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Intracellular mechanisms mediating the inhibition of apoB-containing lipoprotein synthesis and secretion in HepG2 cells by avasimibe (CI-1011), a novel acyl-coenzyme A: cholesterol acyltransferase (ACAT) inhibitor

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Abstract

We have studied the cellular and molecular mechanisms involved in the suppression of apoB secretion from HepG2 cells following incubation with avasimibe (CI-1011), a novel inhibitor of acyl-coenzyme A: cholesterol acyltransferase (ACAT). Cellular lipid analysis revealed that avasimibe significantly decreased the synthesis of cholesterol and cholesteryl ester, and, at higher doses, of triglyceride. Time-course trypsin protection assays revealed that avasimibe induced the accumulation of translocationally arrested apoB intracellularly. Pulse-chase studies showed that the treatment with avasimibe induced a >75% decrease in apoB secretion relative to control, but initially enhanced the protein stability and cellular accumulation of apoB. Subcellular fractionation of microsomes further confirmed the accumulation of secretion-incompetent apoB-lipoproteins in the endoplasmic reticulum (ER) and Golgi compartments of avasimibe-treated HepG2 cells. Although incubation of drug-treated cells with carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132), a potent proteasome inhibitor, increased cellular apoB (70%), it failed to increase apoB secretion. Drug treatment induced an accumulation of secretion-incompetent apoB-containing lipoprotein particles, the majority of which demonstrated a density in a range similar to that of high-density lipoprotein. However, studies in permeabilized cells demonstrated that, at longer chase times, intracellularly accumulated apoB was eventually degraded, indicating that the inhibition of degradation may be transient. Oleate treatment of avasimibe-treated cells partially restored apoB secretion but not to the levels seen in control cells. In summary, we hypothesize that avasimibe acutely blocks the secretion of apoB and its associated lipoproteins from HepG2 cells, transiently enhancing its membrane association and cellular accumulation with eventual intracellular degradation of accumulated apoB.

Keywords: ACAT; Avasimibe; Lipoproteins; Liver; Cholesteryl ester; Apolipoprotein B; Degradation

1. Introduction

ACAT (E.C. 2.3.1.26) catalyzes the formation of cholesteryl esters from cholesterol and long-chain fatty acyl-coenzyme A [1,2]. It has been suggested that ACAT plays a role in hepatic cholesterol homeostasis by preventing excessive accumulation of free cholesterol in intracellular membranes [3–5]. Cholesteryl esters are also found in the core of VLDL particles assembled and secreted by mammalian hepatocytes. There is a growing body of evidence on the role of the core and surface lipid components of VLDL in protecting apoB from cellular degradation and facilitating its maturation and subsequent secretion from hepatocytes. Failure to mobilize sufficient lipid to complete either of these stages in the assembly process results in apoB degradation [6]. The intracellular availability of phospholipid [7], of triglyceride [8–12], and of cholesterol esters [13–15] all have been suggested as important determinants of hepatic apoB secretion. There are different functional pools of cholesterol in the hepatocyte, including a metabolically active pool, an ACAT substrate pool, and a cholesteryl ester pool [16]. The role of ACAT and cholesteryl esters in apoB secretion has been controversial.

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Abbreviations: ACAT, acyl-coenzyme A: cholesterol acyltransferase; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; apoB, apolipoprotein B; CSK, cytoskeletal; DTT, dithiothreitol; ER, endoplasmic reticulum; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PMSF, phenylmethylsulfonyl fluoride; VLDL, very-low-density lipoprotein.

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Cholesteryl ester is believed to be an important substrate for microsomal triglyceride transfer protein (MTP) [17] and may have a role in the protection of newly synthesized apoB from degradation [18–22]. *In vitro* studies have shown that ACAT inhibitors reduce apoB100 secretion in primary rabbit hepatocytes [18,23], primary rat hepatocytes [13], and HepG2 cells [19–21,24–27]. Several *in vivo* studies in miniature pigs [28–30], rabbits [31–33], rats [31,32], hamsters [32], and monkeys [22,32,34] have confirmed these *in vitro* observations. In contrast, several studies have argued against any regulatory role of cholesteryl ester in apoB secretion. For example, Graham and colleagues [35], using HepG2 cells and two different ACAT inhibitors, showed that cholesterol esterification is not essential for apoB secretion. Wu and colleagues [11] have reported that blocking of the stimulatory effect of oleic acid on apoB secretion in HepG2 cells occurred without affecting the stimulation of cholesteryl ester synthesis. They concluded that in HepG2 cells cholesteryl ester synthesis is not rate limiting for apoB secretion. Similar conclusions were reported by Furukawa and Hirano [10] and Sato *et al.* [36]. Ooyen *et al.* [37] reported decreased intracellular degradation and increased apoB secretion following incubation of HepG2 cells with an ACAT inhibitor (Sandoz 58-035). There are several possible explanations for the above discrepancies. Since most of the studies were conducted using ACAT inhibitors with completely different physico-chemical and structural properties, it is difficult to compare the efficacy of different ACAT inhibitors without knowing their bioequivalency and any possible pleiotropic effects (for structure–activity reviews, refer to [38,39]). Additional discrepancies may arise from the choice of animal models and cell types (primary versus cell line) with different cholesterol and cholesteryl ester pool sizes. Furthermore, analysis of newly synthesized versus the total mass of cholesteryl ester may be another source of contradictory observations.

In the present study, we have investigated molecular mechanisms involved in the synthesis, stability, assembly, and secretion of apoB100-containing lipoproteins in HepG2 cells following incubation with avasimibe, a novel ACAT inhibitor. Avasimibe has been shown to lower plasma total cholesterol in chow-fed rats [40], casein-fed rabbits [40], and hypercholesterolemic hamsters [41], and to decrease lipoprotein(a) and apoB100 levels both *in vivo* and *in vitro* in cynomolgus monkeys [34]. More recently, in miniature pigs, Burnett *et al.* [30] reported that avasimibe significantly decreased plasma VLDL-apoB levels and VLDL-apoB secretion as well. From the same laboratory, Wilcox *et al.* [27] reported a significant dose-dependent reduction of apoB secretion in HepG2 cells in the presence of avasimibe. In the present study, we further investigated the intracellular mechanisms by which ACAT inhibition influences the assembly and secretion of apoB-containing lipoproteins, focusing on the intracellular mechanisms of action of this inhibitor.

2. Materials and methods

2.1. Materials

HepG2 cells (ATCC HB 8065) were obtained from the American Type Culture Collection. Fetal bovine serum (certified grade) and cell culture medium were from Life Technologies Inc. Culture dishes and plates were obtained from Sarstedt. BSA, leupeptin, pepstatin, PMSF, trypsin (tissue culture grade), soybean trypsin inhibitor, albumin and α1-antitrypsin antiserum, rabbit anti-goat IgG, cycloheximide, puromycin, and other common laboratory reagents were from the Sigma, and ALLN were obtained from Calbiochem. Trasylol (aprotinin) was from Bayer. Ultrapure electrophoresis reagents were from Bio-Rad. [^{3}H]Oleate, [^{3}H]acetate, and [^{35}S]methionine protein labeling mixtures were purchased from DuPont, Canada. Prestained protein standards (rainbow markers) and Amplify were purchased from Amersham International. Polyclonal apoB antibodies were obtained from Medix-Biotech and purified in the laboratory. Immunoprecipitin (*Staphylococcus aureus* extract) was prepared in the laboratory.

2.2. Cell culture

Monolayer cell cultures were maintained in α-MEM in culture flasks or multiwell dishes containing 10% fetal bovine serum. Cells were grown in 35- or 100-mm dishes at 37°, 5% CO_2 in complete medium (α-MEM, 10% fetal bovine serum). Cultures were allowed to reach 80–100% confluency before experiments were performed.

2.3. Analysis of cellular lipid synthesis

To assess the rate of cholesterol and cholesteryl ester synthesis, HepG2 cells were pulsed for 6 hr with 5 μCi/mL of [^{3}H]acetate. Triglyceride synthesis was monitored by labeling cells for 6 hr with 5 μCi/mL of [^{3}H]oleate bound to BSA. Labeled cells were treated with hexane:isopropanol (3:2) to extract total lipid. Extracted total lipid was dried, suspended in hexane, and applied to a thin-layer chromatogram. The TLC plates were developed using a two-solvent system to separate polar lipids with chloroform:methanol:acetic acid:formic acid:H_2O (70:30:12:4:2) and neutral lipids with petroleum ether:ethyl ether:acetic acid (90:10:1). The lipids were stained with iodine vapor and identified based on comparison to known lipid standards (Sigma). The spots identified on the TLC plates were cut and counted using a scintillation counter.

