, 11,808/21,104; chase I = 30 min, 11,682/16,870; chase I = 45 min, 12,204/18,930; chase I = 60 min, 11,313/22,527; chase I = 75 min, 10,112/13,372; chase I = 90 min, 8,424/9,910. The fall in absolute counts/min in untreated cells (denominators) between 45 and 90 min of chase I results from secretion of apoB during the increasing chase I times. B, the graph depicts the results of three separate experiments (mean ± S.D.) (the S.D. values of the first 3 points were to small to be presented in the graph). The data are presented as the percentage of apoB100 secreted during chase 2 from cells treated with the MTP inhibitor compared with cells treated with only MeSO. The ability of MTP to inhibit apoB100 secretion during chase II diminished significantly as the duration of chase I increased.

We found (Fig. 2A) that if chase I was 0 min (i.e. the medium was changed immediately after radiolabeling), then apoB100 secretion in the presence of CP-10447 during the succeeding 150-min chase II was only 24.1 ± 0.8% of that measured for control cells not treated with CP-10447 during chase II. However, as the duration of chase I progressively increased, i.e. up to 90 min, apoB100 secretion during chase II, in the presence of CP-10447, increased linearly to 50.2 ± 1.4%, 55.9 ± 2.0%, 64.5 ± 5.1%, 69.2 ± 6.1%, 75.6 ± 7.1%, and 85.0 ± 7.6% of the secretion rate of the control cells that were untreated during chase II. In other words, inhibition of apoB100 secretion during chase II decreased from ~76%, if the MTP inhibitor was added immediately after labeling (i.e., chase I was 0 min), to only 15%, if the MTP inhibitor was added 90 min after labeling (Fig. 2B). We also used a higher dose of CP-10447, i.e. 50 μM, and obtained very...
similar results (data not shown). This experiment indicated that an MTP inhibitor gradually loses its effect on the assembly and secretion of apoB100-lipoproteins when it is added to the media at longer and longer times after the synthesis of apoB100 has been initiated. Based on our recent studies (22, 25), when chase I was 60 min or greater, most newly synthesized apoB100 in these HepG2 cells would be almost fully translated, but would still be associated with the translocon, ribosomes, Hsp70, and the proteasome. It appears that this is the pool of apoB100 that has lost its dependence on MTP activity for secretion.

These data are similar to those reported by Rustaeus et al. (8) using brefeldin A (BFA) to inhibit lipoprotein assembly in McA-RH7777 cells during an initial chase period. In that study, the authors found that inhibition of MTP within 15 min of labeling resulted in a marked decrease in apoB100 secretion, but that this effect was significantly reduced if MTP was inhibited after longer chase periods. Their protocol differed from ours in that they used BFA to inhibit the movement of nascent apoB100 from the ER membrane into the ER lumen. The mechanism whereby BFA reversibly inhibits the assembly of apoB100-lipoproteins is unclear, although a recent report from the same group suggests that BFA-mediated inhibition of intracellular vesicular trafficking may be important (26).

Neither Inhibition of MTP nor Inhibition of TG Synthesis Affects ApoB100 Secretion during the Later Stages of Lipoprotein Assembly—Although published data suggested that CP-10447 did not inhibit TG synthesis in HepG2 cells (16), we observed during the present studies that CP-10447 did inhibit TG synthesis in HepG2 cells. HepG2 cells were preincubated with 1.5% BSA for 60 min, then labeled for 2 h with 10 μCi/ml [3H]glycerol in the presence of increasing doses of CP-10447. The results in Fig. 3 demonstrate that synthesis of TG was significantly inhibited by CP-10447 at a concentration of 25 μM, which also effectively inhibited apoB100 secretion (see Fig. 2A, chase I; 0 min). This effect of CP-10447 appeared to be specific, as there was no concomitant inhibition of phospholipid synthesis during these experiments (data not shown). Furthermore, the inhibition of TG synthesis by CP-10447 was dose-dependent. We also labeled HepG2 cells in the presence of 25 μM CP-10447 for 1–6 h, finding that this agent significantly inhibited TG synthesis during that entire time course (data not shown).

