

Inhibition of apolipoprotein B and triglyceride secretion in human hepatoma cells (HepG2)

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Abstract Apolipoprotein B (apoB), the major protein component of triglyceride-rich lipoproteins, is assembled into a lipoprotein particle via a complex, multistep process. Recent studies indicate that triglyceride-rich lipoprotein assembly requires the activity of the heterodimeric protein, microsomal triglyceride transfer protein (MTP). We identified a novel inhibitor of apolipoprotein B secretion using the human hepatoma cell line, HepG2. CP-10447, a derivative of the hypnotic drug methaqualone (Quaalude), inhibited apoB secretion from HepG2 cells with an IC_{50} of $\sim 5 \mu M$. CP-10447 also inhibited apoB secretion from Caco-2 cells, a model of intestinal lipoprotein production. In experiments using [³H]glycerol as a precursor for triglyceride synthesis, CP-10447 (20 μM) inhibited radiolabeled triglyceride secretion by $\sim 83\%$ ($P < 0.0001$) in HepG2 cells and 76% ($P < 0.05$) in Caco-2 cells with no effect on radiolabel incorporation into cellular triglyceride, indicating that CP-10447 inhibited triglyceride secretion without affecting triglyceride synthesis. RNA solution hybridization assay indicated that CP-10447 did not affect apoB or apoA-I mRNA levels. Pulse-chase experiments in HepG2 cells confirmed that CP-10447 inhibited the secretion of apoB (not its synthesis) without affecting secretion of total proteins or albumin and suggested that CP-10447 stimulates the early intracellular degradation of apoB in the endoplasmic reticulum (ER). Further studies demonstrated that CP-10447 is a potent inhibitor of human liver microsomal triglyceride transfer activity ($IC_{50} \sim 1.7 \mu M$) in an in vitro assay containing artificial liposomes and partially purified human MTP. These data suggest that CP-10447 may inhibit apoB and triglyceride secretion by inhibiting MTP activity and stimulating the early ER degradation of apoB. CP-10447 should provide a useful tool for further study of the mechanisms of apoB secretion and triglyceride-rich lipoprotein assembly.—Haghpassand, M., D. Wilder, and J. B. Moberly. Inhibition of apolipoprotein B and triglyceride secretion in human hepatoma cells (HepG2). *J. Lipid Res.* 1996. 37: 1468–1480.

Supplementary key words microsomal triglyceride transfer protein (MTP) • very low density lipoprotein assembly • hepatocyte • triacylglycerol • Caco-2 cells • enterocyte • methaqualone

Apolipoprotein B (apoB) is the major protein component of triglyceride-rich lipoproteins including chylomicrons and very low density lipoproteins (VLDL). Overproduction of triglyceride-rich lipoproteins and plasma accumulation of their remnants (chylomicron remnants, intermediate and low density lipoproteins) are

considered to be atherogenic and may contribute significantly to the morbidity and mortality of coronary heart disease. Consequently, a major focus of lipoprotein research is to understand the mechanism of apoB secretion and its relation to triglyceride-rich lipoprotein assembly.

Assembly of triglyceride-rich lipoproteins by the liver and intestine involves a complex process in which newly synthesized apoB is packaged with hydrophobic core lipids, including triglyceride and cholesteryl ester, and polar surface lipids, such as free cholesterol and phospholipid. Addition of core lipid (predominantly triglyceride) to the nascent lipoprotein particle is thought to occur in conjunction with the translation and translocation of apoB into the endoplasmic reticulum (ER) lumen (1–3). During this process, apoB remains tightly bound to the ER membrane (4, 5). Although additional core lipid and phospholipid are added in the pre-Golgi and Golgi compartments (6, 7), initial assembly in the ER is considered the rate-limiting step in triglyceride-rich lipoprotein secretion (8, 9).

In primary rat hepatocytes and human HepG2 cells, apoB is constitutively expressed and secretion is regulated at the post-translational level (10, 11). Under lipid-poor conditions, a significant proportion of newly synthesized apoB is rapidly degraded in the ER (8, 12, 13). Addition of substrates such as oleate to the culture medium protects nascent apoB from degradation by ER proteases and leads to increased apoB secretion (13–15). Conversely, n-3 fatty acids such as eicosapentaenoic or docosahexaenoic acid increase the degradation of apoB in the ER and decrease apoB secretion from rat hepatocytes (16). Thus, factors that affect initial core lipid addition and ER degradation of apoB may be key regulators of the secretion of triglyceride-rich lipoproteins.

Abbreviations: apoB, apolipoprotein B; CETP, cholesteryl ester transfer protein; ER, endoplasmic reticulum; MTP, microsomal triglyceride transfer protein; PAGE, polyacrylamide gel electrophoresis; VLDL, very low density lipoprotein.

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Microsomal triglyceride transfer protein (MTP) has recently been identified as a necessary factor for the assembly of triglyceride-rich lipoproteins (17). MTP is a heterodimeric protein consisting of a large, 97 kDa subunit and the 58 kDa multifunctional enzyme, protein disulfide isomerase. MTP is localized in the lumen of the ER in liver and intestine, where it transfers triglycerides, cholesteryl ester, and phospholipids between phospholipid membranes (18). Evidence that MTP plays an obligatory role in the assembly of apoB-containing particles comes from patients with abetalipoproteinemia, who synthesize apoB normally but are unable to secrete triglyceride-rich lipoproteins from the liver or intestine (19). These patients lack MTP activity and have been shown to have defects in the gene coding for the large subunit of MTP (17, 20, 21). Demonstration that MTP expression permits triglyceride and apoB secretion in transfected cells that do not normally secrete apoB further supports a critical role of MTP in triglyceride-rich lipoprotein assembly (22, 23).

In the present report, we describe a novel inhibitor of apoB secretion, CP-10447, a derivative of the hypnotic and anti-convulsive drug methaqualone (commonly known as Quaalude). CP-10447 inhibited apoB and triglyceride secretion from HepG2 and Caco-2 cells and also potently inhibited human liver MTP activity in an artificial liposome assay. These data suggest that inhibition of MTP activity may be the underlying mechanism for the inhibition of apoB secretion by CP-10447.

EXPERIMENTAL PROCEDURES

Materials

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 76 and used between passages 80 and 98. Caco-2 cells were subcultured from stocks obtained from F. Jeffrey Field (University of Iowa) and used between passages 50 and 60. Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids, L-glutamine, and gentamicin sulfate were obtained from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). Transwell filters were from Costar (Cambridge, MA). Ninety-six well Nunc-Maxisorb plates were obtained from Marsh Biomedical Products (Rochester, NY). Monoclonal antibodies for apolipoprotein A-I (Catalog No. MAB011) and apoB (Cat. No. MAB012) and polyclonal antibodies for apoB (Cat. No. AB742) were obtained from Chemicon (Temecula, CA). Monoclonal antibody C1.4 used in immunoblotting was a generous gift from Drs. Gustav Schonfeld and Elaine Krul (Washington University, St. Louis, MO). [³⁵S]methionine and [³H]glycerol were purchased from

Amersham (Arlington Heights, IL). Superose-6 gel filtration columns and FPLC equipment were from Pharmacia (Piscataway, NJ). cDNAs for apoA-I and apoB used in the RNase protection assay were obtained from Dr. Jan Breslow, Rockefeller University, New York, NY. BCA protein assay kit was purchased from Pierce (Rockford, IL). Ready Safe™ and Aquasol™ scintillation cocktails were obtained from Beckman (Fullerton, CA) and NEN/DuPont (Boston, MA), respectively. CP-10447, 4'-bromo methaqualone, was chemically synthesized. Organic solvents were purchased from Fisher Scientific (Pittsburgh, PA). All other reagents were obtained from Sigma (St. Louis, MO).