2.4. Metabolic labeling of intact HepG2 cells

HepG2 cells were preincubated in methionine-free MEM at 37° for 1 hr and pulsed with 100 μCi/mL of [^{35}S]methionine for 1 hr. Following the pulse, the cells

were washed twice and chased in complete medium supplemented with 10 mM methionine. At various chase times, duplicate or triplicate dishes were harvested, and cells were lysed in solubilization buffer (PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM leupeptin, 2 μg/mL of ALLN). The lysates were centrifuged for 10 min at 4° in a microcentrifuge, and supernatants were collected for immunoprecipitation.

To assess the effects of different conditions on apoB synthesis and secretion, cells were incubated with or without 10 μM avasimibe (DMSO was used as a negative control) or with or without 360 μM oleate conjugated with BSA (oleate:BSA ratio 8:1). Oleate–BSA complex was prepared as follows. A total of 150 mg of fatty acid-free BSA was added to 10 mL of pre-warmed methionine-free MEM, or hepatocyte attachment medium, or Williams' E medium and mixed and allowed to incubate for 5 min at 37° until BSA was dissolved completely. The medium–BSA solution was then sterilized by syringe-filtration (0.45 μm). The filtered solution was diluted by adding 40 mL of additional medium. A solution of oleic acid was prepared by dissolving 34 mg of the oleic acid in 500 μL of anhydrous ethanol. A volume of 75 μL of the prepared oleate stock solution was added in 50 mL of medium-BSA solution. The mixture was then either incubated overnight while constantly rotating at room temperature or for 2 hr at 37°. By using this protocol, the final concentration of oleate in solution was 360 μM with an oleate:BSA ratio of 8:1. The oleate–BSA complex was added to the culture medium and incubated with the HepG2 cells for a period of 18 hr.

2.5. Permeabilization of HepG2 cells

HepG2 cells cultured in 35-mm dishes were depleted of methionine by incubation in methionine-free MEM for 1 hr at 37° under 5% CO_2. Cells were pulsed with 100 μCi/mL of [^{35}S]methionine for 1 hr and then were permeabilized as described [42]. Briefly, pulsed cells were washed twice and then were incubated in CSK (0.3 M sucrose, 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM Na-free EDTA, and 10 mM PIPES, pH 6.8) containing 50 μg/mL of digitonin for 10 min. Permeabilized cells were then washed three times in CSK buffer and chased in CSK buffer for different time periods. All steps were conducted in the presence and absence of avasimibe. At various intervals, duplicate or triplicate dishes were washed, solubilized, and subjected to immunoprecipitation.

2.6. Trypsin digestion of permeabilized HepG2 cells

To determine the translocation status of apoB within the ER membrane, we used a protocol previously developed in our laboratory [42,43]. Briefly, HepG2 cells pretreated with and without avasimibe (10 μM) were pulsed for 15 min and chased for various periods of time. At each time point, cells were permeabilized by incubating in CSK buffer containing 75 μg/mL of digitonin for 5 min. Then cells were subjected to trypsin (200 μg/mL) treatment for 10 min at room temperature. After trypsinization, proteolysis was stopped by adding a 10-fold excess of soybean trypsin inhibitor, and cells were solubilized for immunoprecipitation. To prevent any residual trypsin activity, a protease inhibitor mixture was included in all the steps.

2.7. Subcellular fractionation of ER and Golgi compartments

To investigate the effect of avasimibe on intracellular trafficking of newly synthesized apoB-containing lipoproteins, we used a subcellular fractionation protocol to separate the ER and Golgi compartments. Briefly, HepG2 cells were pulsed with 100 μCi/mL of [^{35}S]methionine and chased for 0 and 2 hr in the presence and absence of the ACAT inhibitor. Cells were homogenized and subjected to sucrose gradient ultracentrifugation as described [44]. Gradients were separated into 12 fractions and immunoprecipitated for apoB. Fractions 1–6 and 7–12 corresponded to ER and Golgi compartments, respectively, based on organelle-specific enzyme marker measurements. NADPH-cytochrome *c* activity and *N*-acetylglucosamine phosphotransferase activity were assayed as organellar-specific markers for ER and Golgi, respectively, as described [44].

2.8. Analysis of luminal and membrane-associated apoB pools

Isolation of the microsomal fraction and separation of the luminal and membrane components by carbonate extraction and ultracentrifugation were performed as described [44–47]. Membrane and luminal fractions were then diluted with 800 μL of a solubilization buffer containing 360 μL of 5 × C buffer (250 mM Tris–HCl, pH 7.4, 750 mM NaCl, 25 mM EDTA, 5 mM PMSF, 5% Triton X-100) and 410 μL of PBS supplemented with 450 KIU/mL of Trasylol, 5 mM PMSF and subjected to immunoprecipitation, SDS–PAGE, and fluorography.

2.9. Immunoprecipitation, SDS–PAGE, and fluorography

Immunoprecipitation was performed as described previously [42,48]. Immunoprecipitates were washed with wash buffer (10 mM Tris–HCl, pH 7.4, 2 mM EDTA, 0.1% SDS, 1% Triton X-100) and were prepared for SDS–PAGE by resuspension and boiling in 100 μL of electrophoresis sample buffer. Proteins were separated on SDS-polyacrylamide gels, which then were fixed, stained, and soaked in Amplify (Amersham), before being dried and exposed to DuPont autoradiographic film at

−80° for 1–4 days. The apoB bands were excised from the gel, digested in hydrogen peroxide/perchloric acid, and associated radioactivity was quantitated by liquid scintillation counting.

3. Results

3.1. Effect of avasimibe on cellular lipid synthesis

To assess the effects of avasimibe on the synthesis of lipids, HepG2 cells were incubated for 18 hr in the presence of drug concentrations ranging from 0 to 10 μM. The cells were labeled with either [^{3}H]acetate or [^{3}H]oleate bound to BSA. Radiolabeled lipid fractions were extracted from the cells and analyzed by TLC and scintillation counting. The results revealed that in the presence of avasimibe, the levels of intracellular newly synthesized triglyceride, free cholesterol, and cholesteryl esters decreased dramatically in a concentration-dependent manner (Fig. 1). The maximum response was observed at 10 μM avasimibe, which resulted in near complete inhibition of lipid synthesis. Interestingly, during the incubation period, incorporation of labeled oleate into triglyceride increased initially at 1 μM avasimibe ($26.6 \pm 10.1\%$, $P = 0.04$) but decreased sharply at 5 and 10 μM. The IC_{50} concentration of the drug for inhibition of cholesterol ester synthesis was found to be approximately 2 μM. At this concentration, there was no inhibition of triglyceride synthesis. Thus, higher concentrations of avasimibe were required to observe inhibition of triglyceride synthesis.

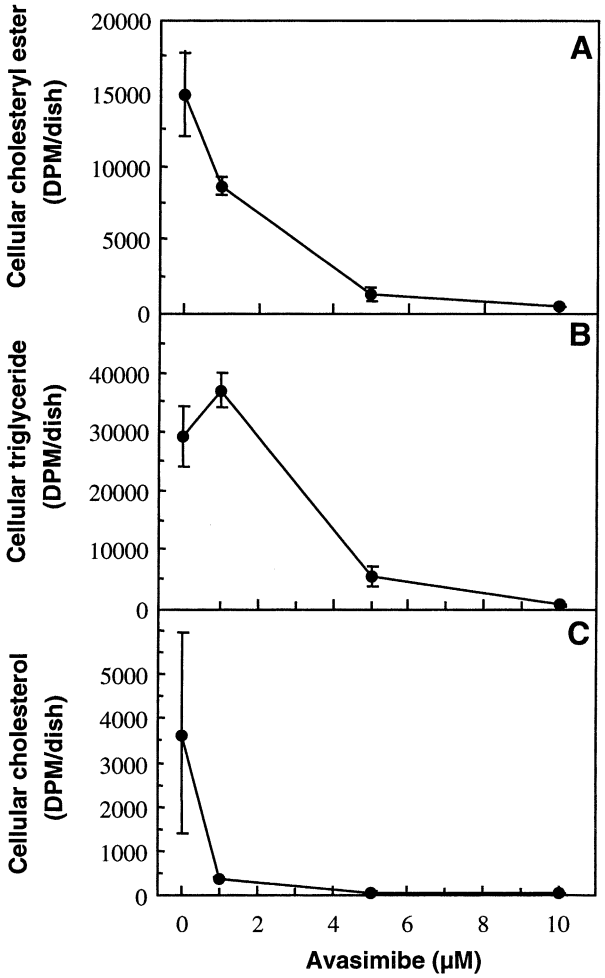

Fig. 1. Effect of avasimibe on cellular lipid synthesis. HepG2 cells were incubated for 18 hr in the presence of 0–10 μM avasimibe followed by a 6-hr pulse in the presence of 5 μCi/mL of [^{3}H]acetate (panel A: cholesteryl ester; panel C: free cholesterol) or [^{3}H]oleate bound to albumin (panel B: triglyceride). Total cellular lipid was extracted and separated by TLC as described in Section 2. Lipids of interest were visualized with iodine vapor, cut from the plate, and quantitated by liquid scintillation counting. Data shown are total dpm per culture dish (means ± SD, $n = 3$).

