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Apolipoprotein(a) Synthesis and Secretion from Hepatoma Cells Is Coupled to Triglyceride Synthesis and Secretion*

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Apolipoprotein(a) (apo(a)) is synthesized and secreted from liver cells and represents one of the two major protein components of the atherogenic lipoprotein, Lp(a). Little is known, however, of the factors that regulate the secretion of this protein. We have undertaken an analysis of the response to oleate supplementation in stable clones of HepG2 and McA-RH7777 cells expressing either a 6 K-IV or 17 K-IV isoform of apo(a). These cell lines were examined by pulse-chase analysis and each demonstrated an increase (range 2–6-fold) in apo(a) secretion following supplementation with 0.8 mM oleate. Microsomal membranes, prepared from HepG2 cells expressing a 6 K-IV apo(a) isoform, demonstrated that oleate supplementation increased the apparent protection of apo(a) from protease digestion, suggesting that alterations in the translocation efficiency of apo(a) may accompany the addition of oleate. Cells incubated with brefeldin A demonstrated increased recovery of the precursor form of apo(a) with oleate supplementation, suggesting that alterations in post-translational degradation may also contribute to the observed increase in apo(a) secretion following oleate addition. To further characterize the oleate-dependent increase in apo(a) secretion, cells were incubated with an inhibitor of the microsomal triglyceride transfer protein. These experiments demonstrated a dose-dependent decrease in apo(a) secretion from both cell lines. Furthermore, addition of either the microsomal triglyceride transfer protein inhibitor or triacsin C, an inhibitor of acyl-CoA synthase, completely abrogated the oleate-dependent increase in apo(a) secretion. Taken together, these data provide evidence that apo(a) secretion from hepatoma cells may be linked to elements of cellular triglyceride assembly and secretion.

Apolipoprotein(a) (apo(a))[1] is a highly polymorphic glycoprotein whose mRNA is expressed virtually exclusively in the liver of humans and higher primates (1–3). In humans, apo(a) circulates largely in association with low density lipoprotein, in the form of a hybrid lipoprotein referred to as Lp(a). The association between apo(a) and the major protein moiety of low density lipoprotein, namely apolipoprotein B100 (apoB100), is mediated through covalent linkage of a single unpaired cysteine residue in apo(a) to a complementary unpaired cysteine in the extreme carboxyl terminus of apoB100 (4, 5). Interest in the biology of this lipoprotein species is driven by the observation that elevated levels of Lp(a) in the plasma of humans is associated with increased risk for atherosclerotic heart and peripheral vascular disease (2, 3). In addition, while the molecular and physiologic basis for this observation is currently unresolved, the consensus appears to be that plasma levels of Lp(a) are largely determined by hepatic synthesis and not by alterations in catabolism (6, 7). Nevertheless, while plasma levels of Lp(a) may vary by 3 log-orders between individuals, the levels for each individual appear to be quite constant and subject to little variability in response to diet and to pharmacologic agents which lower plasma cholesterol levels (8). Accordingly, there is heightened interest in the possibility that understanding the mechanisms which regulate secretion of apo(a) and apoB100 from hepatocytes may result in progress toward a rational therapeutic target for elevated plasma levels of Lp(a) (9).

The predominant mechanism underlying individual differences in plasma Lp(a) levels relates to variations at the *APO(a)* locus (reviewed in Ref. 8) with an inverse correlation observed between plasma levels and the number of kringle IV (K-IV) repeats (10). White and colleagues (11) have demonstrated, using baboon hepatocytes, that this inverse correlation could be accounted for by differences in the rate and extent to which apo(a) isoforms were processed through the endoplasmic reticulum (ER). Specifically, these workers demonstrated that smaller isoforms undergo a more complete and rapid exit from the ER than was observed with larger isoforms (11). Among the implications to emerge from these studies is that a crucial restriction point in the secretion of apo(a) from hepatocytes may be topologically linked to early processing of this glycoprotein within the ER. Indeed, this prediction was confirmed in further studies by White and colleagues (12) in which tunicamycin treatment of baboon hepatocytes was found to eliminate apo(a) secretion, in conjunction with a virtually complete block in the exit of the precursor apo(a) from the ER. Recent studies from our laboratory have extended these findings with the demonstration that the extent of the exit block of apo(a) from the ER is dependent on the number of K-IV repeats and may reflect differences in the binding of apo(a) to candidate chaperone proteins (13).

In this study, we examine the effects of alterations in cellular triglyceride assembly and secretion on the synthesis and secretion of apo(a) from stable clones of transfected hepatoma cells (14), expressing either a 6 K-IV or a larger, 17 K-IV, apo(a) isoform. The data demonstrate an increase in apo(a) secretion of both isoforms following oleate supplementation. The mechanisms underlying this response to oleate supplementation may involve alterations in the efficiency of apo(a) translocation, coupled with changes in post-translational degradation of

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[1] The abbreviations used are: apo(a), apolipoprotein(a); Lp(a), lipoprotein(a); apoB100, apolipoprotein B100; ER, endoplasmic reticulum; MTP, microsomal triglyceride transfer protein; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; DMEM, Dulbecco's modified Eagle's medium; BFA, brefeldin A; PAGE, polyacrylamide gel electrophoresis.

apo(a). Furthermore, these studies demonstrate that the oleate-mediated increase in apo(a) secretion can be blocked by inhibition of either microsomal triglyceride transfer protein (MTP) or acyl-CoA synthase, suggesting that the secretion of apo(a) may be linked to elements of complex lipid assembly and/or secretion. Taken together, these data provide evidence that hepatic secretion of apo(a) may be related to the synthesis and secretion of triglyceride-rich lipoproteins.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium, methionine- and cysteine-free DMEM, and fetal bovine serum were all obtained from Life Technologies, Inc. (Gaithersburg, MD). Water-soluble oleic acid, fatty acid-free bovine serum albumin, and proteinase K were purchased from Sigma. MTP inhibitor, BMS-197636-02, was a gift from Dr. Richard Gregg (Bristol-Myers Squibb, Princeton, NJ). Triacsin C was obtained from Biomol Research Laboratory, Inc. (Plymouth Meeting, PA). Protein G-agarose was obtained from Boehringer Mannheim. Goat anti-apo(a) antiserum was purchased from Biodesign Int. (Kennebunk, ME). This antibody exhibits no reactivity toward human apoB100 (14, 15). Rabbit anti-human apoB was purchased from Calbiochem (La Jolla, CA). Sheep anti-rat albumin was obtained from ICN Immunologicals (Lisle, IL). Tran^{35}S-label (1000 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Rabbit anti-human apoA-I was generated in our laboratory using apoA-I isolated from plasma high density lipoprotein as the immunogen (16). Reagents for gel electrophoresis were purchased from Life Technologies, Inc. All other chemicals and reagents were of the highest grade available and purchased from Sigma.