Cell culture

HepG2 cells were cultured in DMEM containing 2 mM L-glutamine, 5.5 mM glucose, 10% heat-inactivated FBS, and 40 µg/ml gentamicin sulfate. Cells were grown in plastic flasks or multi-well plates in a humidified 37°C incubator and allowed to attach for at least 24 h prior to each experiment. Experiments were initiated when cells reached at least 60% confluence (~1 × 10⁵ cells/cm²). Stocks of Caco-2 cells were cultured on plastic flasks in DMEM containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, 25 mM glucose, 20% heat-inactivated FBS, and 40 µg/ml gentamicin sulfate. Caco-2 cells were maintained in a humidified incubator aerated with 10% CO₂. For experiments examining apoB and triglyceride secretion, Caco-2 cells were plated on 24-mm diameter Transwell filters at a density of ~5 × 10⁵ cells/filter and grown for approximately 14 days with apical and basolateral media changes every second day. For incubations of Caco-2 cells with CP-10447, drug was added to both apical and basolateral media. In oleate studies, supplemented serum-free media was used consisting of DME/Ham's F-12, 25 mM HEPES, 17.5 mM glucose, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 35 mg/l human transferrin, 5 mg/l bovine pancreatic insulin, 20 µM ethanolamine, 25 µM sodium selenite, and 40 µg/ml gentamicin (11).

Assay conditions

CP-10447 was dissolved in DMSO at a concentration of 40–50 mM and diluted to appropriate concentrations in media prior to incubation with cells. DMSO at an equivalent concentration was added to control wells. The resulting DMSO concentration was 0.2% at 100 µM CP-10447 and 0.02% at 10 µM CP-10447. In control experiments, cellular morphology and lipoprotein secretion were unaffected at DMSO concentrations up to 1%. Cells were typically incubated with CP-10447 for 6 or 24 h in complete medium containing FBS. Media were collected and centrifuged to remove cellular debris and assayed immediately or frozen at -70°C. When

applicable, cells were harvested and assayed for protein by the Lowry method (24) or BCA protein assay (25). Cells were lysed for apolipoprotein or lipid measurements as described below.

Apolipoprotein B and A-I ELISA

Secreted apoA-I and apoB levels were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (26). The antibodies used were repeatedly shown to be specific in both immunoblotting and immunoprecipitation experiments. HepG2 and Caco-2 cell conditioned media were diluted 1/10 for apoB and 1/100 for apoA-I ELISA. ApoB in the form of ultracentrifugally isolated LDL and purified apoA-I (Sigma) were used for generating standard curves for the apoB and apoA-I ELISA, respectively.

FPLC analysis

Separation of lipoproteins from conditioned media was performed using fast protein liquid chromatography (FPLC) gel filtration as previously described (27). Conditioned media samples (1 ml) were injected into an FPLC system and eluted from two Superose 6 columns connected in series. Elution buffer consisted of physiological saline (pH 8.2) containing 1 mM EDTA. Fractions (1 ml each) were assayed for apoB and apoA-I by ELISA as described above.

Lipid analysis

HepG2 and Caco-2 cells cultured on 24-well plates or Transwell filters were preincubated with 20 μ M CP-10447 or DMSO and then pulse-labeled with 4–5 μ M [3 H]glycerol (19.4 Ci/mmol) in the presence or absence of drug. Media and cells were harvested at the end of 6 h and radiolabeled lipids were extracted in chloroform–methanol 2:1. Radiolabeled lipids were separated by thin-layer chromatography in petroleum ether–diethyl ether–acetic acid 80:20:0.5 on silica gel plates, and bands corresponding to triglyceride or phospholipids were cut and counted in a Beckman scintillation counter using Aquasol™ scintillation cocktail.

RNase protection assay

mRNA quantitation by RNA solution hybridization was performed as previously described (26). Riboprobes were synthesized by *in vitro* transcription of antisense RNA from cDNA probes in the presence of [32 P]UTP. Subsequently, RNA samples extracted from HepG2 cells were hybridized to radiolabeled riboprobes for 3.5 h at 63°C. After hybridization, samples were treated with RNase A and T1 for 40 min. RNase-resistant RNA-RNA hybrids were TCA-precipitated, collected on glass wool filters, and counted for radioactivity.

Oleate treatment

Oleic acid was complexed to BSA according to previously published work (11). HepG2 or Caco-2 cells, plated in 24-well Costar plates, were incubated in the presence or absence of 0.81 mM oleate with or without 20 μ M CP-10447 for 24 h in serum-free media. ApoA-I and apoB secretion were determined as described above. In addition, triglyceride secretion was determined by labeling Caco-2 cells with [3 H]glycerol as described above.

Pulse chase studies

HepG2 cells were preincubated with or without CP-10447 for 2 h, pulsed for 10 min with 120 μ Ci of [35 S]methionine (~1300 Ci/mmol) in methionine-free DMEM without serum, and chased with complete media containing a 10-fold excess concentration of unlabeled methionine. At each time point, media were collected and cell extract was obtained after incubation of cells in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μ g/ml PMSF, 1 μ g/ml aprotinin, and 1% NP-40) for 20 min. Cell extract and media were spun to remove cellular debris, and apoB was immunoprecipitated as previously described (26). Immunoprecipitated samples were either directly counted in Ready Safe™ scintillation cocktail or run on a 3–13% SDS-PAGE gradient gel, dried, and exposed using autoradiography. Total protein synthesis was determined by incubating aliquots of media or cells with 20% cold trichloroacetic acid overnight at 4°C, followed by precipitation, washing, solubilization, and scintillation counting of the protein pellet.

MTP activity assay

Human hepatic microsomes, used as the source of MTP activity, were isolated by the procedure of Wetterau and Zilversmit (28). Briefly, human liver chunks were frozen at -80°C, thawed on ice, minced, and rinsed several times with ice-cold 0.25 M sucrose. All subsequent steps were performed on ice. A 50% homogenate in 0.25 M sucrose was prepared using a Potter-Elvehjem Teflon pestle. The homogenate was diluted 1:1 with 0.25 M sucrose and centrifuged at 10,000 *g* for 20 min at 4°C. The pellet was resuspended in sucrose and recentrifuged at 10,000 *g* for 20 min. Supernatants were combined and the microsomes were pelleted by centrifugation at 105,000 *g* for 75 min. The supernatant was discarded and the microsomal pellet was suspended in a minimal volume of 0.25 M sucrose, diluted to 3 ml per gm starting liver weight with 0.15 M Tris-HCl, pH 8.0. This suspension was divided into 12 fractions, and centrifuged at 105,000 *g* for 75 min. Supernatants were discarded and the microsomal pellets were stored frozen at -80°C until needed. A thawed pellet was sus-

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ended in 12 ml of cold 50 mM Tris-HCl, 50 mM KCl, 5 mM MgCl (pH 7.4), and 1.2 ml of a 0.54% deoxycholate (pH 7.4) solution was added slowly with mixing to disrupt the microsomal membrane. After a 30-min incubation on ice with gentle mixing, the suspension was centrifuged at 105,000 *g* for 75 min. The supernatant, containing the soluble MTP protein, was dialyzed for 2–3 days with 4 changes of assay buffer (150 mM Tris-HCl, 40 mM NaCl, 1 mM EDTA, 0.02% NaN₃, pH 7.4). The partially purified human liver MTP was stored at 4°C and diluted 1:5 with assay buffer just before use. MTP preparations showed no notable loss of transfer activity with storage up to 30 days.