3.2. Effect of avasimibe on apoB synthesis, secretion, and degradation

To determine the effect of avasimibe on the synthesis and secretion of apoB, HepG2 cells were incubated overnight with 10 μM avasimibe and then pulsed with [^{35}S]methionine and chased for 0 and 2 hr. Immunoprecipitated apoB was analyzed by SDS–PAGE and fluorography. Fig. 2 shows the effect of the ACAT inhibitor on cellular, medium, and total apoB remaining. In avasimibe-treated cells, cellular apoB at 0 hr showed a $36.9 \pm 2.2\%$ decrease ($P = 0.027$) compared with control, indicating significant inhibition of synthesis by the drug. Surprisingly, in drug-treated cells, cellular apoB remaining after the 2-hr chase period was 2-fold more than that in the control cells ($P = 0.001$), suggesting intracellular accumulation of apoB in the presence of avasimibe. Secretion of apoB in drug-treated cells decreased $75.6 \pm 5.7\%$ ($P = 0.01$). This drastic decrease in apoB secretion can be attributed partly to the possible suppression of apoB synthesis and also to its accumulation in avasimibe-treated cells. Examination of total apoB remaining after the 2-hr chase revealed that, in control cells, $65.9 \pm 1.9\%$ (4148 ± 358 and 1414 ± 80 cpm per dish at $t = 0$ and 2 hr, respectively; $P = 0.004$) of radiolabeled apoB was degraded, while in avasimibe-treated cells only $20.2 \pm 10.1\%$ (2618 ± 91 and 2089 ± 264 cpm per dish at $t = 0$ and 2 hr, respectively; $P = 0.06$) was degraded. It appears that in HepG2 cells avasimibe increased the stability of cellular apoB, causing its accumulation inside the cells.

To determine whether there might be differences between the 18-hr treatments used in the preceding experiments and a more acute exposure of the cells to avasimibe, a set of experiments was performed using a 90-min pretreatment time. The results showed that synthesis of apoB was not affected significantly by the drug under these conditions, whereas inhibition of secretion and intracellular accumulation of apoB were still observed (data not shown). These results suggest that at least these effects of the ACAT inhibitor are likely to be mediated by a relatively fast-acting mechanism.

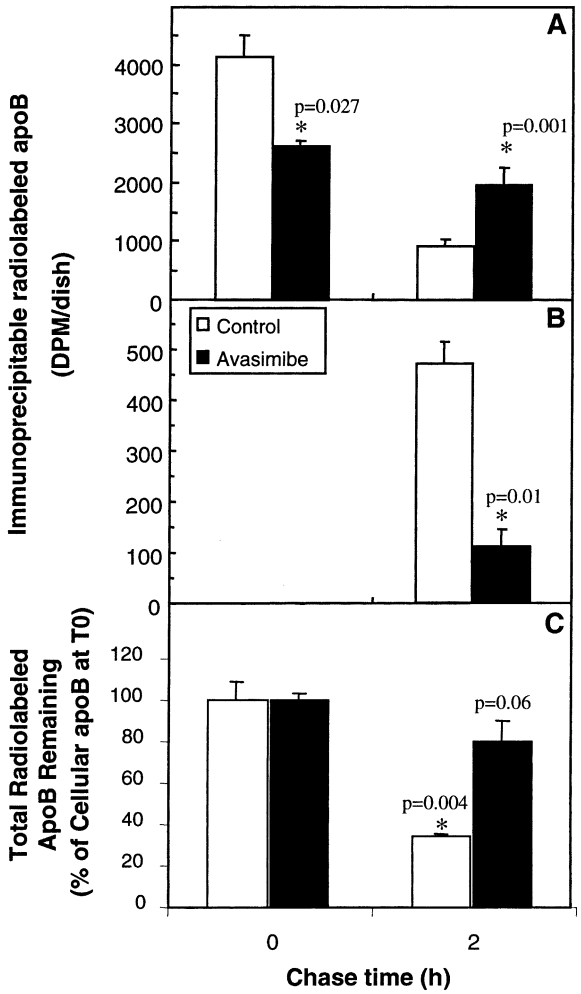

Fig. 2. Effect of avasimibe on apoB synthesis, secretion, and degradation. Overnight incubation of HepG2 cells in the presence or absence of 10 μM avasimibe was followed by a pulse with [^{35}S]methionine and a chase of 0 and 2 hr. Medium and cells were harvested, immunoprecipitated with an anti-human apoB antibody, and run on a SDS-polyacrylamide gel. Full-length apoB was visualized by fluorography and quantitated by excision from the gel, digestion, and liquid scintillation counting. The figure shows radiolabeled apoB immunoprecipitated from cells (panel A) and medium (panel B). Panel C shows total apoB remaining in control and avasimibe-treated cells at 0 and 2-hr chase time points. Data are means ± SD, $n = 3$.

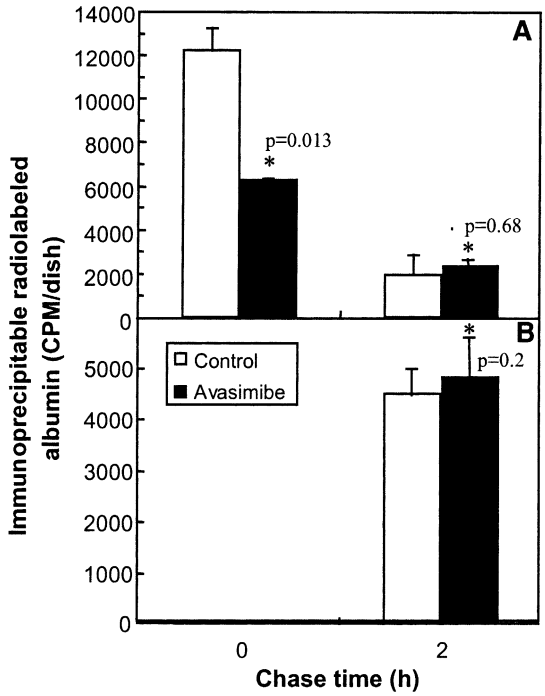

Fig. 3. Effect of avasimibe on albumin synthesis and secretion. Overnight incubation of HepG2 cells in the presence or absence of 10 μM avasimibe was followed by a pulse with [^{35}S]methionine and a chase of 0 and 2 hr. Medium and cells were harvested, immunoprecipitated with an anti-human albumin antibody, and run on a SDS-polyacrylamide gel. Full-length apoB was visualized by fluorography and quantitated by excision from the gel, digestion, and liquid scintillation counting. The figure shows radiolabeled albumin immunoprecipitated from cells (panel A) and medium (panel B). Data are means ± SD, $n = 3$.

To investigate any global effects of avasimibe treatment, the effects of the drug on the synthesis and secretion of two hepato-specific proteins, albumin and α1-antitrypsin, were evaluated. HepG2 cells were incubated in the presence and absence of avasimibe and then labeled with [^{35}S]methio- hr. Cellular and secreted albumin and α1-antitrypsin were isolated by immunoprecipitation and quantitated as for apoB. Fig. 3 illustrates that, although avasimibe had no effect on albumin secretion, the drug significantly inhibited albumin synthesis at 0 hr (48.5 ± 0.3% decrease relative to the control; 12310 ± 1400 and 6345 ± 39 cpm per dish in control and avasimibe-treated cells, respectively; $P = 0.013$). Cellular levels of albumin after the 2-hr chase rebounded to the control value (1970 ± 913 and 2421 ± 272 cpm per dish in control and avasimibe-treated cells, respectively; $P = 0.68$). Similar results were obtained for α1-antitrypsin (data not shown). The reduced level of albumin synthesis was not due to avasimibe-induced cell death as no significant cell loss was observed. However, when cells were pretreated for only 90 min before pulse-chase labeling, no significant effects could be observed on albumin and α1-antitrypsin synthesis or secretion (data not shown). This suggests that short-term pretreatment of HepG2 cells with avasimibe does not affect the synthesis and/or secretion of hepato-specific proteins.

3.3. Effect of avasimibe on the translocation efficiency of apoB

The following experiments were undertaken to determine whether inhibition of apoB secretion might be due to an alteration in translocational efficiency. Translocational status of newly synthesized apoB was assessed based on accessibility to trypsin in permeabilized HepG2 cells. This protocol has been used previously in our laboratory to investigate apoB translocation in HepG2 cells [42,43], McRH7777 cells [49], and primary hamster hepatocytes [50]. HepG2 cells were pretreated with and without 10 μM avasimibe, pulse-chased, and permeabilized with digitonin.