Cell Culture—Stable clones of HepG2 and McA-RH7777 cells expressing a recombinant apo(a) containing 6 K-IV-like domains were generated as described previously (14). McA-RH7777 cells were also stably transfected with an apo(a) expression construct (pRK5ha17) encoding 17 K-IV repeats. The pRK5ha17 expression plasmid (17) encodes for K-IV repeats 1–5, a fusion kringle consisting of repeats 6 and 26, followed by K-IV repeats 27–37 and kringle V. HepG2 cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, 100 mg/ml streptomycin, and 100 units/ml penicillin. McA-RH7777 cells were grown in DMEM containing 4 mM L-glutamine and 10% fetal bovine serum.

Pulse-Chase Studies—Transfected HepG2 and McA-RH7777 cells were grown to 90% confluence in T-25 flasks. On the day of the experiment, the cells were washed twice with phosphate-buffered saline, preincubated for 1 h in methionine- and cysteine-free DMEM without serum, pulse-labeled in the same medium containing 250 μCi/ml Tran^{35}S-label, and chased in complete medium containing 3 mM cysteine and 10 mM methionine for the times indicated in the figure legends. Preincubation, pulse, and chase media contained various additives as defined for each experiment. Where indicated, brefeldin A (BFA) was added at 5 μg/ml and was included in preincubation, pulse, and chase media. Where indicated, oleate was added at a final concentration of 0.8 mM. For experiments examining the effects of the MTP inhibitor (BMS) and triacsin C, the cells were preincubated in methionine- and cysteine-free DMEM for 1 h and then labeled in the same media containing BMS or triacsin C for 4 h. BMS and triacsin C were dissolved in dimethyl sulfoxide at a concentration of 100 and 1 mg/ml, respectively, and diluted to appropriate concentrations in media just prior to incubation with cells. Dimethyl sulfoxide, at identical final concentrations, was added alone to control cells. At the indicated times following radiolabeling, media were collected on ice and adjusted to a final concentration of the following protease inhibitors (100 mM leupeptin, 450 mM aprotinin, 2 mM pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). The cells were washed three times with ice-cold phosphate-buffered saline and subsequently lysed in cold lysis buffer (100 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.1% SDS) containing protease inhibitors and, for HepG2 cells, 100 mM ϵ-aminocaproic acid. Cell lysates and media were clarified by centrifugation at 10,000 rpm at 4 °C for 5 min to remove cellular debris and immunoprecipitations were conducted as described below. Incorporation of radioactivity into total protein was determined by trichloroacetic acid precipitation of cell lysates, in all cases demonstrating comparable values between control and experimental groups (data not shown).

Immunoprecipitations—Both medium and lysates were precleared by incubation with protein G-agarose for 2–3 h at 4 °C. Aliquots were immunoprecipitated with saturating quantities of anti-apo(a), anti-apoB, anti-apoA-I, or anti-albumin antisera. After overnight incubation at 4 °C, protein G-agarose beads were added and the incubation continued for another 2–3 h at 4 °C. The final pellet was washed four times in immunoprecipitation wash buffer (50 mM Tris, pH 7.4, 0.65 M NaCl, 10 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), two times in water, and boiled for 10 min in SDS sample buffer (4% SDS, 20% glycerol, 0.001% bromphenol blue, 125 mM Tris, pH 6.8, and 100 mM dithiothreitol). After centrifugation, the supernatant was analyzed by SDS-PAGE and fluorography. Quantitation was conducted using a PhosphorImager (SI, Molecular Dynamics) and the ImageQuant software. In order to average data from different experiments, where the absolute incorporation rates are divergent, immunoprecipitable apo(a) is presented as a percent of maximum intracellular apo(a), which was determined in each experiment.

Microsome Preparation and Proteinase K Digestion—Transfected HepG2 cells were preincubated for 1 h in cysteine- and methionine-free DMEM containing 1.5% fatty acid-free bovine serum albumin in the presence or absence of 0.8 mM oleate. The cells were then pulse-labeled for 10 min and chased for 10 min in the same media. Membranes were prepared by Dounce homogenization as described by Shelness and colleagues (18, 19). Briefly, the cells were washed two times with ice-cold phosphate-buffered saline and scraped in hypotonic buffer (10 mM HEPES, pH 7.4) containing protease inhibitors (as above). After a 15-min incubation on ice, the cells were homogenized by 50 up-and-down strokes using a tight-fitting (type B) pestle. The homogenate was immediately adjusted to 250 mM sucrose and subjected to two rounds of centrifugation (700 $\times$ *g* for 10 min at 4 °C) to ensure complete removal of unbroken cells. The supernatant was then centrifuged at 200,000 $\times$ *g* for 15 min using an S120-AT2 rotor (Sorvall RC M120 EX ultracentrifuge). The pellet was homogenized in a final volume of 750 μl of resuspension buffer (10 mM HEPES, pH 7.4, 250 mM sucrose, 10 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, and 10 mM $CaCl_2$) using a type B Dounce with 10 up-and-down strokes. Aliquots of equal volume from the final suspension were processed immediately on the day of preparation. Each received either no addition (control), 0.30 mg/ml proteinase K, or 0.30 mg/ml proteinase K plus 1% Triton X-100 (20). After incubation on ice for 30 min, protease inhibitors (200 μg/ml aprotinin, 2.5 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml pepstatin) were added and the samples incubated on ice for an additional 15 min. All samples were adjusted to a final concentration of lysis buffer (25 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100) containing protease inhibitors as above and the membranes solubilized on ice for 30 min. The samples were then immunoprecipitated as described above.

Statistical Comparisons—Where indicated in the text, comparisons were made using Student's *t* test for paired or unpaired samples using the InStat statistical package, version 2.0 (GraphPad Software, San Diego, CA).