Donor and acceptor liposomes were prepared by the procedure described by Wetterau et al. (17). Donor liposomes were prepared under nitrogen by bath sonication of a dispersion containing 447 μM egg phosphatidylcholine, 83 μM bovine heart cardiolipin, and 0.91 μM [¹⁴C]triolein (110 Ci/mol). The lipids in chloroform were added in the proper amounts and dried under a nitrogen stream before hydrating with assay buffer. Sonication was performed at room temperature. Acceptor liposomes were prepared under nitrogen by bath sonication of a dispersion containing 1.3 mM egg phosphatidylcholine, 2.6 μM triolein, and 0.5 nM ³H-labeled egg phosphatidylcholine (50 Ci/mol) in assay buffer. The donor and acceptor liposomes were centrifuged at 160,000 *g* for 2 h at 7°C. The top 80% of the supernatant, containing small unilamellar liposomes, was carefully removed and stored at 4°C until used for transfer assays.

MTP activity was determined by adding 200 μl of assay buffer containing either 5% BSA (control) or 5% BSA plus various concentrations of CP-10447 to a mixture containing 50 μl donor liposomes, 100 μl acceptor liposomes, and 150 μl of partially purified human liver MTP. The BSA, liposomes, and MTP were diluted in assay buffer (total 500 μl) and incubated at 37°C for 45 min. Triglyceride transfer was terminated by the addition of 300 μl of a 50% (w/v) DEAE cellulose suspension in assay buffer. After 4 min of agitation, the donor liposomes, bound to the DEAE cellulose, were selectively sedimented by low speed centrifugation. An aliquot of the supernatant containing the acceptor liposomes was counted and the ¹⁴C and ³H counts were used to calculate the percent recovery of acceptor liposomes and the percent triglyceride transfer using first order kinetics.

CETP activity assay

Partially purified cholesteryl ester transfer protein (CETP) was prepared from cell culture media conditioned by Chinese hamster ovary cells expressing full-length human CETP using a single butyl Toyopearl column as described elsewhere (29). The purity of this

preparation of CETP was approximately 30%. CETP activity was determined exactly as for MTP except that partially purified CETP (0.25 μg) was used in place of MTP and the incubation was carried out for 2 h.

Other methods

Secretion of high and low molecular weight forms of apoB (B-100 and B-48) by Caco-2 cells was examined by immunoblotting of aliquots of media using a monoclonal antibody as previously described (30). Bands representing apoB-100 and apoB-48 on the immunoblot (2 h exposure) were quantitated by scanning densitometry using Molecular Analyst™ software (Bio-Rad Laboratories, version 2.0). Statistical analysis was performed by Student's *t* test using Statview statistical software package for Macintosh or by ANOVA using JMP® software (version 2). Results are reported as means ± standard deviation unless otherwise stated.

RESULTS

CP-10447, a bromine derivative of methaqualone (Fig. 1A), inhibited apoB secretion from HepG2 cells with an IC₅₀ of ~5 μM when incubated for 24 h in complete media (Fig. 1B). In contrast to apoB, apoA-I secretion from HepG2 cells was not affected at CP-10447 concentrations up to 50 μM. When examined by phase contrast microscopy, morphology of attached cells was not affected at any concentration up to a maximum of 200 μM. The secretion of apoB was linear for at least 6 h, and the secretion of apoA-I was linear for at least 12 h (Fig. 2). A decrease in apoB secretion was evident within 2 h after the addition of CP-10447 (10 μM), and this decrease remained significant at all subsequent times. Levels of apoB detected at times earlier than 1.5 h were at the lower limit of detection by ELISA assay and were therefore not significantly different when cells were incubated with CP-10447. Based on these data, the rate of apoB secretion between 3 and 11 h was 60% lower in cells incubated with 10 μM CP-10447 compared with controls. CP-10447 also inhibited secretion of metabolically labeled apoB by approximately 70% at 6 h, in good agreement with results from the ELISA (data not shown). Total protein secretion and cellular protein synthesis were unaffected by CP-10447 as determined by TCA precipitation and scintillation counting of radiolabeled proteins.

The majority of apoB secreted by HepG2 cells is not associated with VLDL but resides instead within triglyceride-enriched, LDL-sized particles (31). In order to determine whether CP-10447 affected the size of the lipoprotein particles containing apoB, HepG2-conditioned media were fractionated by gel filtration and

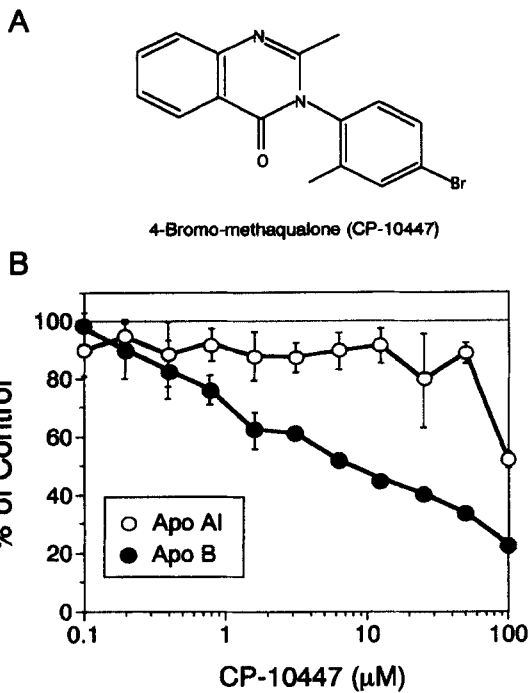


Fig. 1. Panel A: Chemical structure of CP-10447. Panel B: Inhibition of apoB secretion by CP-10447 in HepG2 cells. HepG2 cells were seeded at ~50,000 cells/well in 96-well plates and cultured as described in Experimental Procedures. After 48 h, media were replaced with complete media containing increasing concentrations of CP-10447 diluted in DMSO. After 24 h, conditioned media were collected and apoB and apoA-I mass were determined by ELISA. Values are means \pm standard deviation, $n = 4$ for each point. Results presented are characteristic of more than three separate experiments.

assayed by ELISA. Consistent with published results, apoB secreted by HepG2 cells was found in the LDL-size range after FPLC gel filtration (Fig. 3A). CP-10447 (10 μ M) dramatically decreased the amount of apoB present in lipoproteins in the LDL size range. Interestingly, the amount of apoB associated with very small LDL (shoulder in Fig. 3A) was less affected by CP-10447. There was no consistent effect of CP-10447 on the amount or size of apoA-I-containing lipoproteins (Fig. 3B).

The formation of small, dense apoB-containing particles by HepG2 cells is presumably due to the relatively low rates of triglyceride secretion by these cells, in spite of normal cellular contents of triglyceride (32). To determine whether inhibition of apoB secretion by CP-10447 was associated with a decrease in triglyceride synthesis or secretion, we radiolabeled HepG2 cells with [3 H]glycerol in the presence of CP-10447, and isolated radiolabeled lipids by thin-layer chromatography. CP-10447 (20 μ M) inhibited radiolabeled triglyceride secretion from HepG2 cells by more than 83% without affecting cellular triglyceride content (Fig. 4). Because the amount of secreted triglyceride is small relative to total triglyceride (<10%), this degree of triglyceride secretion

inhibition was not expected to affect (i.e., increase) cellular triglyceride levels. Similar results were obtained when oleate was used as the label instead of glycerol (data not shown). These data are consistent with an inhibition of hepatic triglyceride-rich lipoprotein secretion independent of changes in cellular triglyceride synthesis.