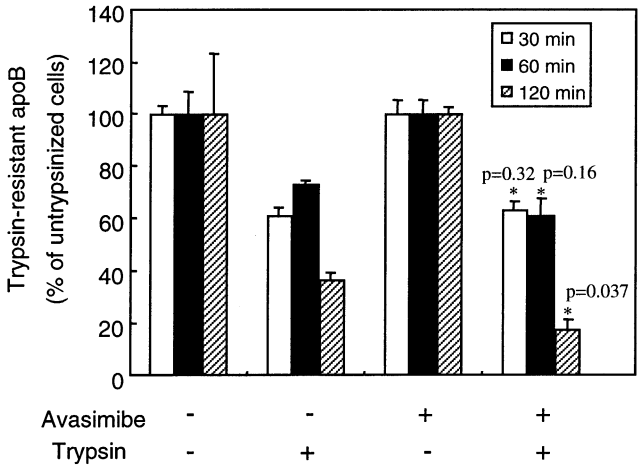

Fig. 4. Effect of avasimibe on translocational status of apoB. Pretreatment of HepG2 cells with 10 μM avasimibe was followed by [^{35}S]methionine pulse-labeling and chase periods of 30, 60, or 120 min. All cells were permeabilized in CSK buffer containing 75 μg/mL of digitonin, and half were treated with 200 μg/mL of trypsin. After arresting proteolysis with soybean trypsin inhibitor, cells were harvested in solubilizing buffer and subjected to immunoprecipitation with an anti-human apoB antibody. Intact, full-length apoB was isolated by SDS–PAGE, visualized by fluorography, and quantitated by liquid scintillation counting. Data are expressed relative to control cells not treated with avasimibe and not exposed to trypsin (mean percent of control ± SEM, $n = 3$). The P values represent comparisons between the levels of trypsin-resistant apoB in cells treated without or with avasimibe at different time points (30, 60, and 120 min).

Trypsin protection assays were then performed as described in Section 2, and translocational efficiency was calculated as the percentage of intact, trypsin-resistant apoB in trypsin-treated cells relative to total apoB recovered from control cells that were permeabilized but not exposed to trypsin. This assay was carried out at chase times of 30–120 min (Fig. 4). After a 30-min chase, although avasimibe caused a $23.6 \pm 3.5\%$ ($P = 0.027$) decrease in cellular apoB (compared with the control cells without trypsin), the percentage of trypsin-resistant apoB was found to be similar under both conditions (61.1 ± 3.2 and $63.4 \pm 3.4\%$ in control and avasimibe-treated cells, respectively; $P = 0.32$), suggesting no significant difference in translocation efficiency at this time point. In control cells after a 60-min chase, $73.1 \pm 0.8\%$ of radiolabeled apoB was found to be trypsin-resistant, indicating an increase in apoB translocation across the ER membrane (compared with the 30-min data). In avasimibe-treated cells, the percentage of trypsin-resistant apoB was not significantly different from the level after 30 min ($61.5 \pm 5.7\%$ compared with $63.4 \pm 3.4\%$). After a 120-min chase, avasimibe-treated cells contained $18.0 \pm 3.8\%$ trypsin-resistant apoB, indicating exposure of greater than 80% of apoB to the cytosolic face of the microsomal membrane. In contrast, control cells had a significantly higher percentage of trypsin-resistant apoB after the 120-min chase ($37.5 \pm 5.4\%$; $P = 0.037$ relative to avasimibe-treated cells). Thus, it appears that translocational efficiency of apoB is not affected by avasimibe treatment at the initial phase of apoB transit through the secretory pathway. However, there may be a greater proportion of apoB remaining, associated with the cytosolic face of the microsomes in avasimibe-treated cells, at longer chase times. This may suggest a reduced ability of newly synthesized and membrane-associated apoB to enter a secretion competent pool in the presence of avasimibe.

3.4. Effect of avasimibe on apoB transit through the ER and Golgi compartments

To further investigate the effect of avasimibe on intracellular biogenesis of apoB, we used a subcellular fractionation protocol to separate the ER and Golgi compartments. HepG2 cells were pulsed and chased for 0 and 2 hr. Cells were homogenized and subjected to sucrose gradient centrifugation. Gradients were separated into 12 fractions, and apoB was immunoprecipitated. Fig. 5 depicts apoB isolated from the ER (fractions 1–6) and Golgi (fractions 7–12). At 0 hr in both control and avasimibe-treated cells, most of the apoB resided in the ER

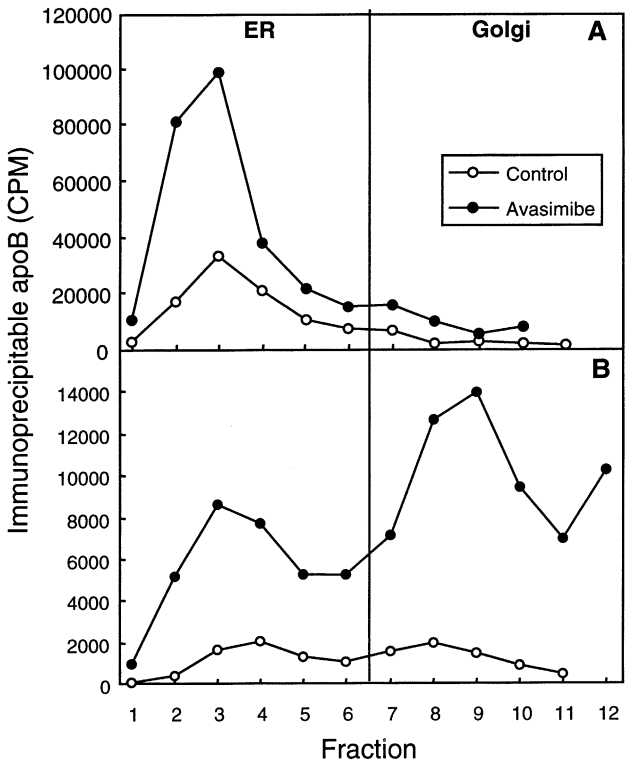

Fig. 5. Effect of avasimibe on apoB transit through the ER and Golgi compartments. Cells were pulsed and chased for 0 (panel A) and 2 hr (panel B) with and without 10 μM avasimibe pretreatment. Cell homogenates were subjected to sucrose gradient centrifugation as described in Section 2. Gradients were fractionated, and each fraction was immunoprecipitated with an anti-human apoB antibody. SDS–PAGE, fluorography, and scintillation counting allowed quantitation of apoB associated with each fraction. Fractions 1–6 contain contents isolated from the ER, while fractions 7–12 represent the Golgi compartment. Shown is a representative experiment of two independent experiments.

compartment with a peak at fraction 3. In all ER fractions, apoB radioactivity was markedly higher in avasimibe-treated cells relative to control cells. After a 2-hr chase, less radioactivity was recovered from the fractions of control cells, suggesting that most of the apoB was secreted or degraded (Fig. 5B). In contrast, after a 2-hr chase in ACAT inhibitor-treated cells, there was an accumulation of radiolabeled apoB in both ER and Golgi, with a higher level in the latter compartment.

3.5. Effect of carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) on the degradation of apoB

The involvement of the proteasome in apoB turnover in the presence of an ACAT inhibitor was studied using MG132, a potent proteasome inhibitor. The proteasome is reported to be responsible for the degradation of apoB that is associated with the ER membrane or retro-translocated back to the cytosol from the ER lumen. Both control and avasimibe-treated cells were incubated in the presence of MG132 for 15 min before the pulse, and the protease inhibitor was also present during the pulse and chase. Immunoprecipitable apoB recovered under each condition is shown in Fig. 6. At 0 hr, MG132 increased cellular apoB in control cells more than 2.3-fold, suggesting significant protection of apoB against proteasomal degradation ($55{,}975 \pm 767$ and $127{,}226 \pm 3337$ cpm per dish, without and with MG132, respectively; $P = 0.008$). The extent of protection against degradation by MG132 in avasimibe-treated cells at 0 hr was 1.7-fold ($58{,}029 \pm 10{,}790$ and $103{,}197 \pm 4440$ cpm per dish, without and with MG132, respectively; $P = 0.017$). Interestingly, although the presence of MG132 in avasimibe-treated cells increased recovery of newly synthesized apoB, it failed to restore it completely to the level of control cells + MG132, suggesting the possible involvement of mechanisms other than proteasomal degradation in reducing cellular apoB levels. After a 2-hr chase, MG132 increased cellular apoB in control (from 7516 ± 1208 to $51{,}699 \pm 4006$ cpm per dish; 6.9-fold) and avasimibe-treated cells (from $36{,}685 \pm 16{,}210$ to $87{,}835 \pm 24{,}800$ cpm per dish; 2.4-fold), respectively. Expressed as a percentage, the level of radiolabeled apoB degraded was $72.9 \pm 8\%$ (−MG132) versus $46.8 \pm 2.9\%$ (+MG132) ($P = 0.003$) in control cells and $30.4 \pm 4.5\%$ (−MG132) versus $13.9 \pm 7.1\%$ (+MG132) ($P = 0.045$) in avasimibe-treated cells. Thus, MG132 stimulated secretion of apoB more than 2-fold in control cells but significantly suppressed apoB secretion in avasimibe-treated cells.