RESULTS

Oleate Supplementation Increases Apo(a) Secretion from Transfected HepG2 Cells—HepG2 cells expressing a 6 K-IV apo(a) isoform were examined by pulse-chase analysis in the presence or absence of 0.8 mM oleate. As demonstrated in Fig. 1, there was a sustained ~2-fold increase in the accumulation of newly synthesized apo(a) in the media of the oleate supplemented cells at 60 and 120 min of chase (Fig. 1, *A, right panel*, and *C*). Note that intracellular accumulation of newly synthesized apo(a) and its rate of decline were comparable in both control and oleate-supplemented cells (Fig. 1*B*), suggesting that the increase in apo(a) delivery into the media noted with oleate supplementation reflects alterations in secretion efficiency. By way of confirmation of the effects of oleate supplementation on lipoprotein secretion, lysates and media from these cells were also examined for apoB100 synthesis and secretion. The results demonstrate the expected increase in apoB100 accumulation both within lysates (Fig. 1*D*) and also in the media of oleate-supplemented cells (Fig. 1*E*). In addition, the data in Fig. 1*A* demonstrate an increase in the abundance of apoB100 coimmunoprecipitating with apo(a) in the media of oleate-supplemented cells. Taken together, these results indicate that oleate supplementation produces a rapid increase in the secretion of newly synthesized apoB100 as previously dem-

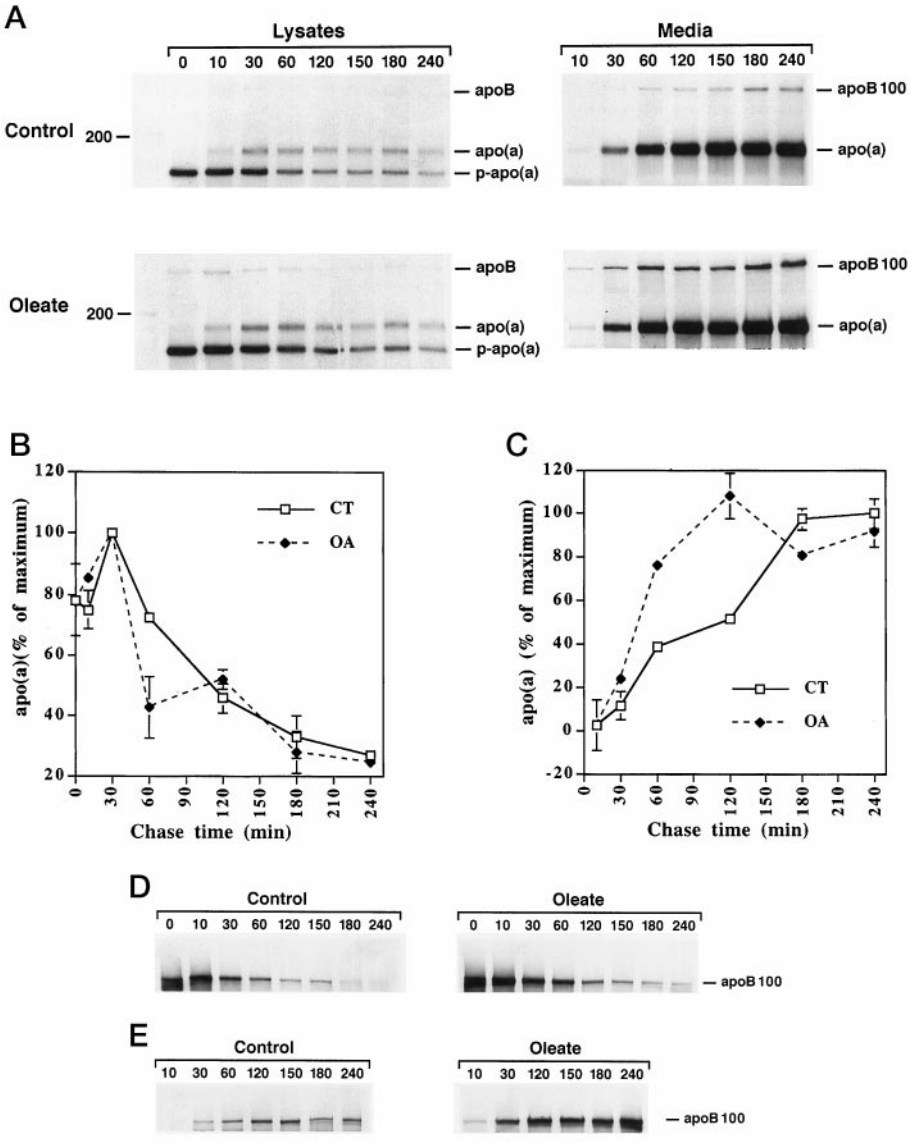

FIG. 1. **Effect of oleate supplementation on intracellular accumulation and secretion of a 6 K-IV apo(a) isoform and of apoB100 from transfected HepG2 cells.** HepG2 cells expressing a 6 K-IV apo(a) isoform were preincubated in serum-free medium for 60 min, pulse-labeled for 10 min with 250 μCi/ml Tran^{35}S-label, and chased for 0–240 min. Preincubation, pulse, and chase media each contained either 1.5% bovine serum albumin alone (*CT*) or 1.5% bovine serum albumin in addition to 0.8 mM oleate (*OA*). *A*, cell lysates (*left panels*) and media (*right panels*) were collected at the indicated times of chase, apo(a) immunoprecipitated, and analyzed by 4–12% SDS-PAGE. *B*, averaged mean ± S.E. of intracellular apo(a) accumulation at the indicated chase times. *C*, averaged mean ± S.E. of secreted apo(a) at the indicated times. In both *panels B* and *C*, the data are expressed as a percentage of maximum apo(a) immunoprecipitated from the cells (30 min of chase). The values represent three independent experiments. *D*, intracellular apoB100 accumulation following oleate supplementation. *E*, secretion of apoB100 following oleate supplementation. Both panels are representative illustrations of triplicate independent experiments.

onstrated, but further demonstrate a temporally similar increase in apo(a) secretion into the media of transfected HepG2 cells.

Oleate Supplementation Increases Apo(a) Secretion from Hepatoma Cells Independent of Its Association with ApoB100—Our previous studies with HepG2 cell clones expressing a 6 K-IV isoform indicated that a portion of newly synthesized apo(a) is found in the media in association with apoB100 (14). In addition, previous studies with HepG2 cells expressing larger isoforms of apo(a), as well as studies of primary baboon hepatocytes, have demonstrated that apo(a) is competent to associate with newly synthesized apoB100 secreted into the media (21–23). Accordingly, experiments were conducted to examine whether the modulation of apo(a) secretion following oleate supplementation was mediated through its association with apoB100, a protein whose secretion has been previously shown, and confirmed above (Fig. 1, *D* and *E*), to be regulated by oleate supplementation (24–27). Pulse-chase experiments were conducted in stably transfected rat hepatoma McA-RH7777 cells expressing a 6 K-IV apo(a) isoform. This cell line was selected since rat apoB100 contains a tyrosine corresponding to the free cysteine at position 4326 in human apoB100 (28), and thus does not form a covalent complex with apo(a). The data again demonstrate the earlier appearance of the mature, processed form of apo(a) in lysates of oleate-supplemented cells (Fig. 2*A, left panel*). In addition, the findings demonstrate a similar pattern of intracellular apo(a) decline in control and oleate-supplemented cells (Fig. 2*B*), coupled with a sustained 6–7-fold increase in apo(a) secretion at 120 min of chase (Fig. 2, *A, right panel*, and *C*), suggesting again that oleate supplementation is associated with increased secretion efficiency of apo(a). The findings further suggest that the increase in apo(a) secretion noted following oleate supplementation occurs independent of a *requirement* for covalent association with apoB100.

