Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge

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Abstract Secretion of hepatic apoB lipoproteins removes excess triglyceride from the liver. However, the mechanism by which synthesis of apoB, which occurs on the rough endoplasmic reticulum, is coordinated with synthesis of triglyceride, which takes place in the smooth endoplasmic reticulum, is not known. To examine this question, we have manipulated intracellular synthesis of triglyceride and cholesteryl ester in HepG2 cells and determined the impact of these maneuvers on apoB secretion. Since cholesteryl ester is the only major lipid class synthesized in the rough endoplasmic reticulum, our hypothesis was that, in response to a fatty acid challenge, synthesis of cholesteryl ester rather than synthesis of triglyceride would be the immediate trigger to apoB secretion. Oleate complexed to bovine serum albumin caused intracellular triglyceride synthesis to increase 6-fold and cholesteryl ester synthesis to increase almost 3-fold, while apoB secretion into the medium increased by 2.5-fold ($P < 0.0125$) at all time points between 4 and 24 h. Addition of acylation stimulating protein to the medium further stimulated both triglyceride and cholesteryl ester synthesis (58% and 108%, respectively) above oleate alone and this resulted in a 50% increase in apoB secretion ($P < 0.0025$). By contrast, both progesterone and 2-bromooctanoate inhibited triglyceride and cholesteryl ester synthesis and these effects were associated with reduced apoB secretion. Lovastatin inhibited cholesteryl ester synthesis (45%, $P < 0.0025$); however, at the doses used, triglyceride formation was unaffected. Under these circumstances, apoB secretion was reduced by 25% ($P < 0.05$). Similarly, 58-035 (an inhibitor of acyl CoA:cholesterol acyltransferase) on the one hand reduced cholesteryl ester synthesis markedly (59%, $P < 0.005$), but on the other, increased triglyceride synthesis though not statistically significantly (65%, P NS), and again this resulted in decreased apoB secretion (25%, $P < 0.005$). Control experiments established that changes in low density lipoprotein catabolism did not contribute importantly to the quantity of apoB in the medium. Taken together, the data indicate that, at least in HepG2 cells, there are parallel changes in cholesteryl ester synthesis and apoB secretion and suggest that it is cholesteryl ester synthesis, not triglyceride synthesis, that is the immediate regulator of apoB secretion when these cells are exposed to an increased influx of fatty acids. However, alternative or additional regulatory mechanisms, such as, for example, a role for acylation of apoB, are not excluded by these studies.—**Cianflone, K. M., Z. Yasruel, M. A. Rodriguez, D. Vas, and A. D. Sniderman.** Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge. *J. Lipid Res.* 1990. **31:** 2045–2055.

Supplementary key words triglyceride • acylation stimulating protein

The risk of premature coronary artery disease is, to a large extent, a function of the number of LDL particles in plasma. LDL particle number is, in turn, a function of the rate at which these particles are formed and the rate at which they are catabolized. Both mechanisms may be perturbed by disease with LDL particle number rising as a consequence. Thus the LDL particle number may rise because the LDL catabolic pathway has failed either due to a defect in the ligand, such as in the familial defective B-100 syndrome (1), or to any of a series of faults within the pathway itself, all of which present clinically as familial hypercholesterolemia (2). On the other hand, overproduction of hepatic apoB lipoproteins such as occurs in hyperapobetalipoproteinemia can also lead to an elevated LDL particle number (3), and as a consequence, increased risk of premature coronary disease (4). The immediate pathogenesis of disorders that produce an elevated LDL particle number due to impaired catabolism is now well understood; the pathogenesis of disorders of overproduction is not.

It is known that long chain fatty acids can increase the synthesis and secretion of triglyceride by the liver (5–7). However, even though most observers report a simultaneous increase in apoB secretion (5–9), the mechanism by which this occurs has not yet been explicated. Since synthesis of triglyceride occurs in the smooth endoplasmic reticulum whereas synthesis of apoB occurs in the rough endoplasmic reticulum, the purpose of the present studies was to *identify a mechanism that could result in coordinate regulation* of these two processes, one synthesis of a lipid, the other synthesis of a protein. To do so, we have used the human hepatoma cell line, HepG2. Our hypothesis was that synthesis of cholesteryl ester — a process that occurs predominantly in the rough endoplasmic reticulum (10) — is a critical link between triglyceride synthesis and apoB secretion from the hepatocyte.

Abbreviations: PBS, phosphate-buffered saline; BSA, bovine serum albumin; ACAT, acyl CoA:cholesterol acyltransferase; ASP, acylation stimulating protein; HPTLC, high performance thin-layer chromatography; LDL, low density lipoproteins.

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MATERIALS AND METHODS

Tissue culture

HepG2 cells obtained from the American Tissue Culture Collection (Rockville, MD) were routinely grown in Minimum Essential Medium supplemented with 10% fetal calf serum and 100 IU penicillin-streptomycin in 75-cm^2 flasks with 15 ml medium in a 37°C incubator with 5% CO_2. Flasks were subcultured every 7 days with a split ratio of 1:3. The cells were dislodged from the culture flask with 0.25% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) for 5 min at 37°C. For experiments, cells were plated out at a density of 1.3×10^4 cells per cm^2 in 17-mm dishes (24-well plates) or 60-mm dishes.