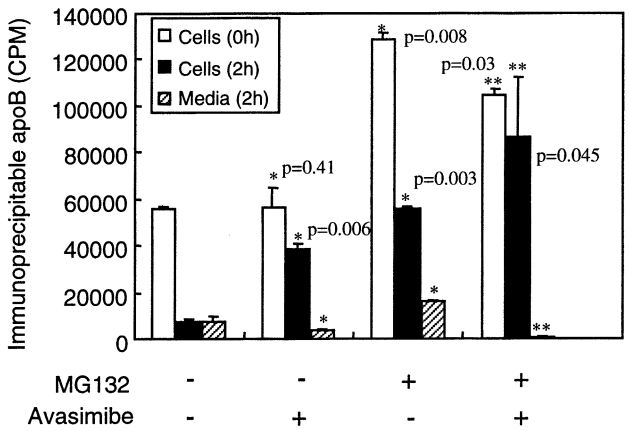

Fig. 6. ApoB synthesis and secretion in the presence of avasimibe and MG132. Cells treated in the presence and absence of avasimibe were exposed to MG132 15 min before the beginning of the pulse. Both drugs were also present during the pulse and subsequent 0 and 2-hr chase. Cells were harvested at each time point, and medium was collected after 2 hr. All samples were analyzed for apoB content by SDS–PAGE, fluorography, and liquid scintillation counting. Radiolabeled apoB isolated from cells at 0 and 2 hr were compared with apoB secreted into the culture medium after 2 hr. Data are means ± SEM, from two independent experiments, each performed in duplicate. The *P* values marked with one asterisk (∗) represent comparisons between cells treated under various conditions and control/untreated cells. The *P* values marked with two asterisks (∗∗) show comparisons between avasimibe-treated versus avasimibe + MG132-treated cells. The *P* values for apoB at the 2-hr chase compare the total radiolabeled apoB in cells plus medium (total apoB) under various conditions.

3.6. Effect of avasimibe on apoB turnover in permeabilized cells

Intact HepG2 cells incubated with and without avasimibe were pulsed, permeabilized with digitonin, and then chased in CSK buffer, in the presence and absence of avasimibe, for 0, 90, and 180 min (Fig. 7). In control cells, there was significant degradation of newly synthesized apoB after 90 min ($25.3 \pm 7.0\%$ of initial apoB remaining), while in avasimibe-treated cells the extent of degradation was significantly ($P = 0.008$) less than control ($90.3 \pm 27.7\%$ remaining). After 180 min of chase, apoB in avasimibe-treated cells was reduced to $29.4 \pm 8.0\%$,

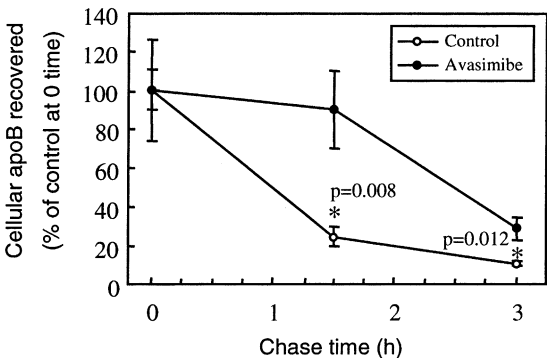

Fig. 7. Effect of avasimibe on apoB turnover in permeabilized cells. Intact HepG2 cells incubated with and without avasimibe were pulsed, permeabilized with digitonin, and then chased in CSK buffer, in the presence and absence of avasimibe, for 0, 90, and 180 min. Full-length apoB remaining after each chase time was quantitated after immunoprecipitation, SDS–PAGE, fluorography, and scintillation counting. Data are means ± SEM, from two independent experiments, each performed in duplicate. The *P* values compare percent apoB remaining between control and avasimibe-treated cells at different chase times.

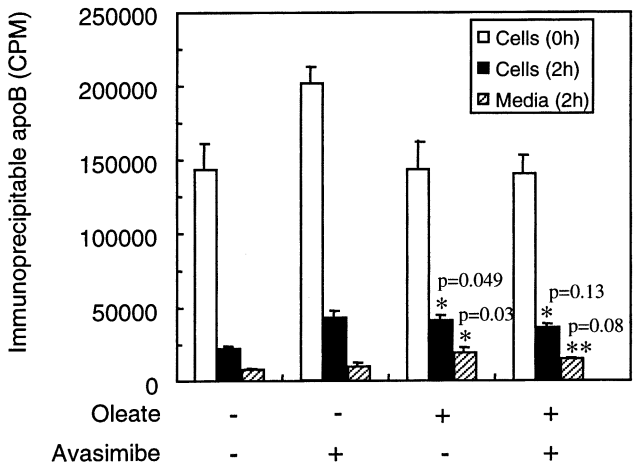

Fig. 8. Effect of avasimibe in oleate-treated HepG2 cells. Control and avasimibe-treated cells were also treated in the presence and absence of oleate. Medium and cells were harvested after a pulse and chase of 0 and 2 hr. Data reflect full-length apoB quantitated from cells at 0 and 2 hr as well as from medium at 2 hr. All treatments contained BSA as a vehicle control as it was used to deliver oleate (means ± SEM, from two independent experiments performed in duplicate). The P values show comparisons between control cells treated with or without oleate as well as avasimibe-treated cells with or without oleate.

which was still significantly higher than the level of $11.2 \pm 0.8\%$ seen in control cells ($P = 0.012$). Overall, these data indicated avasimibe-induced enhancement in the stability of apoB in permeabilized HepG2 cells.

3.7. Effect of avasimibe on oleate-treated cells

To examine the effect of avasimibe on oleate-stimulated cells, control and avasimibe-treated cells were incubated in the presence and absence of oleate. Cells were then pulsed and chased for 0 and 2 hr. Fig. 8 shows radiolabeled apoB recovered under different conditions. At 0 hr, there was no significant difference in cellular apoB except in avasimibe-treated cells (without oleate), in which cellular apoB was approximately 40% greater than that in control cells (144,387± 23,473, 201,822 ± 16,193, 143,589 ± 27,655, and 141,363 ± 16,189 cpm per dish in BSA-, avasimibe + BSA-, BSA + oleate-, and BSA + oleate + avasimibe-treated cells, respectively). At the 2-hr chase, avasimibe increased cellular apoB about 2-fold compared with the control ($P = 0.03$), suggesting apoB accumulation in drug-treated cells. Addition of oleate in control and avasimibe-treated cells showed opposite effects on cellular apoB at the 2-hr chase. As depicted in Fig. 8, oleate treatment of control cells increased both the cellular apoB remaining at 2 hr ($P = 0.049$) and secreted apoB ($P = 0.03$), when compared with control cells not treated with oleate. However, in avasimibe-treated cells, oleate caused a non-significant $16.2 \pm 5.3\%$ decrease in cellular apoB ($P = 0.13$) with a trend towards an increase in apoB secretion ($P = 0.08$). The percentage of apoB degradation in control cells dropped from 78.4% without oleate to 56.7% in the presence of oleate, whereas in the avasimibe-treated cells it declined slightly from 72.7 to 62.9% in the absence and presence of oleate, respectively.

3.8. Effect of avasimibe on the density profile of apoB100-containing lipoproteins

To investigate the effect of avasimibe on the assembly of apoB-containing lipoproteins, HepG2 cells were pulsed, chased for 10 and 120 min, and used to prepare microsomes. Luminal contents as well as culture medium at each chase time were subjected to sucrose gradient ultracentrifugation and immunoprecipitation. Fig. 9 shows the distribution of luminal apoB-containing lipoproteins. Fractions 1–5 represent high-density apoB lipoprotein (apoB-containing lipoprotein with a density similar to that of HDL, peak density 1.065–1.170 g/mL), fractions 6–10 represent the lower density apoB lipoprotein particles (LDL-apoB, peak density 1.011–1.045 g/mL), and the top fractions (11 and 12) contain very-low-density apoB lipoprotein particles (density < 1.011 g/mL) (see [45,51]). Results depicted in Fig. 9A indicate that at 0 hr, in the microsomal lumen of both control and avasimibe-treated cells, radiolabeled apoB was distributed in two different