Because CP-10447 inhibits both MTP lipid transfer activity (referred to as “MTP activity”) (16) and TG synthesis (Fig. 3), we studied other drugs, which inhibited either MTP activity only or TG synthesis only. We chose the Bristol Myers Squibb MTP inhibitor, BMS-197636-01, which only inhibits MTP activity (27), and the fatty acyl-CoA synthetase inhibitor, triacsin C, which inhibits only TG synthesis (28). We followed the general protocol depicted in Fig. 1, but chose the two extremes...
of chase I: 0 or 90 min. In addition, [3H]glycerol was added to the media in chase II to determine the role of newly synthesized TG under each condition. The results, depicted in Fig. 4A, showed that if chase I was 0 min, CP-10447 (25 μM) not only reduced apoB100 secretion in chase II (24.7 ± 1.3% of control cells not treated with CP-10447 during chase II), it also reduced cellular TG synthesis (22.3 ± 2.1% of control) and secretion of newly synthesized TG (20.0 ± 2.0% of control) during chase II. By comparison, although BMS-197636-01 (0.1 μM) when added after a chase I of 0 min, similarly reduced the secretion of both apoB100 (26.1 ± 0.1% of control) and newly synthesized TG (26.1 ± 4.1% versus control) during chase II, it did not affect TG synthesis during chase II at all (114.8 ± 6.8% of control). However, when triacsin C (4 μM) was added after a 0-min chase I, it acted like CP-10447 and reduced apoB100 secretion (46.1 ± 7.4% of control), TG synthesis (49.2 ± 6.6% of control), and secretion of newly synthesized TG (62.1 ± 4.9% of control) during chase II. These studies demonstrated that inhibition of either MTP activity or TG synthesis (or both) during the co-translational and early stages of apoB100-lipoprotein assembly (chase I equal to 0 min) results in significant inhibition of both apoB100 and TG secretion. Furthermore, inhibition of TG secretion during chase II was observed with the MTP inhibitors whether or not they inhibited TG synthesis.

In sharp contrast, if chase I was 90 min (Fig. 4B), none of the three reagents, CP-10447, BMS-197636-01, or triacsin C, significantly impaired apoB100 secretion during chase II: Inhibition was only 16.0, 19.2, and 13.9%, respectively, compared with cells not receiving any of these treatments during chase II. Importantly, when chase I was 90 min, the effects of each reagent on both TG synthesis and secretion during chase II were identical to those observed when chase I was 0 min. These results showed very clearly that during the later stages of apoB100-lipoprotein assembly (chase I equal to 90 min), when apoB100 is still associated with the translocon (22), secretion of apoB100 is unaffected even when both MTP activity and new TG synthesis are effectively inhibited. Of particular interest was the concomitant finding that secretion of newly synthesized TG (as measured by [3H]glycerol labeling of TG during chase II) was inhibited to the same degree by all reagents irrespective of the length of chase.

Thus, although apoB100 secretion during the late post-translational period was independent of MTP activity, the integration of newly synthesized TG molecules as components of the core of the apoB100-lipoproteins was clearly dependent on the presence of MTP activity during this time period.

Although our results for apoB100 in HepG2 cells are compatible with the recent studies of Rustaeus et al. (8) in which membrane-associated, poorly lipidated apoB100-lipoproteins are converted to VLDL by oleic acid treatment of McA-RH7777 cells, those investigators did not determine whether newly synthesized TG was needed for the conversion of lipid-poor apoB100-lipoproteins to VLDL. Effects of MTP inhibition on apoB100 secretion that were similar to ours were also reported by Wang et al. (21). In their study of McA-RH7777 cells, inhibition of MTP before radiolabelling with [35S]methionine/cysteine was associated with a 90% inhibition of apoB100 se-
creatin, whereas inhibition of MTP following a 2-h radiolabeling period reduced apoB100 secretion by only 45%. Although their protocol differed from ours in that there was no “chase I,” the fact that the radiolabeling protocol used by Wang et al. (21) lasted 2 h means that a significant proportion of apoB100 molecules were in the later stages of intracellular processing prior to the addition of the MTP inhibitor. We believe, therefore, that the diminished effect of the MTP inhibitor on apoB100 secretion observed by Wang et al. after a long radiolabeling period partially mimics our protocol when chase I was 90 min. Overall, therefore, our results extend the findings in McA-RH7777 cells by both Rustaeus et al. (8) and Wang et al. (21) to HepG2 cells. However, we have also demonstrated that the change from MTP-dependent to MTP-independent secretion occurs in the absence of brefeldin A and/or olate supplementation.