Like hepatocytes, enterocytes also package and secrete apoB in triglyceride-rich particles. However, unlike human hepatocytes, human enterocytes secrete triglyceride-rich lipoproteins containing a truncated version of apoB, apoB-48, which is produced by a unique RNA editing mechanism (33). In order to determine whether CP-10447 inhibited secretion of apoB and triglyceride-rich lipoproteins from enterocytes and whether secretion of apoB-48 was also inhibited, we studied the effect of CP-10447 in the model enterocyte cell line, Caco-2. Caco-2 cells cultured on permeable supports secrete lipoproteins and apolipoproteins predominantly into the basolateral or serosal compartment. Under conditions that we have previously defined, differentiated Caco-2 cells secrete approximately 50% of

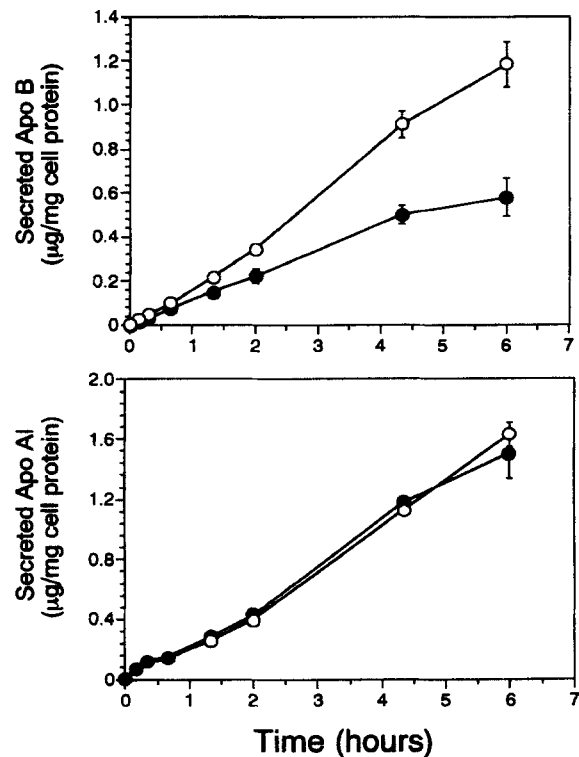


Fig. 2. Time course of apoB and apoA-I accumulation in response to CP-10447. HepG2 cells were seeded at ~50,000 cells/well in 96-well plates. After 48 h, media were replaced with complete media (open circles) or complete media containing 10 μ M of CP-10447 (closed circles). At the times indicated, media samples were collected, and apoB and apoA-I levels were measured by ELISA as described above. Cells were lysed using 0.2 N NaOH and protein levels were determined using BCA protein assay; $n = 3 \pm$ standard deviation for each point.

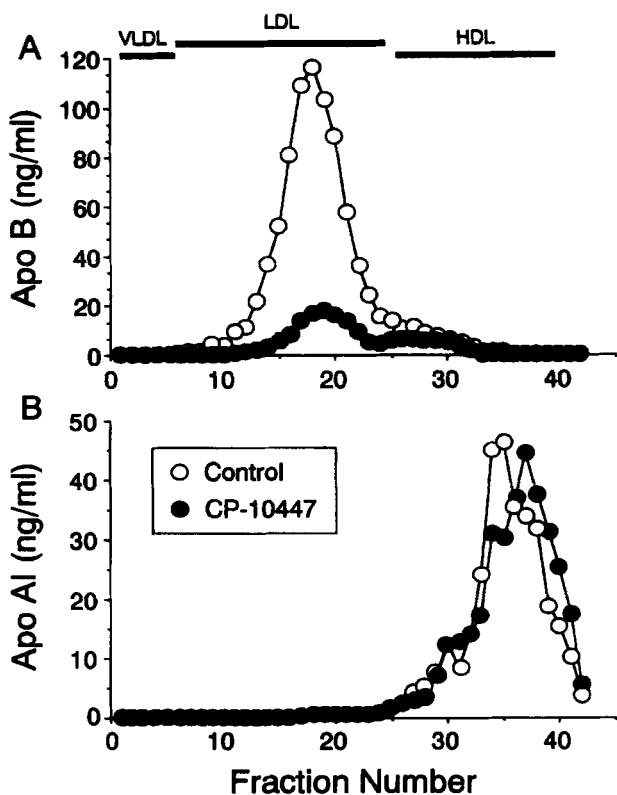


Fig. 3. Effect of CP-10447 on accumulation of LDL size lipoproteins from HepG2 cells. Subconfluent HepG2 cells were treated with or without $10 \mu\text{M}$ CP-10447 for 24 h. Conditioned media were separated by FPLC and fractions were tested for apoB (panel A) and apoA-I (panel B) by ELISA. Bars above the graph indicate the fractions that contain VLDL, LDL, and HDL in a human plasma sample separated by the FPLC under the same conditions.

apoB as B-48 (30), the remainder being secreted as apoB-100. In Caco-2 cells grown on Transwell filters, CP-10447 (at $30 \mu\text{M}$) inhibited secretion of total apoB (determined by ELISA) by 55% into the basolateral medium ($P < 0.01$, $n = 3$) and by ~58% into the apical medium ($P < 0.01$). Secretion of apoB into the apical medium accounted for approximately 30–35% of the total apoB secretion regardless of the presence or absence of CP-10447. ApoA-I secretion from Caco-2 cells was not affected at concentrations of CP-10447 up to $400 \mu\text{M}$ (data not shown). Interestingly, when the two molecular weight forms of apoB were differentiated by SDS-PAGE and immunoblotting (Fig. 5), it was found that secretion of apoB-100 into the basolateral media was inhibited by 41% (6.79 ± 0.75 vs. 11.57 ± 1.26 arbitrary densitometric units; $P < 0.01$) whereas apoB-48 secretion was not significantly inhibited (11.63 ± 0.90 vs. 11.45 ± 2.05 arbitrary densitometric units). These data suggest that CP-10447 may be unable to inhibit the assembly and secretion of apoB-48-containing lipoproteins and that differing mechanisms may be involved in

the assembly of apoB-100- and apoB-48-containing lipoproteins by Caco-2 cells.