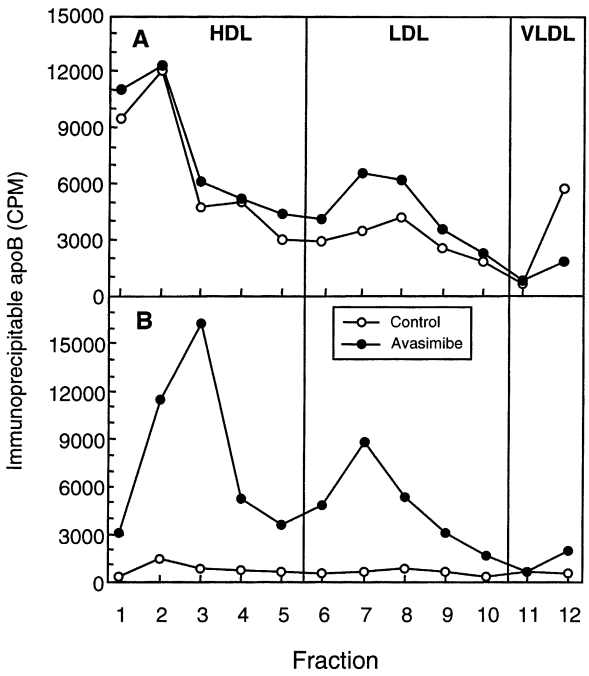

Fig. 9. Effect of avasimibe on the assembly and secretion of apoB-containing lipoproteins. HepG2 cells treated with and without avasimibe were pulsed with [^{35}S]methionine and chased for 10 (panel A) and 120 min (panel B). Medium was collected, and microsomes were isolated from harvested cells. The luminal contents of the microsomes were extracted with carbonate, and these contents, were subjected to sucrose gradient ultracentrifugation to separate HDL-like (fractions 1–5), LDL-like (fractions 6–10), and VLDL-like lipoprotein particles (fractions 11 and 12). Radiolabeled apoB associated with each fraction was quantitated by immunoprecipitation, SDS–PAGE, fluorography, and scintillation counting. These data are representative of two independent experiments.

density regions of the gradient (LDL and HDL) with the majority of nascent apoB being present in the secretion incompetent-HDL particles with the peak at fraction 2. Interestingly, at the 2-hr chase time, the luminal apoB-containing lipoprotein pattern was completely different for control and avasimibe-treated cells (Fig. 9B). After the 2-hr chase, neither radiolabeled apoB-containing HDL (secretion-incompetent) nor LDL-like particles (secretion-competent) were detected in the ER lumen of the control, indicating either gradual conversion of HDL-like particles (by recruiting more lipid) to secretion-competent LDL or their degradation (or a combination of both). Analysis of medium from control cells revealed a high level of apoB secretion in the form of LDL particles (data not shown). Analysis of avasimibe-treated cells revealed no change in apoB-containing lipoprotein particle profile between the 0 and 2-hr chase, suggesting drug-induced inhibition of apoB secretion, cellular apoB stabilization, as well as lack of lipid recruitment by the nascent lipoproteins leading to their cellular accumulation.

4. Discussion

The body of data concerning the effects of avasimibe is small, but there have been studies using other ACAT inhibitors including: Dup 128, Sandoz 58-035, CL277,082, 447C88, FCE 27677, and NTE-122. In the present study, overnight incubation of HepG2 cells with avasimibe significantly decreased intracellular levels of newly synthesized cholesterol and cholesteryl ester, and at higher doses triglyceride synthesis was also inhibited. This observation on cholesterol and cholesteryl ester is in agreement with the findings of Wilcox *et al.* [27]. They reported similar effects on HepG2 cells following a 19–24-hr incubation with avasimibe and Dup 128, another ACAT inhibitor. In contrast to our findings, they observed no effect on cellular triglyceride synthesis as assessed by [^{14}C]oleate labeling, although triglyceride secretion as measured by either [^{14}C]acetate or [^{14}C]oleate labeling was shown to be inhibited. Other investigators have also made contrasting observations on the effect of ACAT inhibitors on cellular lipid biosynthesis. Benoist and Grand-Perret [52] using Sandoz 58-035 found a reduction in cholesteryl ester mass and synthesis without any effect on cellular cholesterol and triglyceride, or apoB secretion. However, Avramoglu *et al*. [21], using the same ACAT inhibitor in HepG2 cells, reported reduction in newly synthesized cellular cholesteryl ester and concluded that both newly synthesized and stored cholesteryl ester are important determinants of apoB secretion from HepG2 cells. In contrast, Graham and colleagues [35], using two different ACAT inhibitors (447C88 and CL277,082), found that while both drugs inhibited cholesterol esterification, only one of the two inhibitors (CL277,082) significantly reduced triglyceride and phosphatidylcholine secretion from HepG2 cells. Interestingly, in another study, incubation of HepG2 cells with Sandoz 58-035 resulted in increased cellular triglyceride and cholesterol while decreasing cholesteryl ester content [37]. Furthermore, in an *in vivo* feeding study, Burnett *et al.* [30] found that in miniature pigs avasimibe treatment induced a dose-dependent increase in liver triglyceride accumulation. Since we have not measured triglyceride mass in our current study, we cannot rule out the possibility that cellular triglyceride mass levels may not be changed in parallel with the rate of cellular triglyceride synthesis (which was decreased under the conditions of this study).

In the present study, avasimibe was found to inhibit apoB secretion significantly. This inhibition was observed after both short-term (90 min) and long-term (overnight) incubations. Overnight treatment also suppressed apoB synthesis, whereas short-term incubation did not. Long-term incubation with avasimibe appears to cause a general suppression of protein synthesis as evidenced by decreased synthesis of both albumin and α1-antitrypsin. Wilcox *et al.* [27] reported no change in apoB mRNA levels in HepG2 cells following a 24-hr incubation with avasimibe. Thus, it is possible that the lower level of radiolabeled apoB observed at the end of the pulse period in our study was due to a slower protein translation in avasimibe-treated cells. Interestingly, the avasimibe-induced inhibition of apoB secretion coincided with a significant decline in intracellular synthesis of both core lipoprotein lipids, triglyceride and cholesterol ester. The inhibition of triglyceride synthesis was unexpected but it might explain, at least partially, the reduction in apoB secretion since the link between triglyceride availability and apoB secretion has been well established [9,52–56]. It is also important to note that at the IC_{50} concentration (~2 μM) for inhibition of cholesteryl ester synthesis, there was no significant effect on triglycerides, and higher doses were required for inhibition of triglyceride synthesis.

Surprisingly, we found that cellular degradation of apoB was attenuated drastically during short-term treatment with the drug. Inhibition of secretion together with higher cellular stability of apoB caused its accumulation inside the cells under these conditions. In contrast to our findings, Wilcox *et al.* [27] found an accelerated rate of apoB degradation in avasimibe-treated HepG2 cells. The reasons for these contrasting findings are unclear but may be related to the different cell culture conditions employed. In addition, our conclusions are based on classical pulse-chase studies, whereas Wilcox *et al.* [27] derived their conclusions based on multicompartmental modeling of pulse-chase data. It should also be noted that we did observe increased degradation of accumulated apoB in avasimibe-treated permeabilized HepG2 cells following a longer chase time. In support of our findings of an initial inhibition of apoB degradation, there is one report in the literature indicating suppression of apoB degradation in HepG2 cells by Sandoz 58-035 [37].

To further study the apoB secretory pathway under these conditions, we examined translocation of newly synthesized apoB across the ER membrane. According to our findings, although avasimibe did not initially affect the rate of apoB translocation across the ER membrane, it appeared to impair posttranslational movement of apoB out of the membrane-associated pool such that, after a 2-hr chase period, more than 80% of apoB was still exposed on the cytosolic face of the ER. This translocation-arrested apoB would ordinarily be targeted for degradation by the proteolytic machinery of the cell, particularly the proteasome system, but our data indicate that at least part of this pool remains intact under conditions of short chase times.

Recently, Post *et al.* [57] reported that in rat hepatocytes avasimibe stimulated bile acid synthesis and increased cholesterol 7α-hydroxylase mRNA and protein levels. Hepatocytes treated with avasimibe showed a shift from storage and secretion of cholesteryl esters to conversion of cholesterol into bile acids. The enzyme that links these two metabolic pathways is the liver-specific cholesterol-7α-hydroxylase [58,59]. Studies in Chinese hamster ovary (CHO) cells expressing apoB53 and cholesterol-7α-hydroxylase found that apoB53 was not degraded and accumulated in cells while still associated with the ER membrane [58]. The mechanism of this protection from degradation was found to be an inhibition of apoB ubiquitination, an early step in proteasome-mediated degradation. This effect of the expression of cholesterol-7α-hydroxylase was reversed by oxysterols [58]. Interestingly, oxysterols are true ACAT activators (refer to the review by Chang *et al.* [60]). These findings provide a possible link between the sterol-status of cells, altered by the expression of cholesterol-7α-hydroxylase, and the ubiquitin-dependent proteasomal degradation of apoB, although direct evidence requires further studies.