Oleate Supplementation Increases Secretion of a 17 K-IV Apo(a) Isoform from Transfected McA-RH7777 Cells—In order to determine whether the findings with the 6 K-IV isoform can be extended to the larger isoforms of apo(a) which are found in human populations, further studies were undertaken using McA-RH7777 cells expressing a 17 K-IV apo(a) isoform. Pulse-chase studies were conducted to examine the time course of intracellular processing and secretion of the larger isoform in the presence or absence of 0.8 mM oleate (Fig. 3). The data indicate a similar pattern of intracellular accumulation of apo(a) in the presence or absence of oleate (Fig. 3, *A, left panel*, and *B*). However, there was an increase in apo(a) secretion efficiency with the earlier appearance of apo(a) and a sustained

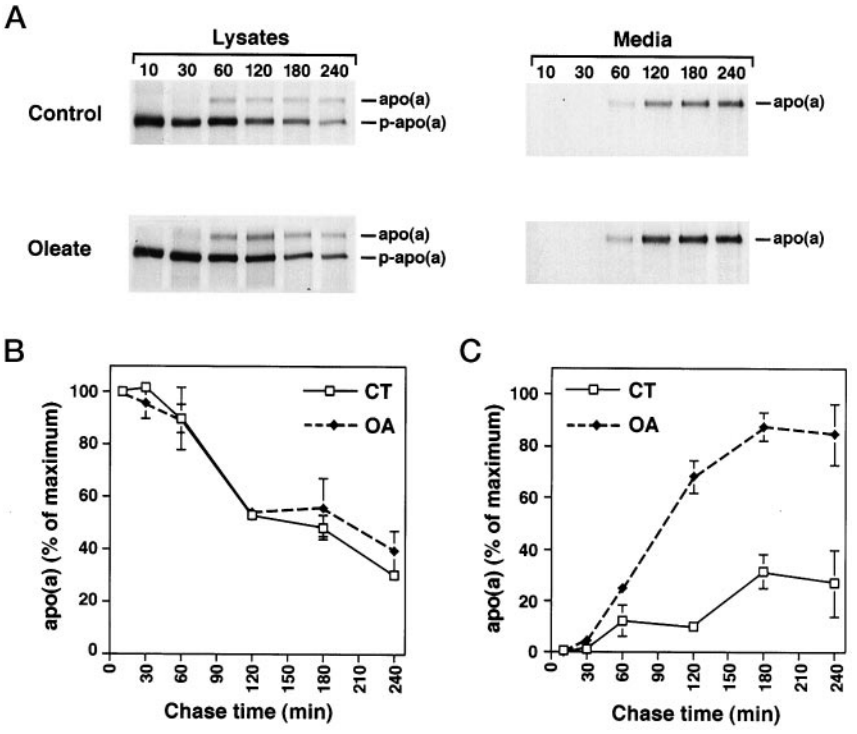

FIG. 2. **Effect of oleate supplementation on intracellular accumulation and secretion of a 6 K-IV apo(a) isoform from McA-RH7777 cells.** McA-RH7777 cells expressing a 6 K-IV apo(a) isoform were preincubated in serum-free media for 60 min, pulse-labeled for 10 min using 250 μCi/ml Tran^{35}S-label, and chased in fresh media containing either 1.5% bovine serum albumin (*CT*) or 1.5% bovine serum albumin, 0.8 mM oleate (*OA*) for 10–240 min. *A*, cell lysates (*left panels*) and media (*right panels*) were collected at the indicated times of chase, apo(a) immunoprecipitated, and analyzed by 4–12% SDS-PAGE. *B*, averaged mean ± S.E. of intracellular apo(a) accumulation at the indicated chase times. *C*, averaged mean ± S.E. of secreted apo(a) at the indicated times. In both *panels B* and *C*, the data are expressed as a percentage of maximum apo(a) immunoprecipitated from the cells (10 min of chase), the values derived from three independent experiments.

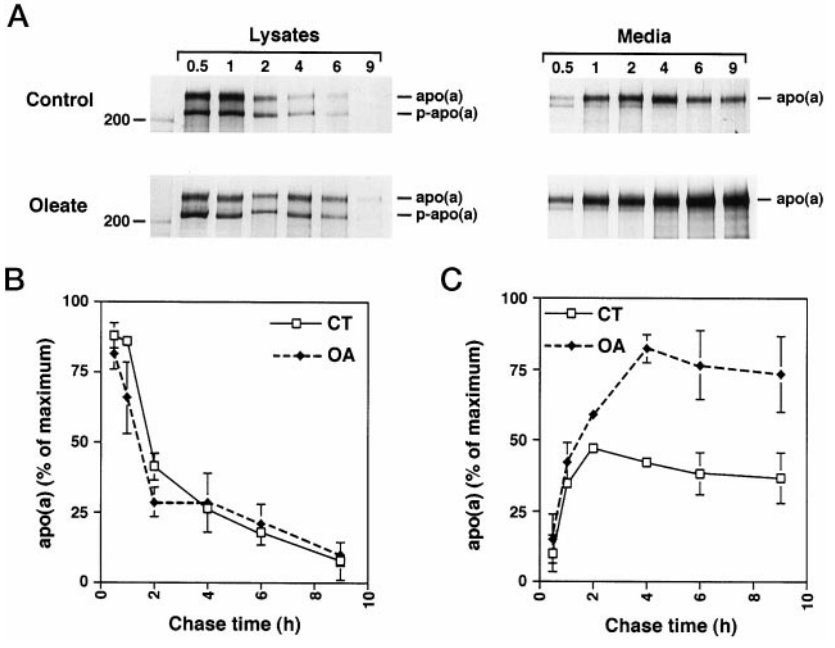

FIG. 3. **Effect of oleate supplementation on intracellular accumulation and secretion of a 17 K-IV apo(a) isoform from McA-RH7777 cells.** McA-RH7777 cells expressing a 17 K-IV apo(a) isoform were preincubated in serum-free media for 1 h, pulse-labeled with 250 μCi/ml Tran^{35}S-label for 1 h, and chased in fresh media containing either 1.5% bovine serum albumin (*CT*) or 1.5% bovine serum albumin, 0.8 mM oleate (*OA*) for 0.5–9 h. *A*, cell lysates (*left panels*) and media (*right panels*) were collected at the indicated times of chase, apo(a) immunoprecipitated, and analyzed by 4–12% SDS-PAGE. *B*, averaged mean ± S.E. of intracellular apo(a) accumulation at the indicated chase times. *C*, averaged mean ± S.E. of secreted apo(a) at the indicated times. In both *panels B* and *C*, the data are expressed as a percentage of maximum apo(a) immunoprecipitated from the cells (10 min of chase), the values derived from three independent experiments.