Neither Inhibition of MTP nor Inhibition of TG Synthesis/Secretion in the Later Stages of Lipoprotein Assembly Changes the Density Pattern of ApoB100-lipoproteins—Our results (Fig. 4) indicate that, although neither MTP inhibitors nor the TG-synthesis inhibitor, triacsin C, affected the secretion of apoB100 that has been in the cell for a significant length of time (chase I, 90 min), they all certainly inhibited the secretion of newly synthesized TG during that period. This observation raised the following question: what was the density of the apoB100-lipoproteins secreted in these experiments?

To initially address this question, we used our general protocol in HepG2 cells, except that chase I was either 0 or 60 min (preliminary experiments demonstrated that the same differences versus chase I equal to 0 min were obtained when chase I was either 60 or 90 min, so only 60 min was compared with 0 min for convenience). After chase I, the medium was removed and new medium was applied during chase II, which was still 150 min. The medium of chase II was collected and subjected to sucrose-gradient ultracentrifugation to determine the density of the secreted lipoproteins. Each of 12 fractions (1 ml/fraction) was used to isolate apoB100 by immunoprecipitation and then further analyzed by SDS-PAGE. We thereby determined the distribution of apoB100-lipoproteins secreted during chase II, when chase I was either 0 or 60 min. The fluorograph of apoB100 in each fraction demonstrated that, whether chase I was 0 or 60 min, apoB100-lipoproteins were secreted over the same wide range of density during chase II (Fig. 5A). The overall decrease in the intensities of the bands for all density fractions when chase I was 60 min, compared with when chase I was 0 min, results from the secretion of some apoB100 during the 60-min chase I period (data not shown). With either duration of chase I, however, the majority of secreted apoB100-lipoproteins isolated during chase II floated in the range of IDL/LDL, although some VLDL and HDL were present as well.

The finding of a similar distribution of apoB100 across the full lipoprotein density range secreted during chase II irrespective of the length of chase I speaks against the possibility that the MTP-independent secretion we observed after 60–90 min of chase I resulted from the formation of fully formed, TG-rich apoB100-lipoproteins during that chase I period. Rustaeus et al. (8) demonstrated in McA-RH7777 cells that after two 30-min chase periods, when VLDL formation (after addition of oleate) was MTP-independent, the majority of the apoB100 still came from the same membrane-associated, lipid-poor pool that had earlier been dependent on MTP for the addition of bulk lipid. Therefore, we also studied microsome-associated apoB100-lipoproteins after different durations of chase I using the protocol of Rustaeus et al. (8). As mentioned earlier, ALLN was present during pretreatment, labeling, and chase. HepG2 cells were pulsed with [35S]methionine/cysteine for 20 min, then chased either 10 or 60 min.apoB100 was extracted from microsomes after each of these chase I periods. After 10 min of chase I, a period of time adequate for completion (or near completion (Ref. 25)) of the translation of most 35S-labeled apoB100, ∼92% of microsome-associated apoB100-lipoproteins were in the form of poorly lipidated HDL-density particles (Fig. 5B). After 60 min of chase I, ∼80% of the extracted apoB100-lipoproteins were still in the form of poorly lipidated HDL-density particles. These results confirm, in HepG2 cells, the findings of Rustaeus et al. (8) in McA-RH7777 cells. Furthermore, they indicate that the TG-enriched VLDL, IDL, and LDL density apoB100-lipoproteins that are secreted long after labeling of the cells (i.e. during chase II after a long chase I; see Fig. 5A), when secretion is independent of both MTP activity and new TG synthesis, are not the products of early, rapid conversion of lipid-poor apoB100 to TG-rich apoB100-lipoproteins within the ER (during chase I). Indeed, a comparison of secreted apoB100-lipoproteins (Fig. 5A) with intracellular apoB100-lipoproteins (Fig. 5B), irrespective of the duration of chase I, makes it clear that secretion must occur very quickly after conversion of HDL-density particles to VLDL/IDL/LDL-density particles.