Our data on the assembly of apoB-containing lipoproteins also suggest poor lipidation of these particles in avasimibe-treated cells. Most of the radiolabeled apoB recovered in the ER lumen was associated with particles in the density range of HDL, while in control cells most radiolabeled apoB was either secreted or degraded. It is noteworthy that in avasimibe-treated cells even apoB-containing lipoproteins with the density of LDL were not secretion-competent but remained confined inside the secretory pathway. To study the role of lipidation on apoB stability and secretion, we incubated control and avasimibe-treated cells with oleate. The effects of avasimibe and oleate on cellular apoB were not additive, and apoB accumulation in avasimibe-treated cells was abrogated, in part, by oleate treatment. This suggests that oleate can potentially reverse the short-term blocking action of avasimibe on apoB degradation and secretion. One possible explanation for these observations may be that oleate, by increasing ACAT activity [61], was able to neutralize the blocking effect of avasimibe and restore apoB secretion. On the other hand, oleate treatment might reverse the avasimibe-induced inhibition of triglyceride synthesis, enhance triglyceride availability, and thus induce assembly and secretion of apoB lipoproteins.

We also studied the effects of MG132, a potent proteasome inhibitor, on apoB biogenesis in the presence and absence of avasimibe. Interestingly, MG132 increased the cellular level of apoB in both control and avasimibe-treated cells, indicating that the proteasomal system is active in the presence of avasimibe. The apoB rescued by MG132 was not secretion-competent and accumulated inside the ACAT inhibitor-treated cells. Since the effects of avasimibe and MG132 on apoB stability (2-hr level) appear to be additive, it also argues in favor of two distinct mechanisms being affected by the two drugs. Our findings on apoB degradation in permeabilized cells appear to support the above hypothesis. In avasimibe-treated cells, apoB was significantly more stable than control cells (about 7-fold) at 90 min, but this enhanced stability was reversed almost completely by 180 min. These observations indicate possible transient inhibition of ER-localized proteases by the drug.

In summary, avasimibe appears to exert its blocking effects on apoB secretion from HepG2 cells through novel mechanisms. Prolonged association with the microsomal membrane and impaired lipidation of nascent apoB, together with suppressed cellular protein turnover systems, lead to transient accumulation of secretion-incompetent apoB-containing lipoproteins inside the cell. Further investigation is required to better elucidate the nature of these mechanisms.

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References

[1] Goodman DS, Deykin D, Shiratori T. The formation of cholesterol esters with rat liver enzymes. J Biol Chem 1964;239:1335–45.
[2] Mukherjee S, Kunitake G, Alfin-Slater RB. The esterification of cholesterol with palmitic acid by rat liver homogenates. J Biol Chem 1968;230:91–6.
[3] Cheng D, Chang CC, Qu X, Chang TY. Activation of acyl-CoA: cholesterol acyltransferase by cholesterol or by oxysterol in a cell-free system. J Biol Chem 1995;270:685–95.
[4] Chang CCY, Chen J, Thomas MA, Cheng D, Del Priore VA, Newton RS, Pape ME, Chang T-Y. Regulation and immunolocalization of acyl-CoA: cholesterol acyltransferase in mammalian cells as studied with specific antibodies. J Biol Chem 1995;270:29532–40.
[5] Spector AA, Mathur SN, Kaduce TL. Role of acylcoenzyme A: cholesterol *O*-acyltransferase in cholesterol metabolism. Prog Lipid Res 1979;18:31–53.

[6] Yao Z, Tran K, McLeod RS. Intracellular degradation of newly synthesized apolipoprotein B. J Lipid Res 1997;38:1937–53.
[7] Yao ZM, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. J Biol Chem 1988;263:2998–3004.
[8] Pullinger CR, North JD, Teng BB, Rifici VA, Ronhild de Brito AE, Scott J. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. J Lipid Res 1989;30:1065–77.
[9] Wu X, Sakata N, Dixon J, Ginsberg HN. Exogenous VLDL stimulates apolipoprotein B secretion from HepG2 cells by both pre- and post-translational mechanisms. J Lipid Res 1994;35:1200–10.
[10] Furukawa S, Hirano T. Rapid stimulation of apolipoprotein B secretion by oleate is not associated with cholesteryl ester biosynthesis in HepG2 cells. Biochim Biophys Acta 1993;1170:32–7.
[11] Wu X, Sakata N, Lui E, Ginsberg HN. Evidence for a lack of regulation of the assembly and secretion of apolipoprotein B-containing lipoprotein from HepG2 cells by cholesteryl ester. J Biol Chem 1994;269:12375–82.
[12] Gibbons GF, Khurana R, Odwell A, Seelaender MC. Lipid balance in HepG2 cells: active synthesis and impaired mobilization. J Lipid Res 1994;35:1801–8.
[13] Brown A, Wiggins D, Gibbons GF. Manipulation of cholesterol and cholesteryl ester synthesis has multiple effects on the metabolism of apolipoprotein B and the secretion of very-low-density lipoprotein by primary hepatocyte cultures. Biochim Biophys Acta 1999;1440:253–65.
[14] Thompson GR, Naoumova RP, Watts GF. Role of cholesterol in regulating apolipoprotein B secretion by the liver. J Lipid Res 1996;37:439–47.
[15] Watts GF, Naoumova R, Cummings MH, Umpleby AM, Slavin BM, Sonksen PH, Thompson GR. Direct correlation between cholesterol synthesis and hepatic secretion of apolipoprotein B-100 in normolipidemic subjects. Metabolism 1995;44:1052–7.
[16] Synouri-Vrettakou S, Mitropoulos KA. Acyl-CoA: cholesterol acyltransferase. Transfer of cholesterol to its substrate pool and modulation of activity. Eur J Biochem 1993;133:299–307.
[17] Jamil H, Dickson Jr. JK, Chu CH, Lago MW, Rinehart JK, Biller SA, Gregg RE, Wetterau JR. Microsomal triglyceride transfer protein. Specificity of lipid binding and transport. J Biol Chem 1995;270:6549–54.
[18] Tanaka M, Jingami H, Otani H, Cho M, Ueda Y, Arai H, Nagano Y, Doi T, Yokode M, Kita T. Regulation of apolipoprotein B production and secretion in response to the change of intracellular cholesteryl ester contents in rabbit hepatocytes. J Biol Chem 1993;268:12713–8.
[19] Musanti R, Giorgini L, Lovisolo PP, Pirillo A, Chiari A, Ghiselli G. Inhibition of acyl-CoA: cholesterol acyltransferase decreases apolipoprotein B-100-containing lipoprotein secretion from HepG2 cells. J Lipid Res 1996;37:1–14.
[20] Dashti N. The effect of low density lipoproteins, cholesterol, and 25-hydroxycholesterol on apolipoprotein B gene expression in HepG2 cells. J Biol Chem 1992;267:7160–9.
[21] Avramoglu RK, Cianflone K, Sniderman AD. Role of the neutral lipid accessible pool in the regulation of secretion of apoB100 lipoprotein particles by HepG2 cells. J Lipid Res 1995;36:2513–28.
[22] Carr TP, Hamilton Jr. RL, Rudel LL. ACAT inhibitors decrease secretion of cholesteryl esters and apolipoprotein B by perfused livers of African green monkeys. J Lipid Res 1995;36:25–36.
[23] Rea TJ, DeMattos RB, Homan R, Newton RS, Pape ME. Lack of correlation between ACAT mRNA expression and cholesterol esterification in primary liver cells. Biochim Biophys Acta 1996;1299:67–74.
[24] Cianflone KM, Yasruel Z, Rodriguez MA, Vas D, Sniderman AD. Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge. J Lipid Res 1990;31:2045–55.
[25] Riddell D, Bright CP, Burton BJ, Bush RC, Harris NV, Hele D, Moore UM, Naik K, Parrott DP, Smith C, Williams RJ. Hypolipidaemic properties of a potent and bioavailable alkylsulphinyldiphenylimidazole ACAT inhibitor (RP 73163) in animals fed diets low in cholesterol. Biochem Pharmacol 1996;52:1177–86.
[26] Azuma Y, Kawasaki T, Ohno K, Seto J, Yamada T, Yamasaki M, Nobuhara Y. Effects of NTE-122, a novel acyl-CoA: cholesterol acyltransferase inhibitor, on cholesterol esterification and secretions of apolipoprotein B-containing lipoprotein and bile acids in HepG2. Jpn J Pharmacol 1999;79:151–8.
[27] Wilcox LJ, Barrett PH, Newton RS, Huff MW. apoB100 secretion from HepG2 cells is decreased by the ACAT inhibitor CI-1011: an effect associated with enhanced intracellular degradation of apoB. Arterioscler Thromb Vasc Biol 1999;19:939–49.
[28] Huff MW, Telford DE, Barrett PH, Billheimer JT, Gillies PJ. Inhibition of hepatic ACAT decreases apoB secretion in miniature pigs fed a cholesterol-free diet. Arterioscler Thromb 1994;14:1498–508.
[29] Burnett JR, Wilcox LJ, Telford DE, Kleinstiver SJ, Barrett PHR, Huff MW. Inhibition of cholesterol esterification by DuP 128 decreases hepatic apolipoprotein B secretion *in vivo*: effect of dietary fat and cholesterol. Biochim Biophys Acta 1998;1393:63–79.
[30] Burnett JR, Wilcox LJ, Telford DE, Kleinstiver SJ, Barrett PH, Newton RS, Huff MW. Inhibition of ACAT by avasimibe decreases both VLDL and LDL apolipoprotein B production in miniature pigs. J Lipid Res 1999;40:1317–27.
[31] Azuma Y, Kawasaki T, Ikemoto K, Obata K, Ohno K, Sajiki N, Yamada T, Yamasaki M, Nobuhara Y. Cholesterol-lowering effects of NTE-122, a novel acyl-CoA: cholesterol acyltransferase (ACAT) inhibitor, on cholesterol diet-fed rats and rabbits. Jpn J Pharmacol 1998;78:355–64.
[32] Marzetta CA, Savoy YE, Freeman AM, Long CA, Pettini JL, Hagar RE, Inskeep PB, Davis K, Stucchi AF, Nicolosi RJ, Hamanaka ES. Pharmacological properties of a novel ACAT inhibitor (CP-113,818) in cholesterol-fed rats, hamsters, rabbits, and monkeys. J Lipid Res 1994;35:1829–38.
[33] Chiari A, Lovisolo P, Fogliatto G, Fancelli D, Ghiselli G. Correction of dyslipoproteinaemia of casein-fed rabbit by FCE 27677, a potent novel ACAT inhibitor. Pharmacol Res 1996;33:181–9.
[34] Ramharack R, Spahr MA, Sekerke CS, Stanfield RL, Bousley RF, Lee HT, Krause BK. CI-1011 lowers lipoprotein(a) and plasma cholesterol concentrations in chow-fed cynomolgus monkeys. Atherosclerosis 1998;136:79–87.
[35] Graham A, Wood J, Russell LJ. Cholesterol esterification is not essential for secretion of lipoprotein components by HepG2 cells. Biochim Biophys Acta 1996;1302:46–54.
[36] Sato R, Imanaka T, Takano T. The effect of HMG-CoA reductase inhibitor (CS-514) on the synthesis and secretion of apolipoproteins B and A-1 in the human hepatoblastoma Hep G2. Biochim Biophys Acta 1990;1042:36–41.
[37] Ooyen C, Zecca A, Zanelli T, Catapano AL. Decreased intracellular degradation and increased secretion of apo B-100 in Hep G2 cells after inhibition of cholesteryl ester synthesis. Atherosclerosis 1997;130:143–52.
[38] Lee HT, Roark WH, Picard JA, Sliskovic DR, Roth BD, Stanfield RL, Hamelehle KL, Bousley RF, Krause BR. Inhibitors of acylcoenzyme A: cholesterol *O*-acyltransferase (ACAT) as hypocholesterolemic agents: synthesis and structure-activity relationships of novel series of sulfonamides, acylphosphonamides and acylphosphoramidates. Bioorg Med Chem Lett 1998;8:289–94.
[39] Vaccaro W, Amore C, Berger J, Burrier R, Clader J, Davis H, Domalski M, Fevig T, Salisbury B, Sher R. Inhibitors of acyl coenzyme A: cholesterol acyltransferase. J Med Chem 1996;39:1704–19.