~2-fold increase in apo(a) secretion after 4 h of chase following oleate supplementation (Fig. 3, *A, right panel*, and *C*). These findings reinforce the impressions gained above with the 6 K-IV apo(a) isoform and indicate that the effects of oleate supplementation on apo(a) secretion efficiency are not confined to the smaller fragment.

Oleate Supplementation Decreases Protease Susceptibility of Newly Synthesized Apo(a) within Microsomal Membranes—One mechanism considered to account for the increased secretion of apo(a) from hepatoma cells following oleate supplementation was a change in the efficiency of translocation of this large protein into the ER. As an indirect measure of this process, microsomes were prepared from radiolabeled HepG2 cells expressing a 6 K-IV apo(a) isoform and aliquots of equal volume immediately subjected to proteinase K digestion with or without detergent. In these experiments, apparent protection from exogenous protease is used as a surrogate marker of translocation into the lumen of the ER. Proteinase K digestion of control cells demonstrated that the precursor apo(a) was largely (81 ± 2%, n = 3) susceptible to proteolysis in the absence of detergent. Additionally, the previously demonstrated band of coimmunoprecipitating apoB100 (14) was also susceptible to proteinase K digestion (Fig. 4). The addition of oleate prior to the preparation of microsomes resulted in an apparent increase in protection of apo(a) from proteinase K digestion, with only 44 ± 9% (n = 3) of apo(a) accessible to proteolysis in the absence of detergent (Fig. 4). These findings suggest that oleate supplementation may be associated with enhanced translocation of apo(a) across the ER membrane.

Oleate Supplementation Alters Post-translational Degrada-

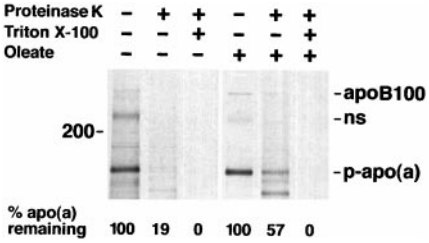

FIG. 4. **Proteinase K digestion of apo(a) in microsomal membranes from transfected HepG2 cells.** HepG2 cells expressing a 6 K-IV apo(a) isoform were preincubated in serum-free medium for 60 min, pulsed for 10 min with 250 μCi/ml Tran^{35}S-label, and chased for 10 min. Preincubation, pulse, and chase media each contained either 1.5% bovine serum albumin alone or 1.5% bovine serum albumin, 0.8 mM oleate (*OA*). Microsomal membranes were prepared and equal aliquots immediately incubated for 30 min on ice in the absence or presence of proteinase K. Triton X-100 (1%) was added with proteinase K where indicated. The samples were immunoprecipitated with anti-apo(a) antibody and analyzed by 4–12% SDS-PAGE and fluorography. The migration of apoB100 and the precursor apo(a) (p-apo(a)) is indicated, along with the 200-kDa molecular mass (MW) marker; *ns*, nonspecific co-immunoprecipitating band. In this representative experiment, the percentage of apo(a) remaining in the samples treated with proteinase K was determined by PhosphorImager, using the control lane in each preparation as 100%.

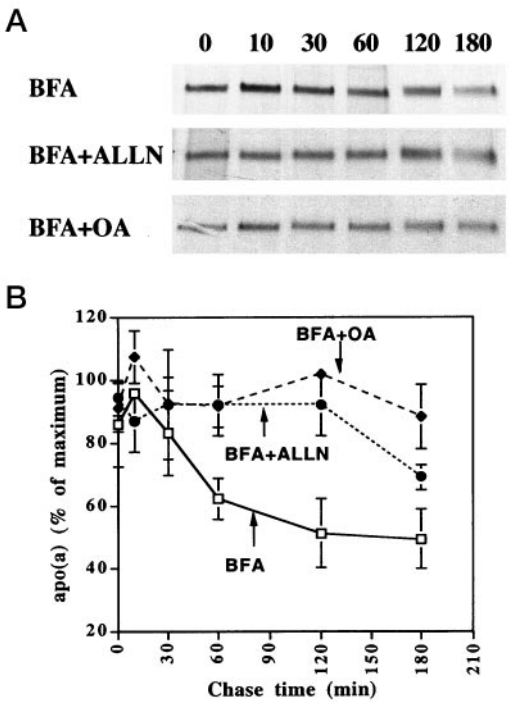

FIG. 5. **Oleate inhibits degradation of a 6 K-IV apo(a) isoform in HepG2 cells treated with brefeldin A.** HepG2 cells expressing a 6 K-IV apo(a) isoform were preincubated in serum-free medium for 60 min, pulsed for 10 min with 250 μCi/ml Tran^{35}S-label, and chased for 0–180 min in serum-free medium. Preincubation, pulse, and chase media contained 1.5% bovine serum albumin + 5 μg/ml BFA, in the presence of either 0.8 mM oleic acid (*BFA + OA*) or 50 μg/ml ALLN (*BFA + ALLN*). *A*, cell lysates were collected after each chase period, apo(a) was immunoprecipitated and analyzed by 4–12% SDS-PAGE. Representative fluorograms are shown from one of three experiments. *B*, data represent the recovery of apo(a) immunoprecipitated at each time point, expressed as a percentage of maximum apo(a) (10 min of chase). Values represent the mean ± S.E. of three independent experiments.

tion of Apo(a)—A further mechanism to explain the increased secretion efficiency and enhanced recovery of newly synthesized apo(a) following oleate supplementation may involve alterations in intracellular degradation. In order to address this possibility, transfected HepG2 cells were treated with BFA, which blocks ER to Golgi processing of apo(a), and results in the accumulation of the precursor form of apo(a) within the cell (14). HepG2 cells expressing a 6 K-IV isoform of apo(a), treated with BFA alone, demonstrated an exponential decline in precursor apo(a) radioactivity, with recovery of approximately 50% of the initial material at 120 min of chase (Fig. 5, *A* and *B*). By contrast, cells treated with either BFA plus 0.8 mM oleate or BFA plus 50 μg/ml ALLN demonstrated almost complete recovery of apo(a) radioactivity at 120 min of chase (Fig. 5, *A* and *B*). These results suggest that the intracellular degradation of apo(a), detected when cells are incubated in BFA alone, is inhibited by the presence of oleate and by the calpain inhibitor ALLN. To validate these conclusions further, specifically with respect to the larger isoform of apo(a), studies were undertaken using McA-RH7777 cells expressing a 17 K-IV apo(a) isoform. The results indicate a decline in intracellular apo(a) recovery in BFA-treated cells, with less than 50% of the initial material remaining at 2 and 4 h of chase (Fig. 6). By contrast, cells treated with BFA plus 0.8 mM oleate revealed a significant increase in precursor apo(a) recovery with 73 ± 8% and 61 ± 5% (n = 3 each) of the initial material remaining at 2 and 4 h of chase, respectively (Fig. 6). Taken together, these results suggest that apo(a) secretion may be regulated through alterations in intracellular, presumably ER, degradation, a process which in turn appears responsive to oleate supplementation.