Consistent with studies using HepG2 cells, CP-10447 also inhibited the basolateral secretion of radiolabeled triglyceride by Caco-2 cells (~82% at $20 \mu\text{M}$) (Fig. 6). Basolateral secretion of phospholipid and cellular triglyceride and phospholipid levels were not affected. However, when [^3H]oleate was used as the lipid precursor, secretion of radiolabeled triglyceride, cholesteryl ester, and phospholipids were all inhibited (data not shown). Levels of intracellular radiolabeled triglyceride, cholesteryl ester, and phospholipid were not affected by CP-10447. Taken together, these data suggest that CP-10447 is able to inhibit secretion of apoB-100-containing triglyceride-rich lipoproteins from Caco-2 cells without affecting the synthesis of cellular lipids.

apoB expression by cultured hepatocytes and enterocytes is constitutive, and secretion or production of apoB is believed to be regulated at the post-transcriptional level (10, 11). However, as the mechanism of inhibition of apoB secretion by CP-10447 was unknown,

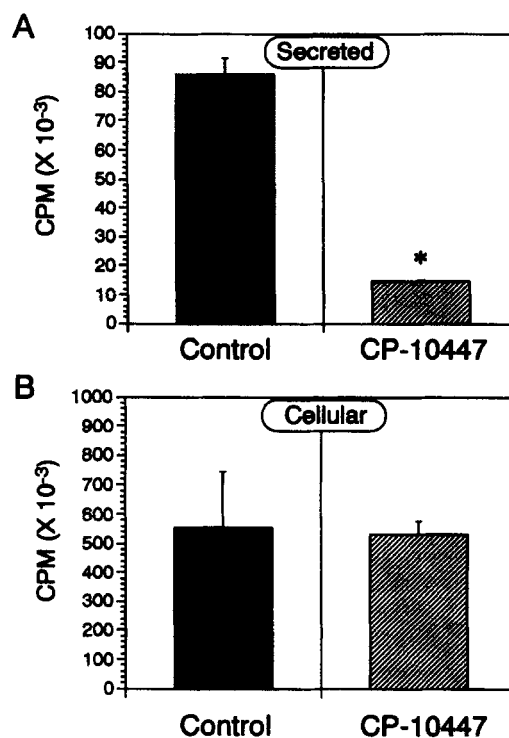


Fig. 4. Effect of CP-10447 on radiolabeled triglyceride secretion and accumulation in HepG2 cells. Subconfluent HepG2 cells grown in 24-well plates were preincubated with or without $20 \mu\text{M}$ CP-10447 for 80 min. Subsequently, cells were pulsed with [^3H]glycerol ($100 \mu\text{Ci}/\text{well}$) in the presence (hatched bars) or absence (solid bars) of CP-10447 for 6 h. Secreted (panel A) and cellular (panel B) lipids were measured as described in Experimental Procedures. Ordinate values must be multiplied by 1,000 to obtain actual cpm incorporation into lipids; $n = 5 \pm$ standard deviation for each point; * $P < 0.0001$.

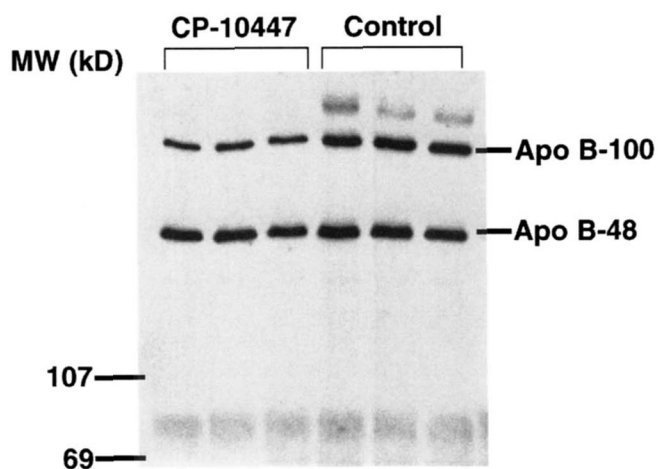


Fig. 5. Immunoblot of apoB secreted into the basolateral medium of Caco-2 cells. Caco-2 cells were grown on Transwell filters as described in Experimental Procedures. Approximately 2 weeks post confluence, complete media containing 30 μ M CP-10447 were added to both the apical and basolateral sides. After 24 h, complete medium was electrophoresed on a 3–6% gradient polyacrylamide gel, transferred to nitrocellulose by Western blotting, and immunoblotted with a monoclonal antibody to apoB (C1.4). Antibody-antigen complexes were detected using 125 I-labeled goat anti-mouse IgG and autoradiographed for 2 h. Molecular weight standards are indicated on the left and the migration of apoB-100 and apoB-48 on the right.

we addressed whether inhibition of apoB secretion by CP-10447 was associated with a decrease in apoB transcription by measuring apoB mRNA levels using a sensitive RNase protection assay. ApoB and apoA-I mRNA levels were determined from subconfluent HepG2 cells grown in 100-mm dishes and incubated for 24 h with 20 μ M CP-10447. CP-10447 did not significantly alter cellular levels of apoB or apoA-I mRNA (96 \pm 4% of control for apoB and 89 \pm 12% of control for apoA-I, $P > 0.05$ [NS]). Media samples from the same plates from which RNA was extracted showed a marked decrease in apoB levels in the presence of CP-10447. This experiment ruled out the possibility that inhibition of apoB secretion by CP-10447 was the result of an inhibition of apoB transcription.

A major mechanism of post-translational regulation of apoB secretion is through its intracellular degradation in the endoplasmic reticulum (12–14). In HepG2 cells, up to 70–80% of newly synthesized apoB is degraded by ER proteases within the first 20 min after its synthesis (14). Fatty acids such as oleate protect nascent apoB from degradation and hence increase apoB secretion by increasing its transport through the secretory pathway (14, 15). To determine whether CP-10447 affects the degradation of newly synthesized apoB, HepG2 cells were pulse-labeled for 10 min with [35 S]methionine and chased with medium containing 10-fold excess unlabeled methionine for 0, 5, 10, 15, 20, and 40 min. A

significantly greater intracellular degradation, represented by a significant decrease in the intracellular concentration of radiolabeled, newly synthesized apoB, was observed as early as 5 min after the pulse ($1.46 \pm 0.151 \times 10^6$ dpm/mg cell protein vs. $2.0 \pm 0.091 \times 10^6$ dpm/mg cell protein; $P < 0.05$; Fig. 7, upper panel). This decrease remained significant at 10 min ($P < 0.001$), 15 min ($P < 0.005$), and 20 min ($P < 0.001$) but not at 40 min after the pulse. Secretion of newly synthesized, radiolabeled apoB was not affected at 20 min but was significantly inhibited at 40 min after the pulse ($P < 0.001$). There was no apoB detected in the culture medium prior to 20 min. Immunoprecipitated samples of intracellular apoB were also run on SDS-PAGE to confirm the direct counting technique (lower panel, Fig. 7). Degradation and secretion of total cellular proteins (based on radiolabel incorporation into total TCA precipitable proteins) was less rapid and was not affected by CP-10447 (data not shown).

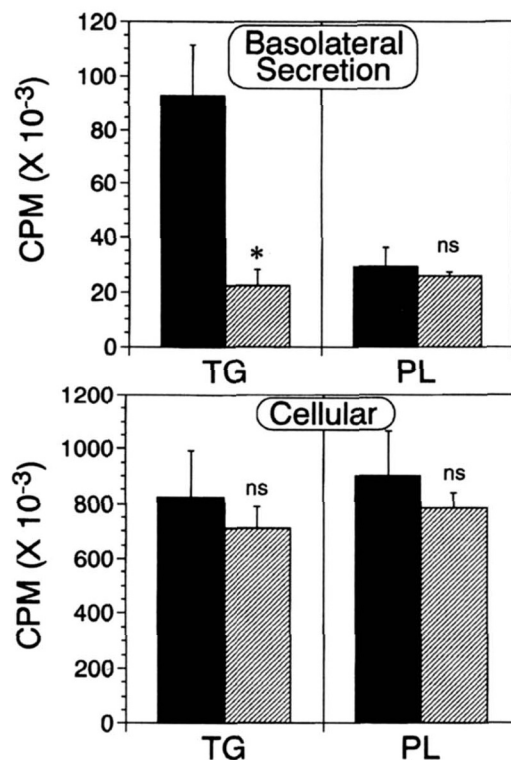


Fig. 6. Effect of CP-10447 on radiolabeled triglyceride and phospholipid secretion and accumulation in Caco-2 cells. Post-confluent Caco-2 cells grown on Transwell filters were preincubated for 1 hr with or without 20 μ M CP-10447. Subsequently, cells were incubated for 6 hours with [3 H]-glycerol (85 μ Ci/well) added to the apical medium in the presence (hatched bars) or absence (solid bars) of CP-10447 added to both the apical and basolateral media. Basolateral and cellular lipids were measured as described in Experimental Procedures. Ordinate values must be multiplied by 1,000 to obtain actual CPM incorporated into lipids; $n = 3 \pm$ standard deviation; * $P < 0.01$; ns, not significant.