To demonstrate directly that neither MTP activity nor new TG synthesis is required for the secretion of TG-rich apoB100-lipoproteins during the later stages of lipoprotein assembly, we explored the effects of the MTP inhibitor, BMS 197636-01, and the inhibitor of TG synthesis, triacsin C, on the density distribution of apoB100-lipoproteins secreted from HepG2 cells during this period. As expected, if chase I was 0 min (control) (Fig. 6A), either BMS 197636-01 or triacsin C significantly reduced the secretion and changed the density distribution of apoB100-lipoproteins secreted during chase II. The proportion of apoB100 that was secreted as VLDL/IDL/LDL during chase II was greatly reduced when either MTP activity or new TG synthesis was inhibited immediately after radiolabeling. However, if chase I was 60 min (Fig. 6B), neither inhibitor had an effect on either the quantity or the density distribution of
apoB100-lipoproteins secreted in chase II. The effect of CP-10447 on apoB100-lipoprotein density distribution was similar to that of BMS 197636-01 (data not shown). It is important to reiterate that, under these conditions (chase I equal to 60 min), secretion of newly synthesized TG was inhibited by each drug. The lack of effect on the density of secreted apoB100 must mean that previously synthesized TG was available for secretion despite inhibition of MTP and/or TG synthesis during chase II.

![Fig. 7](http://www.jbc.org/)

**Fig. 7.** When oleic acid is added during the later stages of lipoprotein assembly in HepG2 cells, it stimulates the secretion and shifts the density of apoB100-lipoproteins even when MTP activity or TG synthesis is inhibited. HepG2 cells were treated as described in Fig. 5A, except that either 0.4 mM oleic acid (OA) (A), or OA + BMS-197636-01 (0.1 μM) (B), or OA + triacsin C (4 μM) (C), was present during chase II. Media were collected after chase II and analyzed by sucrose gradient ultracentrifugation and apoB100-immunoprecipitation. The results are representative of repeated experiments; the addition of OA after 60 min of chase I caused a shift to more TG-enriched apoB100-lipoproteins even when either MTP activity or TG synthesis was inhibited.

apoB100-lipoproteins secreted in chase II. The effect of CP-10447 on apoB100-lipoprotein density distribution was similar to that of BMS 197636-01 (data not shown). It is important to reiterate that, under these conditions (chase I equal to 60 min), secretion of newly synthesized TG was inhibited by each drug. The lack of effect on the density of secreted apoB100 must mean that previously synthesized TG was available for secretion despite inhibition of MTP and/or TG synthesis during chase II.

The results presented in Figs. 5 and 6 indicate clearly that, in HepG2 cells, the assembly and secretion of VLDL/IDL/IDL particles is initially dependent on both MTP activity and concurrent TG synthesis but that this dependence diminishes and then disappears over time. However, under basal conditions (incubation of cells in BSA), apoB100-lipoproteins secreted from HepG2 cells are mostly in the IDL and LDL density range (only modestly lipated), raising the possibility that the secretion we have observed when MTP activity and/or TG synthesis has been inhibited under basal conditions may not be relevant to the in vivo situation where predominantly VLDL is assembled and secreted. Indeed, Wang et al. (27) recently concluded that MTP activity is required at all stages of the assembly and secretion of VLDL from McA-RH7777 cells. Because oleic acid is known to stimulate the secretion of both total apoB100 and more buoyant triglyceride-rich apoB100-lipoproteins from HepG2 cells (23), we examined the effects of MTP inhibition and/or inhibition of concurrent TG synthesis on the ability of oleic acid to stimulate the secretion of lipid-enriched apoB100-lipoproteins during the later stages of lipoprotein assembly. As expected, oleic acid alone increased the total apoB100 secretion in the late stage by ~40% and shifted the secreted apoB100 to more TG-rich particles (Fig. 7A). However, oleic acid also increased total apoB100 secretion and shifted the density distribution of secreted apoB100-lipoproteins to more TG-rich lipoprotein particles, in the presence of either BMS 197636-01 (Fig. 7B) or triacsin C (Fig. 7C).