Alterations in Triglyceride Secretion Modulate Secretion of Apo(a)—The findings presented above raise the question of whether the changes observed in apo(a) secretion following oleate supplementation are related to important elements of triglyceride assembly and secretion by hepatoma cells. Accordingly, apo(a) secretion was determined in transfected hepatoma cells following inhibition of MTP activity by means of a potent competitive inhibitor, previously shown to decrease apoB secretion from both HepG2 and McA-RH7777 cells (29–31). Preliminary studies established an effective dose-response range for the inhibition of apo(a) secretion with the MTP inhibitor of 100–150 μM in both HepG2 and McA-RH7777 cells (data not shown). Transfected HepG2 cells expressing a 6 K-IV isoform were then examined by pulse-chase analysis, the data showing that MTP inhibition resulted in a 48 ± 9% (n = 3) reduction in apo(a) secretion at 120 min of chase (Fig. 7*A*). In comparison, the same dose of MTP inhibitor produced a 96 ± 4% (n = 3) decrease in the secretion of apoB100 (Fig. 7*B*), with no change in the synthesis or secretion of apoA-I (Fig. 7*C*). In addition, there was no change in total protein synthesis following treatment with the MTP inhibitor, as revealed by incorporation of radioactivity into trichloroacetic acid-precipitable material (data not shown).

In order to extend these findings to larger isoforms of apo(a), similar studies were conducted using McA-RH7777 cells expressing a 17 K-IV isoform. These experiments also included triacsin C, an inhibitor of acyl-CoA synthase (32), in order to determine the effects of inhibition of complex lipid synthesis from oleate on apo(a) secretion. Since the studies presented above (Fig. 3) established an optimal time of ~4 h for the detection of an increase in apo(a) secretion into the media following oleate supplementation, this time point was utilized to discriminate alterations in apo(a) secretion with the agents cited above. The results of these studies confirmed a >2-fold increase in apo(a) secretion in the presence of 0.8 mM oleate alone (Fig. 8, *A, top panel*, and *B*). Cells incubated in the presence of the MTP inhibitor demonstrated a marked decrease (>90%) in apo(a) secretion (Fig. 8, *A, top panel*, and *B*). Furthermore, incubation with the MTP inhibitor completely abrogated the effects of oleate supplementation, suggesting that triglyceride secretion is a necessary component of the stimulatory effects on apo(a) secretion noted with oleate supplementa-

tion (Fig. 8). Finally, inhibition of triglyceride synthesis with triacsin C decreased apo(a) secretion by 60% and also abrogated the effects of oleate supplementation (Fig. 8, *A, top panel*, and *B*). Specificity of the effects of MTP inhibition and of treatment with triacsin was evidenced by the absence of a change in albumin secretion under the conditions evaluated. In addition, total protein synthesis, as revealed by incorporation of radioactivity into trichloroacetic acid-precipitable material, was comparable in all groups (data not shown). Taken together, the data strongly suggest that apo(a) secretion from hepatoma cells is linked to important elements of triglyceride assembly and secretion.

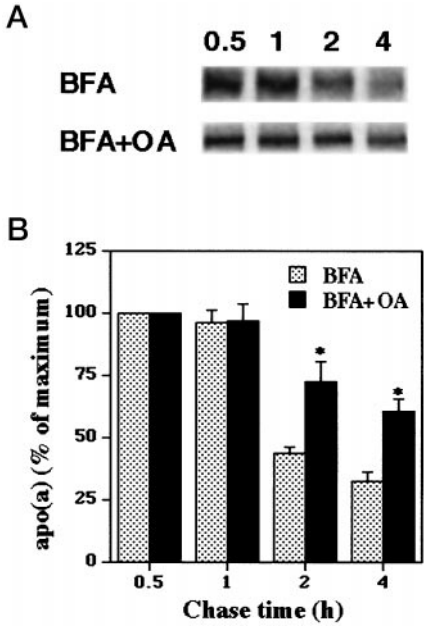

FIG. 6. **Oleate inhibits degradation of a 17 K-IV apo(a) isoform in McA-RH7777 cells treated with brefeldin A.** McA-RH7777 cells expressing a 17 K-IV apo(a) isoform were preincubated in serum-free media for 1 h, pulsed for 1 h with 250 μCi/ml Tran^{35}S-label, and chased for 0.5, 1, 2, or 4 h in fresh media. Preincubation, pulse, and chase media contained 1.5% bovine serum albumin + 5 μg/ml brefeldin A alone (*BFA*) or 0.8 mM oleic acid (*BFA + OA*). *A,* cell lysates were collected after each chase period, apo(a) was immunoprecipitated and analyzed by 4–12% SDS-PAGE, followed by fluorography. A representative immunoprecipitation demonstrates the 17 K-IV precursor apo(a). *B*, the data are presented as a percentage of maximum apo(a) (immunoprecipitated from the cells at 0.5 h of chase), the *bars* representing the mean ± S.E. of three independent experiments. *Asterisks* illustrate statistically significant differences between BFA − and BFA + oleate-treated (*, $p < 0.05$).

DISCUSSION

Plasma levels of Lp(a) in humans are primarily regulated through alterations in the synthesis of apo(a), a process which reflects the virtually exclusive contribution of hepatic apo(a) production (6, 7, 33). However, despite the clear importance of hepatic synthesis of apo(a) to the maintenance of circulating plasma levels of Lp(a), information concerning the physiological regulation of this process is only now beginning to emerge.

Little direct evidence exists in support of the possibility of dietary regulation of hepatic apo(a) secretion in humans, although studies have demonstrated a variable increase in plasma apo(a) levels following an acute oral fat load (34, 35). Studies in cynomolgous monkeys demonstrated plasma Lp(a) levels to be responsive to alterations in dietary fatty acid content and suggested that this may be mediated through both alterations in hepatic apo(a) mRNA abundance as well as post-translational mechanisms (36). In relation to dietary modulation of apo(a) levels in other animal models, earlier studies in apo(a) transgenic mice demonstrated a significant ~1.5–2-fold increase in plasma apo(a) levels following intake of a high fat diet (37). Although these workers did not specifically investigate the mechanism underlying this increase, the expression of apo(a) in these mice was driven by the murine transferrin promoter, which is not known to be regulated by fat intake. Of course, it remains equally possible that the increase in apo(a) levels in these mice reflects a decrease in its clearance rate, and formal evaluation of the underlying mechanism for this apparent increase in apo(a) levels following high fat intake will be required to resolve this ambiguity. Plasma apo(a) levels in YAC transgenic mice were shown to respond to hormonal changes as