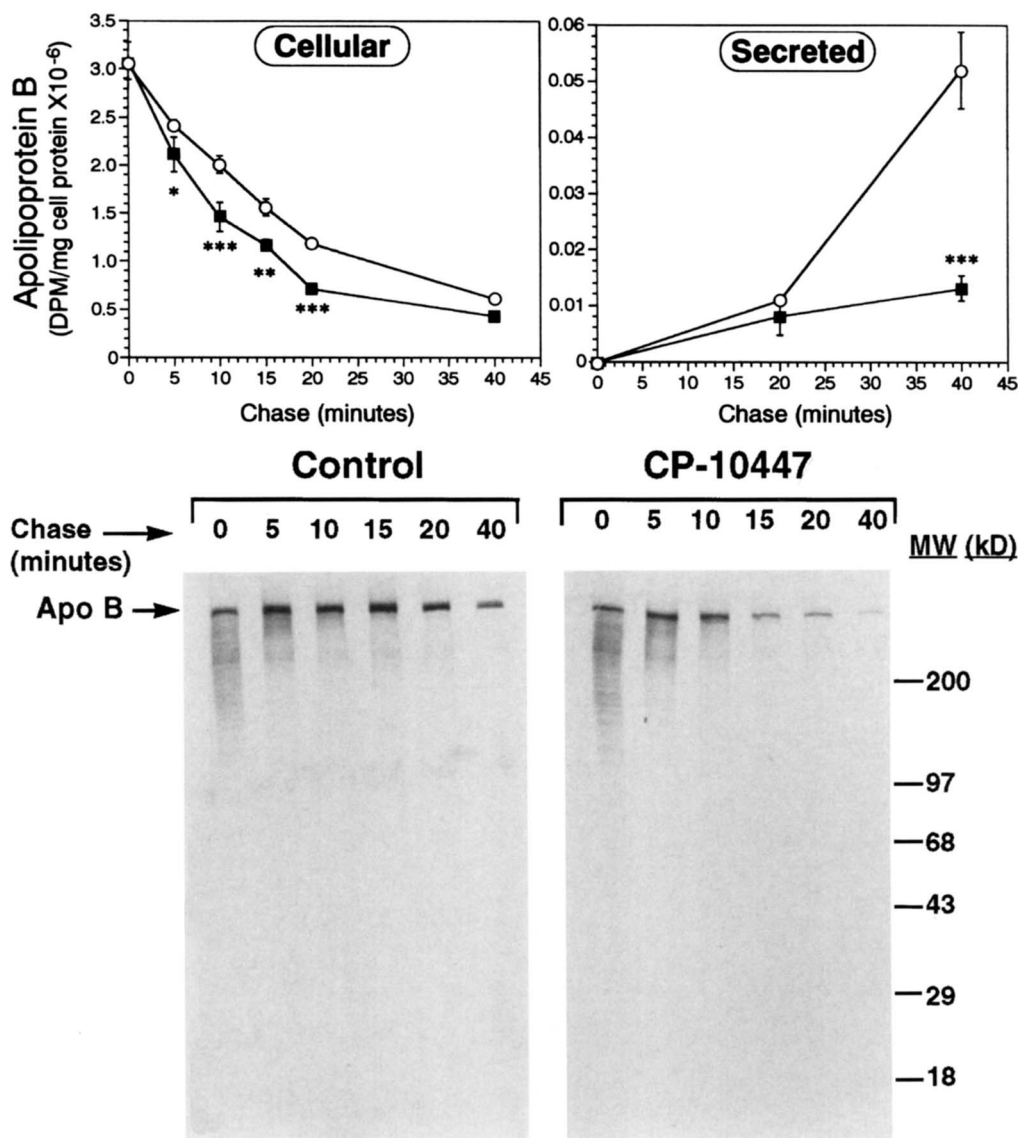


Fig. 7. Effect of CP-10447 on early degradation of intracellular apoB. Subconfluent HepG2 cells were preincubated with or without 40 μ M CP-10447 for 2 h, pulsed for 10 min with [35 S]methionine (120 μ Ci/well), and chased with 10 \times unlabeled methionine for various times in the presence (closed boxes) or absence (open circles) of CP-10447. ApoB was immunoprecipitated from the cell lysate and media samples and counted directly for radioactivity (upper panel). Alternatively, apoB was immunoprecipitated from the cell lysate and run on two SDS-PAGE gels, dried, and exposed to autoradiography (lower panel). Total dpm levels in the upper panels are expressed per mg of cell protein determined by BCA protein assay. Ordinate values must be multiplied by one million to obtain actual dpm of apoB per mg of cell protein; $n = 3 \pm$ standard deviation for each point; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

Oleic acid is known to protect apoB from degradation and to increase its secretion from HepG2 and Caco-2 cells. In order to test whether CP-10447 would prevent the increase in apoB and triglyceride secretion by oleic acid or whether oleate would overcome the inhibitory affect of CP-10447, we measured triglyceride and apoB secretion from HepG2 and Caco-2 cells treated with or without 20 μ M CP-10447 in the presence or absence of 0.8 mM oleic acid complexed to bovine serum albumin

(**Fig. 8** and **Fig. 9**). In the presence of oleic acid, apoB secretion from HepG2 cells was more than doubled ($1.59 \pm 0.17 \mu$ g/well vs. 0.67 ± 0.036) while secretion of apoA-I was not affected, consistent with previous results (10, 11). CP-10447 inhibited apoB secretion from oleate-treated cells by a similar percentage as in non-oleate-treated cells (76.2% inhibition in the presence of oleate vs. 67.2% inhibition in the absence of oleate). Similar results were obtained in Caco-2 cells. Oleate increased

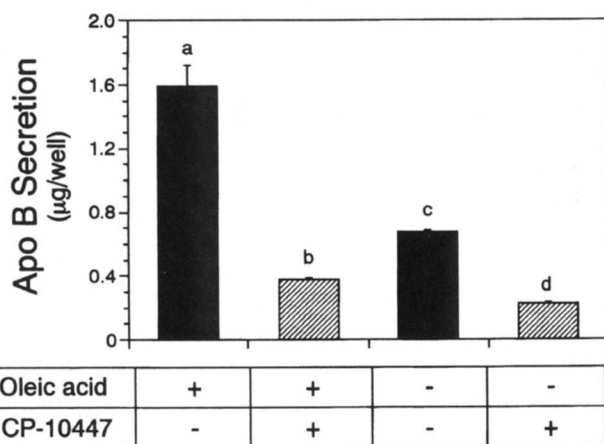


Fig. 8. Effect of oleic acid on the inhibition of apoB secretion by CP-10447 in HepG2 cells. HepG2 cells were plated in 24-well plates at $\sim 3 \times 10^5$ cells/well. After 48 h, fresh media were added containing either 0.81 mM oleate complexed to bovine serum albumin (BSA), oleate plus 20 μ M CP-10447, CP-10447 plus BSA equivalent to that in the oleate addition, or BSA alone. After 24 h, apoB and apoA-I levels were determined by ELISA. ApoA-I levels (not shown) were not significantly different for any of the groups tested. Mean values were significantly different by ANOVA (b vs. d, $P < 0.05$; all other pairs, $P < 0.01$); $n = 3 \pm$ standard deviation for each group.