Finally, to demonstrate that our findings were not restricted to HepG2 cells, we repeated the last series of studies in McA-RH7777 cells. Compared with control cells incubated in serum-free DMEM with 1.5% BSA, McA-RH7777 cells incubated in the same medium plus 0.4 mM oleic acid during chase II secreted ~75% more apoB100; the presence of an inhibitor of MTP had no effect on the ability of oleic acid to stimulate
apoB100 secretion (data not shown). More importantly, as depicted clearly in Fig. 8, the density distribution of apoB100-lipoproteins secreted from McA-RH7777 cells, during chase II, when oleic acid was added 60 min after radiolabeling of the cells (chase I equals 60 min) was also not affected by the co-addition of either BMS 197636-01 (top panel) or Pfizer CP-10447. Thus, inhibiting MTP alone (BMS inhibitor; top panel), or inhibiting both MTP and TG synthesis (Pfizer inhibitor; bottom panel), did not reduce the ability of oleic acid to stimulate the assembly and secretion of VLDL by McA-RH7777 cells.

How can our present results be compatible with the recent studies by Wang et al. (27), where the final step in the formation of TG-rich VLDL particles in McA-RH7777 cells appeared to be dependent on MTP activity? Some insight into this issue can be gained from an earlier paper by that group (21), as well as a recent study by Hebbachi et al. (29). In their earlier study, Wang et al. (21) used a protocol in which cells were labeled with [3H]glycerol just prior to inhibition of MTP and then secretion was stimulated by oleic acid. They observed that post-labeling inhibition of MTP was associated with reductions in the incorporation of "pre-labeled TG" into apoB48 VLDL and apoB100 VLDL, although the effect was greater with apoB48 VLDL. There results are compatible with the requirement for MTP activity for the delivery of cytosolic TG to the ER lumen, where it can be added to the lipid-poor apoB100-lipoprotein. In the study by Hebbachi et al. (29), the investigators concluded that inhibition of MTP activity resulted in an inability to incorporate "pre-synthesized" TG in apoB100-lipoproteins. Importantly, Hebbachi et al. (29) also noted that inhibition of MTP resulted in reduced transfer of membrane-bound apoB100 to the lumen of the ER and Golgi. Most relevant to our present results is the recent observations made by Raabe et al. (30) in mice with a liver-specific gene-targeted deletion of MTP. In these animals, there was a complete absence of VLDL-sized lipid droplets in either the ER or the Golgi apparatus. The authors concluded that MTP was necessary for the generation of these droplets and suggested that the TG that was added to lipid-poor apoB100-lipoproteins during the "second step" might need MTP to gain access to the lumen of the ER.

All of the above, we believe, allow for a unified model that is compatible with our results and with the recent studies of Rustaeus et al. (8), and Wang et al. (27). Data from a number of laboratories indicate that MTP activity and concurrent TG synthesis appear to be required for the targeting of nascent apoB100 for translocation and initial lipidation; this statement is supported by tissue culture data (11, 17, 27, 31), by studies in whole animals (30, 32, 33), and by the syndrome of abetalipoproteinemia in humans (14, 15). Based on extraction studies, it appears that the poorly lipidated apoB100 that is generated at this early stage is mainly in the form of an "HDL-like" particle that is still associated with the ER membrane (7, 8, 29). Our demonstrations that nascent apoB100 is still associated with Sec61 after 60 min of chase (22) and that this apoB100 is mainly in a membrane extractable form as a HDL-like particle (Fig. 5) are also consistent with this scheme. What happens next is not fully characterized at the molecular level, but it is apparent that "HDL-like apoB100" is converted to a more TG-rich lipoprotein (VLDL in primary hepatocytes; VLDL, IDL, or LDL in hepatic cell-lines depending on the presence of oleic acid) just prior to secretion. Very recent studies by Asp et al. (26) and Tran et al. (34) indicate that vesicular transport and/or phospholipid hydrolysis may be important in the conversion, or second, step. In the report by Tran et al., the authors suggested that oleic acid stimulation of VLDL assembly and secretion might be linked to the generation of oleoyl-enriched phospholipids which would signal membrane changes important to the movement of a TG-rich droplet into the ER lumen. If, as is generally accepted, MTP activity is required to generate a TG-rich droplet in the ER lumen (21, 27, 29, 30), our finding that MTP inhibition did not affect oleic acid stimulation of VLDL assembly and secretion might argue against such a mechanism.