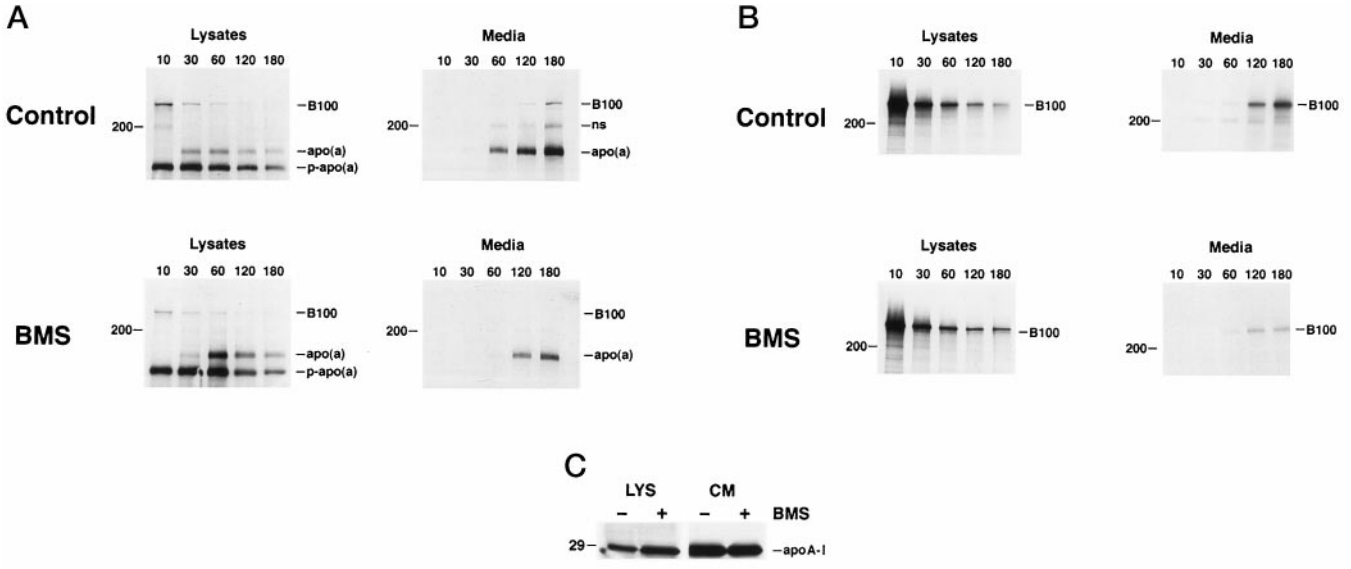

FIG. 7. **Effects of MTP inhibition on apo(a) and apoB100 secretion from transfected HepG2 cells.** HepG2 cells expressing a 6 K-IV apo(a) isoform were preincubated for 1 h in the presence or absence of the MTP inhibitor (BMS-197636), followed by a 10-min pulse and 10–180 min chase in the same media. At the indicated times, lysates and media were immunoprecipitated and analyzed by 4–12% SDS-PAGE. *A*, apo(a) immunoprecipitation. The migration of the precursor (*p-apo(a)*) and secreted protein is indicated; *ns,* nonspecific coimmunoprecipitating band. *B*, apoB100 immunoprecipitation. The migration of apoB100 is indicated. *C*, cells were labeled for 4 h in the presence (*BMS*) or absence of the MTP inhibitor and apoA-I was immunoprecipitated from both the lysates (*LYS*) and media (*CM*). The fluorograms are representative of three independent experiments.

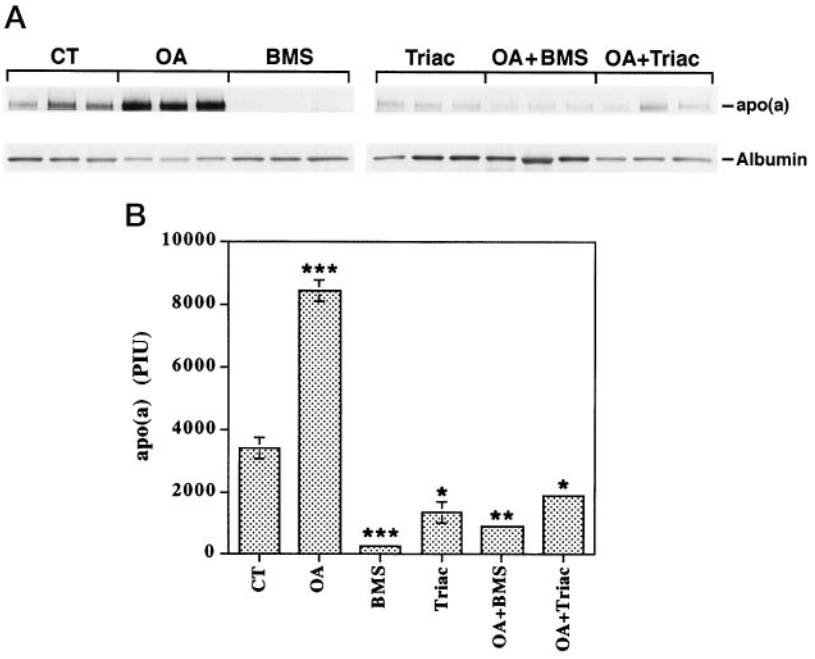

FIG. 8. **Effects of MTP inhibition or triacsin C on 17 K-IV apo(a) secretion from McA-RH7777 cells.** McA-RH7777 cells expressing a 17 K-IV apo(a) isoform were labeled with 250 μCi/ml Tran^{35}S-label for 4 h in the presence of 1.5% bovine serum albumin alone (*CT*), 0.8 mM oleate (*OA*), 150 μM BMS (*BMS*), or 5 μM triacsin C (*Triac*). Separate experiments used combinations of 0.8 mM oleate + MTP inhibitor (*OA + BMS*) or 0.8 mM oleate + 5 μM triacsin C (*OA + Triac*). *A*, culture media was immunoprecipitated for apo(a) or albumin. *B*, data are presented as PhosphorImager units (*PIU*), with *bars* representing the mean ± S.E. of triplicate experiments. *Asterisks* illustrate statistically significant differences between control and experimental groups (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

well as acute-phase inducers, in both instances demonstrating evidence of parallel alterations in hepatic apo(a) mRNA abundance and circulating plasma levels (38). However, Zysow and co-workers (39) more recently demonstrated in these YAC transgenic mice that tamoxifen administration produced a decrease in plasma apo(a) levels which was disproportionate to the alterations in apo(a) mRNA abundance, suggesting that there may be post-transcriptional mechanisms which regulate apo(a) production *in vivo*. Taken together, several independent lines of evidence suggest that the regulation of apo(a) secretion in different animal models may involve mechanisms operating at both transcriptional and post-transcriptional levels.