apoB secretion by 1.6-fold (3.14 ± 0.24 μ g/well vs. 1.93 ± 0.27 μ g/well) in the basolateral media. In Caco-2 cells, the effect of CP-10447 on apoB secretion was greater in non-oleate-treated cells compared to oleate-treated cells (70.6% inhibition in the absence of oleate vs. 46.5% inhibition in the presence of oleate). The effect of oleate and CP-10447 on triglyceride secretion was also assessed in Caco-2 cells (Fig. 9, lower panel). Oleate increased triglyceride secretion into the basolateral media by approximately 18-fold (3489 ± 956 vs. 198 ± 56). Addition of CP-10447 resulted in approximately 90% inhibition of triglyceride secretion in the presence of oleate and 84% in the absence of oleate. Thus, in both HepG2 cells and Caco-2 cells, CP-10447 can inhibit apoB secretion even when secretion of apoB is stimulated by oleic acid.

Microsomal triglyceride transfer protein has been recently implicated to play a major role in initial assembly of apoB-containing particles. We tested the effect of CP-10447 on human liver MTP activity using an artificial liposome assay consisting of [14 C]triolein-labeled donor liposomes and phospholipid acceptor liposomes (17). CP-10447 potently inhibited MTP activity with an IC_{50} of approximately 1.7 μ M in this assay (Fig. 10). In order to rule out the possibility of nonspecific interaction of CP-10447 with liposomes, the effect of CP-10447 on CETP-mediated transfer was measured under the same assay conditions as for MTP. CP-10447 had no effect on CETP-mediated transfer of triglyceride at concentrations as high as 30 μ M. These results combined with the discovery of other hydrophobic analogs of CP-10447

that do not inhibit MTP suggest that CP-10447 is a specific inhibitor of MTP and rule out the possibility of a nonspecific hydrophobic perturbation of liposomes. Furthermore, the effect of CP-10447 analogs on MTP activity correlated with their ability to inhibit apoB secretion. For example, methaqualone hydrochloride, a less potent inhibitor of apoB secretion from HepG2 cells, inhibited apoB secretion with an IC_{50} of ~ 40 μ M (vs. 5 μ M for CP-10447) and inhibited MTP activity with an IC_{50} of ~ 13.2 μ M (vs. 1.7 μ M for CP-10447). These data suggest that inhibition of apoB secretion by CP-10447 may be a result of its ability to directly inhibit human liver MTP activity.

DISCUSSION

CP-10447, a derivative of the hypnotic drug methaqualone, inhibits apoB and triglyceride secretion

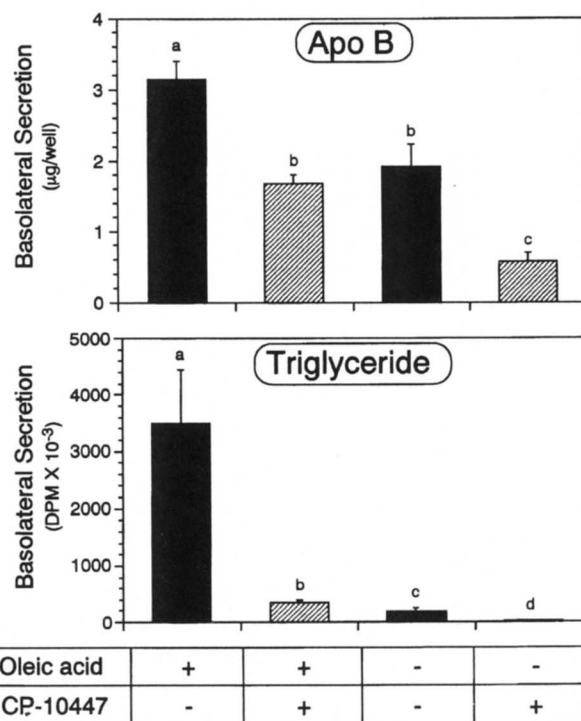


Fig. 9. Effect of oleic acid on the inhibition of apoB and triglyceride secretion by CP-10447 in Caco-2 cells. Confluent Caco-2 cells were incubated for 24 h with serum-free media containing 0.81 mM oleate or BSA control with or without 20 μ M CP-10447. ApoB was determined by ELISA (upper panel). Caco-2 cells were labeled for 6 h with ~ 5 μ Ci/filter with [3 H]glycerol in the presence or absence of oleate and CP-10447. Radiolabeled triglycerides were detected in the basolateral media as described in Experimental Procedures (lower panel). Values on the Y-axis in the lower panel must be multiplied by 1,000 to obtain total dpm incorporated. In each panel, values marked with different letters are significantly different ($P < 0.01$) by ANOVA. Triglyceride data were log transformed prior to analysis. Actual triglyceride counts were a) $3.49 \pm 0.96 \times 10^6$ dpm; b) $3.54 \pm 0.38 \times 10^5$ dpm; c) $1.98 \pm 0.56 \times 10^5$ dpm; and d) $3.1 \pm 0.2 \times 10^4$ dpm; $n = 3 \pm$ standard deviation for each group.

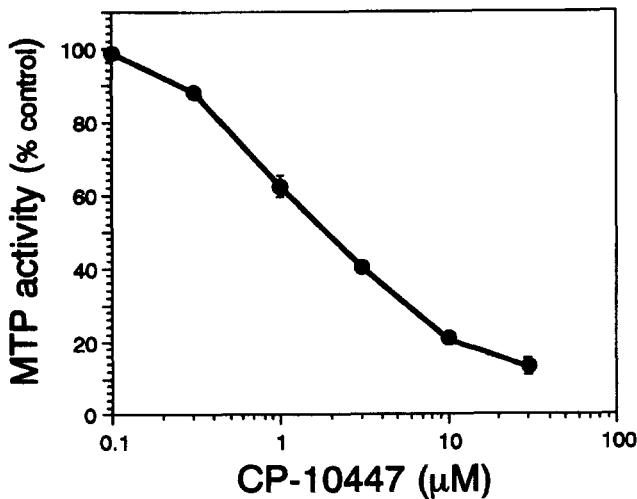


Fig. 10. Effect of CP-10447 on human liver microsomal triglyceride transfer activity. MTP activity was determined by adding 100 μ l of assay buffer containing either 5% BSA (control) or 5% BSA plus various concentrations of CP-10447 ranging from 0.1 to 30 μ M to a mixture of donor liposomes, acceptor liposomes, and partially purified human MTP as described in Experimental Procedures. After incubation at 37°C for 45 min, triglyceride transfer was measured in the acceptor liposomes after precipitation of donor liposomes with DEAE cellulose. Transfer is corrected for nonspecific transfer (typically 2–4% of total transfer); $n = 3$ for each point \pm standard deviation. Results are representative of three independent experiments.

from HepG2 and Caco-2 cells and inhibits triglyceride transfer activity in an assay containing partially purified human MTP. The correlation between the inhibition of apoB and triglyceride secretion and the inhibition of microsomal triglyceride transfer activity in this study is consistent with the hypothesis that inhibition of MTP results in an inhibition of apoB secretion and agrees with published evidence that triglyceride-rich lipoprotein assembly is highly dependent on functional MTP. We speculate that inhibition of MTP activity by CP-10447 is responsible for the inhibition of apoB secretion in HepG2 cells and that pharmacological inhibition of hepatic and intestinal MTP may decrease the formation of atherogenic apoB-containing lipoproteins.