[40] Lee HT, Sliskovic DR, Picard JA, Roth BD, Wierenga W, Hicks JL, Bousley RF, Hamelehle KL, Homan R, Speyer C, Stanfield RL, Krause BR. Inhibitors of acyl-coenzyme A: cholesterol *O*-acyl transferase (ACAT) as hypocholesterolemic agents. CI-1011: an acyl sulfamate with unique cholesterol-lowering activity in animals fed noncholesterol-supplemented diets. J Med Chem 1996;39:5031–4.

[41] Nicolosi RJ, Wilson TA, Krause BR. The ACAT inhibitor, CI-1011 is effective in the prevention and regression of aortic fatty streak area in hamsters. Atherosclerosis 1998;137:77–85.

[42] Adeli K. Regulated intracellular degradation of apolipoprotein B in semipermeable HepG2 cells. J Biol Chem 1994;269:9166–75.

[43] Macri J, Adeli K. Studies on intracellular translocation of apolipoprotein B in a permeabilized HepG2 system. J Biol Chem 1997; 272:7328–37.

[44] Borén J, Wettesten M, Sjöberg A, Thorlin T, Bondjers G, Wiklund O, Olofsson S-O. The assembly and secretion of apoB100 containing lipoproteins in Hep G2 cells. Evidence for different sites for protein synthesis and lipoprotein assembly. J Biol Chem 1990;265:10556–64.

[45] Borén J, Graham L, Wettesten M, Scott J, White A, Olofsson S-O. The assembly and secretion of apoB100-containing lipoproteins in Hep G2 cells. ApoB100 is cotranslationally integrated into lipoproteins. J Biol Chem 1992;267:9858–67.

[46] Boström K, Borén J, Wettesten M, Sjöberg A, Bondjers G, Wiklund O, Carlsson P, Olofsson S-O. Studies on the assembly of apoB100-containing lipoproteins in HepG2 cells. J Biol Chem 1988;263:4434–42.

[47] Boström K, Wettesten M, Borén J, Bondjers G, Wiklund O, Olofsson S-O. Pulse-chase studies of the synthesis and intracellular transport of apolipoprotein B-100 in Hep G2 cells. J Biol Chem 1986;261: 13800–6.

[48] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[49] Cavallo D, McLeod RS, Rudy D, Aiton A, Yao Z, Adeli K. Intracellular translocation and stability of apolipoprotein B are inversely proportional to the length of the nascent polypeptide. J Biol Chem 1998;273:33397–405.

[50] Taghibiglou C, Rudy D, Van Iderstine SC, Aiton A, Cavallo D, Adeli K. Intracellular mechanisms regulating apoB-containing lipoprotein assembly and secretion in primary hamster hepatocytes. J Lipid Res 2000;41:499–513.

[51] Boren J, Rustaeus S, Olofsson S-O. Studies on the assembly of apolipoprotein B-100- and B-48-containing very low density lipoproteins in McA-RH7777 cells. J Biol Chem 1994;269:25879–88.

[52] Benoist F, Grand-Perret T. apoB100 secretion by HepG2 cells is regulated by the rate of triglyceride biosynthesis but not by intracellular lipid pools. Arterioscler Thromb Vasc Biol 1996;16:1229–35.

[53] Jin FY, Kamanna VS, Kashyap ML. Niacin accelerates intracellular apoB degradation by inhibiting triacylglycerol synthesis in human hepatoblastoma (HepG2) cells. Arterioscler Thromb Vasc Biol 1999;19:1051–9.

[54] Kurokawa M, Hirano T, Furukawa S, Nagano S, Adachi M. Similar to oleic acid, eicosapentaenoic acid stimulates apolipoprotein B secretion by inhibiting its intracellular degradation in Hep G2 cells. Atherosclerosis 1995;112:59–68.

[55] Wu X, Shang A, Jiang H, Ginsberg HN. Low rates of apoB secretion from HepG2 cells result from reduced delivery of newly synthesized triglyceride to a ''secretion-coupled'' pool. J Lipid Res 1996;37:1198–206.

[56] Wu X, Shang A, Jiang H, Ginsberg HN. Demonstration of biphasic effects of docosahexaenoic acid on apolipoprotein B secretion in HepG2 cells. Arterioscler Thromb Vasc Biol 1997;17:3347–55.

[57] Post SM, Zoeteweij JP, Bos MHA, de Wit ECM, Havinga R, Kuipers F, Princen HMG. Acyl-coenzyme A: cholesterol acyltransferase inhibitor, avasimibe, stimulates bile acid synthesis and cholesterol 7α-hydroxylase in cultured rat hepatocytes and *in vivo* in the rat. Hepatology 1999;30:491–500.

[58] Du EZ, Fleming JF, Wang SL, Spitsen GM, Davis RA. Translocation-arrested apolipoprotein B evades proteasome degradation via a sterol-sensitive block in ubiquitin conjugation. J Biol Chem 1999;274:1856–62.

[59] Fleming JF, Spitsen GM, Hui TY, Olivier L, Du EZ, Raabe M, Davis RA. Chinese hamster ovary cells require the coexpression of microsomal triglyceride transfer protein and cholesterol 7α-hydroxylase for the assembly and secretion of apolipoprotein B-containing lipoproteins. J Biol Chem 1999;274:9509–14.

[60] Chang TY, Chang CC, Cheng D. Acyl-coenzyme A: cholesterol acyltransferase. Annu Rev Biochem 1997;66:613–38.

[61] Rumsey SC, Galeano NF, Lipschitz B, Deckelbaum RJ. Oleate and other long chain fatty acids stimulate low density lipoprotein receptor activity by enhancing acyl coenzyme A: cholesterol acyltransferase activity and altering intracellular regulatory cholesterol pools in cultured cells. J Biol Chem 1995;270:10008–16.