Our present results, together with prior studies, suggest that the second step in the assembly of apoB100-lipoproteins, which involves the bulk addition of core lipid to the HDL-like apoB100 particle, actually has two components (or steps), one that is MTP dependent and one that is MTP-independent. As noted above, the data from Wang et al. (21, 27), Hebbachi et al. (29), and Raabe et al. (30) indicate that MTP is required for the availability of the ER luminal lipid droplets that will be incor-
Lipids

0.0

oleic acid accelerates the emptying of TG from the secretory pathway suggested by Olofsson already in the ER (30), waiting to fuse with a lipid-poor apoB100-lipoprotein during the later stages of lipoprotein assembly. Our data indicate that the actual addition of lipid to the HDL-like apoB100 particles. On the other hand, our data indicate that the actual addition of lipid to the apoB100-lipoprotein during the later stages of lipoprotein assembly is independent of MTP. Our finding that this final fusion of apoB100 with a lipid droplet is also independent of concurrent TG synthesis indicates, as suggested by others (29), that the droplets are synthesized at an earlier time and are already in the ER (30), waiting to fuse with a lipid-poor apoB100-lipoprotein. This model, which is similar to one suggested by Olofsson et al. (3) depicted in Fig. 9, implies that MTP activity and TG synthesis are critical at two separate stages of apoB100-lipoprotein assembly: first to facilitate the initial translocation of nascent apoB100 across the ER membrane, and second to ensure the availability of a bulk form of core lipid needed for the later stages of lipoprotein assembly. In contrast, the final step in the assembly of the TG-enriched apoB100-lipoprotein, that is the actual addition of bulk core lipids to a minimally lipidated apoB100, seems to be independent of both MTP activity and TG synthesis.

The studies presented here, although extending and unifying prior data, still leave several questions unanswered. For example, does the bulk addition of lipid occur while apoB100 is still associated with the ER membrane, resulting in the “extraction” of apoB100 from that membrane, or after an HDL-like apoB100 particle separates itself from the membrane? Our recent observation that a large proportion of intracellular apoB100 exists in an almost completely translated state that is still associated with ribosomes suggests that addition of bulk lipid may be a “signal” for completion of translation/translocation and extraction of apoB100 from the ER membrane (25). Another unanswered question derives from our finding that concurrent TG synthesis is not required for the oleate-induced increase in the incorporation of core lipid into poorly lipidated apoB100 (Fig. 7). What does oleic acid do, therefore, to stimulate this process? Is it acting via signal transduction at this stage rather than as a component of core lipid? Further studies will be needed to address these important questions.

Note Added in Proof—Zammit and Lankester recently reported that oleic acid accelerates the emptying of TG from the secretory pathway (Zammit, V. A., and Lankester, D. L. (2001) Lipids 36, 607–612).

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The Late Addition of Core Lipids to Nascent Apolipoprotein B100, Resulting in the Assembly and Secretion of Triglyceride-rich Lipoproteins, Is Independent of Both Microsomal Triglyceride Transfer Protein Activity and New Triglyceride Synthesis

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J. Biol. Chem. 2002, 277:4413-4421. doi: 10.1074/jbc.M107460200 originally published online November 9, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107460200

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