The mechanism of apoB secretion inhibition in HepG2 cells by CP-10447 is clearly posttranscriptional, as both apoB mRNA levels and [35 S]methionine incorporation into apoB at the start of pulse-chase experiments were similar in treated and control cells. Agents that are known to affect apoB secretion act via a post-transcriptional mechanism and appear to alter the cellular degradation of apoB. For example, oleic acid stimulates apoB secretion from HepG2 cells by inhibiting its early intracellular degradation, apparently directing more apoB into the secretory pathway (13–15). The increased availability of fatty acid and triglyceride pre-

sumably permits folding of apoB into a conformation that protects it from degradation by ER proteases or, alternatively, allows for continuation of its translocation across the bilayer into the ER lumen (34, 35). Cholesteryl ester also stimulates apoB secretion via a posttranscriptional mechanism that involves cellular apoB degradation (36). Triacsin D, a potent inhibitor of fatty acyl-CoA acyltransferase and triglyceride synthesis, blocks the stimulation of apoB secretion from HepG2 cells by oleic acid and results in a corresponding increase in apoB degradation (37). Eicosapentaenoic and docosahexaenoic acids, fatty acids common in fish oil, increase apoB degradation and decrease apoB secretion from rat hepatocytes in spite of an increase in intracellular triglyceride synthesis (16, 38). Similarly, it has been suggested that insulin inhibits apoB and triglyceride-rich lipoprotein secretion from rat hepatocytes in part due to the stimulation of apoB degradation (39). Furthermore, the choline analog phosphatidylmonomethyl-ethanolamine inhibits VLDL and apoB secretion by inhibiting the translocation of apoB across the microsomal membrane into the lumen, stimulating intracellular degradation and increasing the susceptibility of apoB to ER proteases (40). As with other agents that inhibit apoB secretion, CP-10447 stimulates intracellular degradation of apoB (see Fig. 7). This increased degradation occurs during the first 10 min of chase, consistent with studies that demonstrate rapid, early degradation of apoB in the ER (13). Although these studies do not address the precise location or mechanism of increased apoB degradation or whether CP-10447 prevents translocation of apoB into the ER lumen, our results are consistent with the hypothesis that CP-10447 inhibits apoB secretion posttranscriptionally by blocking the early ER assembly of triglyceride-rich lipoproteins.

Unlike Triacsin D or fatty acid synthesis inhibitors that block hepatic VLDL production (37, 41), CP-10447 does not inhibit apoB secretion by inhibiting the synthesis of fatty acids or triglycerides. Concentrations of CP-10447 that result in an 80% reduction in secreted apoB and triglyceride from HepG2 and Caco-2 cells did not affect the cellular accumulation of radiolabeled triglyceride (see Figs. 4 and 6). In addition, treatment of HepG2 and Caco-2 cells with oleic acid was able to increase apoB and triglyceride secretion even in the presence of CP-10447 (Fig. 8 and 9). Finally, CP-10447 did not affect the incorporation of [3 H]oleate into cellular cholesteryl ester, indicating that CP-10447 does not inhibit de novo synthesis of cholesteryl ester.

The discovery that a defect in microsomal triglyceride transfer protein is the basis for the inability to secrete apoB-containing lipoproteins in abetalipoproteinemia lends support to the hypothesis that substrate delivery to nascent apoB is critical for triglyceride-rich lipopro-

tein assembly (17, 20, 21). MTP, a heterodimeric ER protein, is expressed predominantly in the liver and intestine (20, 21). Cells or tissues that lack MTP are unable to assemble triglyceride-rich lipoproteins, even when made to express apoB (22, 42). In non-hepatic cells transfected with a plasmid encoding truncated apoB, apoB secretion is prevented because of a block in its translocation across the ER membrane (43). However, when MTP is coexpressed with the same truncated apoB, secretion of apoB and triglyceride-rich lipoproteins is able to occur (22, 23). Our results are consistent with the hypothesis that CP-10447 inhibits substrate delivery and core-lipidation of nascent VLDL particles by inhibiting the microsomal triglyceride transfer protein. The IC_{50} for inhibition of MTP activity ($\sim 1.7 \mu\text{M}$) is less than that for inhibition of apoB secretion in both HepG2 cells ($\sim 5 \mu\text{M}$) and Caco-2 cells ($\sim 150 \mu\text{M}$). Proof that inhibition of MTP activity is the cause of inhibition of triglyceride and apoB secretion by CP-10447 will depend on further studies to dissect the intracellular action of MTP, perhaps using heterologous cells coexpressing human apoB and MTP or molecular binding studies using purified MTP.

Although the role of MTP in the initial assembly of apoB is now well documented, its exact function in the addition of bulk lipid to nascent VLDL or chylomicron particles is still poorly understood. It has been reported that nascent lipoproteins in the rough ER of rat liver contain enough lipid to fall in the same diameter range as VLDL (44). Our results are consistent with the hypothesis that MTP may be involved in the addition of lipid to nascent apoB subsequent to translocation from ER. In Caco-2 cells, the inhibition of triglyceride secretion by CP-10447 is greater than inhibition of apoB secretion, suggesting that more lipid-poor particles may be secreted in the presence of the MTP inhibitor. In cultured rat hepatocytes, triglyceride secretion was also inhibited to a greater extent than apoB secretion (unpublished observations). Further study will be necessary to address these observations.

We have observed that CP-10447 is a somewhat less potent inhibitor of apoB secretion in Caco-2 cells than in HepG2 cells. One possibility for this difference may be the difference in the level of MTP expression in liver and intestinally derived cells. In the hamster, MTP activity and mRNA levels in the proximal intestine are reported to be 4- to 10-times that found in the liver (45). Although it is not known whether similar differences in MTP expression and activity exist between Caco-2 and HepG2 cells, this could account for all or part of the difference in potency between cell types. Another possibility for the reduced potency of CP-10447 in Caco-2 cells is the apparent inability of CP-10447 to inhibit secretion of apoB-48 in these cells. Further studies are

underway to investigate this finding and to determine whether CP-10447 may be useful to investigate differences in the assembly of apoB-100- and apoB-48-containing lipoprotein particles.

Overproduction of VLDL apoB, or hyperapobetalipoproteinemia, is believed to be a major factor contributing to the hyperlipidemia of familial combined hyperlipidemia (FCHL) (46, 47). Hyperapobetalipoproteinemia and FCHL have been suggested to account for more than 10% of premature coronary heart disease in Western societies (48), and plasma apoB may be a better predictor of coronary atherosclerosis than total or LDL-cholesterol (49). Such findings on the importance of apoB and triglyceride-rich lipoproteins have led to a search for pharmacological inhibitors of apoB secretion. The ability of CP-10447 to inhibit apoB and triglyceride secretion in both the human HepG2 and Caco-2 cell lines and to potentially inhibit human liver MTP activity in vitro demonstrates the value of this approach for identifying new pharmacological agents to test this hypothesis in animal models and ultimately in humans. ■

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