The major observation of this study is that apo(a) secretion from stably transfected hepatoma cells expressing either a 6 or 17 K-IV isoform, driven by the cytomegalovirus promoter, appears to be responsive to alterations in triglyceride flux produced by oleate supplementation. Although transcriptional control mechanisms have not formally been excluded, the current studies were conceived to examine mechanisms centered on the post-translational processing of apo(a) and its trafficking through the secretory apparatus. This latter pathway of regulation has been examined in a series of studies with baboon hepatocytes and has led to the hypothesis that larger isoforms of apo(a) undergo more prolonged retention in the ER, with a consequent delay and reduced overall efficiency of secretion (11). The current studies provide direct evidence of post-translational regulatory mechanisms which modulate the secretion of apo(a) from hepatoma cells in association with alterations in hepatic lipoprotein assembly and secretion. By corollary, these studies raise important implications concerning the relationship between hepatic lipoprotein production and Lp(a) biogenesis.

Previous studies with HepG2 cells expressing a 6 K-IV isoform of apo(a) demonstrated that a portion of the newly synthesized apo(a)-B100 complexes, which were found in the media of these cells, could be accounted for through intracellular assembly (14). Although the existence of an intracellular complex of apo(a) and apoB100 has yet to be confirmed with the larger isoforms of apo(a), these findings raise the intriguing possibility that elements of intracellular apoB100 assembly and secretion are coordinately regulated with that of apo(a). Of relevance to the current observations, several studies have demonstrated increased apoB secretion from both HepG2 and McA-RH7777 cells following oleate supplementation, findings that could be accounted for by alterations in post-translational degradation (20, 26, 27). The demonstration that oleate supplementation increases secretion of both the 6 K-IV and the 17 K-IV apo(a) isoform is significant since the larger apo(a) protein is representative of the size range found in human populations, while the 6 K-IV isoform contains structural elements restricted to the carboxyl terminus proteolytic fragment of apo(a) (reviewed in Ref. 40). Although there is no evidence that the smaller fragment is a representative product of *intracellular* apo(a) degradation (see Mooser *et al.* (41),[2] it is reasonable to conclude that the structural components of apo(a) which mediate the oleate-responsive increase in secretion are contained within the carboxyl terminus of the protein. The augmentation of apo(a) secretion following oleate supplementation extends to both human and rat hepatoma cells, suggesting that there is no obligate requirement for covalent association with human apoB100 in order to detect an oleate-dependent increase in apo(a) secretion. Nevertheless, these findings do not preclude the possibility that noncovalent association of apo(a) occurs with other proteins involved in lipoprotein assembly and secretion, nor do they eliminate the possibility that oleate supplementation alters the degradation of apoB and apo(a) through a shared mechanism independent of their physical association. Along these lines, indirect support for this latter possibility is suggested by the demonstration that ALLN supplementation results in increased recovery of precursor apo(a) in BFA-treated HepG2 cells expressing a 6 K-IV isoform (Fig. 5). These findings are reminiscent of data reported by Ginsberg and colleagues (42) who showed that apoB100 degradation was inhibited by ALLN in BFA-treated HepG2 cells. It bears emphasis, however, that the findings with respect to ALLN and apo(a) degradation must be considered preliminary and will require extensive investigation and confirmation with the larger isoforms.

The demonstration of increased protection from proteolytic degradation of the 6 K-IV apo(a) isoform in microsomal membranes following oleate supplementation is consistent with the

[2] F. Nassir, D. K. Bonen, and N. O. Davidson, unpublished observations.

earlier appearance of the mature, fully processed intracellular apo(a) in lysates from oleate-supplemented cells. The suggestion that oleate supplementation may accelerate or enhance translocation efficiency of the 6 K-IV isoform, while of potential interest, is unlikely to be the *sole* explanation for the increased secretion of apo(a) following oleate treatment. The most compelling support for this conclusion concerns the proportions of precursor-to-product apo(a) for both the 6 and 17 K-IV isoforms isolated from lysates of control and oleate-supplemented cells, which were generally comparable at all time points. In light of the sustained increase in apo(a) secretion following oleate supplementation, a sustained increase in apo(a) translocation would be anticipated to be accompanied by a corresponding increase in the abundance of the mature form of apo(a) unless there were additional alterations, such as changes in the processing and secretion efficiency, or changes in intracellular degradation. Indeed, the further demonstration that intracellular apo(a) degradation is significantly reduced following oleate supplementation strongly suggests that the regulation of apo(a) secretion may involve mechanisms operating at multiple post-translational restriction points. In this regard, it is again tempting to draw certain parallels with the regulation of apoB secretion from the hepatocyte, which has been variously shown to reflect differences in translocation and both co- and post-translational degradation (43–45).

More direct evidence for the importance of hepatic lipoprotein assembly and secretion in the regulation of apo(a) secretion is provided by the experiments in which inhibition of MTP produced a decrease in apo(a) secretion. In addition, the presence of the MTP inhibitor completely abrogated the oleate-dependent increase in apo(a) secretion. Extending these findings, cells incubated with triacsin C also demonstrated similar effects to those noted for the MTP inhibitor. Taken together, the data strongly suggest that alterations in complex lipid, most plausibly triglyceride, assembly and secretion are associated with corresponding effects on the secretion of apo(a). These findings are not inconsistent with earlier studies of subjects with homozygous abetalipoproteinemia which demonstrated that apo(a) was detectable in the plasma, but at reduced levels (46). Studies of apoB secretion from McA-RH7777 cells treated with an MTP inhibitor prior to metabolic labeling demonstrated that secretion of apoB100 and apoB48 very low density lipoprotein was decreased, while the secretion of apoB48 high density lipoprotein was unchanged (31). Other work has demonstrated that MTP is required for the first step of apoB secretion, perhaps coordinating the assembly of a primordial high density lipoprotein-like lipoprotein precursor particle (29). These findings raise interesting implications for lipoprotein assembly with the different apoB species in rat hepatoma cells, particularly with respect to high density lipoprotein *versus* triglyceride-rich lipoprotein secretion. However, it is currently impossible to extend this paradigm to apo(a) secretion from hepatoma cells since crucial information is lacking concerning the ability of apo(a) to associate with a nascent lipoprotein particle under these different conditions. Indeed, information from baboon hepatocytes suggests that apo(a) is secreted largely unassociated with lipoproteins (23). These data, however, are discordant with our earlier findings in human hepatocytes where ~15% of the secreted apo(a) was associated with very low density lipoprotein (47).

The current studies raise important questions concerning the interaction of apo(a) with elements of the machinery of hepatic lipoprotein assembly and secretion. Given that this protein is normally confined to the liver of humans and higher primate species, its functional relevance is difficult to determine through *in vitro* approaches. Studies using transgenic mice and rabbits may illuminate some aspects of this relationship and possibly guide some therapeutic measures to control high plasma levels of this enigmatic lipoprotein.

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