Experimental conditions

[1-^{14}C]Oleic acid (sp act 52.6 mCi/mmol), [2-^{3}H]glycerol (sp act 200 mCi/mmol), [1-^{14}C]acetic acid, sodium salt (sp act 86.4 mCi/mmol), and ^{125}I radionuclide were purchased from DuPont-New England Nuclear (Canada). Oleic acid (sodium salt), progesterone, 2-bromooctanoate, and fatty acid-free bovine serum albumin Fraction V (BSA) were all obtained from Sigma Chemical Co., (St. Louis, MO). Tissue culture medium was purchased from Flow Laboratories (McLean, VA). Fatty acid was added to the medium at the indicated concentrations complexed to BSA at a ratio of 5:1 according to the method of Van Harken (11).

ASP was isolated by a modification (12) of a previously published method (13). LDL was isolated from normal human plasma (14) and iodinated by the method of McFarlane as modified by Bilheimer, Eisenberg, and Levy (15). Lovastatin was generously provided by Merck (Rahway, NJ) and compound 58-035 by Sandoz (East Hanover, NJ). Progesterone and lovastatin were dissolved in isopropanol and added to the cells in order to deliver the desired amount in a volume of isopropanol not exceeding 0.01% of the total medium volume. Compound 58-035 was dissolved in dimethylsulfoxide and delivered to the cells in a volume not exceeding 0.01% of the total medium volume. 2-Bromooctanoate was dissolved in 0.9% NaCl. Control cells were run in parallel with isopropanol alone or dimethyl sufoxide alone in the presence of 1% BSA.

Prior to experiments, cells were preincubated for 24 h at 37°C in 5% CO_2 in serum-free medium supplemented with 1% BSA. Cells were then changed to the specified medium and incubated for the indicated times at 37°C. At the end of the incubation period the cells were placed on ice and the medium was removed and set aside for analysis. The cells were washed twice with 1 ml of ice-cold PBS with 2 mg/ml BSA, than twice with 1 ml of ice-cold PBS, and the cell lipids were extracted with 2 ml of hexane-isopropanol 3:2 (v/v). After 30 min the extracts were removed and the cells were washed once with an additional 1 ml of hexane-isopropanol 3:2 (v/v) and added to the extract. The soluble cell protein was dissolved in 1 ml of 0.1 N NaOH and collected.

Lipid quantitation

Cell lipid extracts were evaporated under nitrogen and redissolved in a known volume of chloroform-methanol 2:1 (v/v). An aliquot was applied to a thin-layer chromatography plate prewashed in chloroform-methanol 2:1 (v/v). The lipids were separated by development of plates in hexane-ether-acetic acid 75:25:1 (v/v/v); the lipid spots were visualized by exposure to iodine vapor and identified by comparison to reference lipids. The silica gel was scraped into vials containing scintillation cocktail and the radioactivity was counted in a scintillation counter (Beckman Instruments, Fullerton, CA). Aliquots of the medium were also extracted with 5 volumes of heptane-isopropanol 1:1 (v/v) and the organic phase was washed twice with 1 ml isopropanol-heptane 4:1 (v/v) and 1 ml 0.05% KOH to remove the remaining radioactive free oleate. The sample was then processed in the same way as the cell extracts to measure medium triglyceride and cholesteryl ester. [^{3}H]Glycerol (avg. sp act = 440 dpm/pmol) or [^{14}C]oleate (avg. sp act = 5.9 dpm/pmol) incorporation into triglycerides was measured. Cholesteryl ester was determined from the incorporation of [^{3}H]acetate (avg. sp act = 105 dpm/pmol) or [^{14}C]oleate (avg. sp act = 5.9 dpm/pmol).

Cellular lipid mass of cholesterol, triglyceride, and cholesteryl ester were measured by HPTLC (high performance thin-layer chromatography) as modified from Wood, Cornwell, and Williamson (16). Briefly, dilutions of >99.9% pure lipid standards were applied to 20×20 cm analytical 0.25-mm TLC plates in a volume of 5 μl containing 200–3200 ng triglyceride and 50–800 ng of cholesteryl ester and cholesterol. Samples of appropriate dilution and standards were separated in a solvent mixture of hexane–isopropylether–acetic acid 65:35:2 (v/v/v). Plates were dipped in 3% cupric acetate (w/v) in 8% phosphoric acid (v/v) and heated at 175°C for 15 min in an oven. Charred spots were read on a scanning densitometer (Helena Laboratories) at a wavelength of 410 nm and quantified against an internal standard of oleyl alcohol at 200 ng/5 μl in every sample.

HepG2 cells were incubated for 24 h with ^{125}I-labeled LDL under different experimental conditions and total ^{125}I-labeled LDL catabolism was measured as the sum of cell-associated ^{125}I-labeled LDL and ^{125}I-labeled LDL degradation products in the medium. Cell-associated ^{125}I-labeled LDL was determined by counting an aliquot of the soluble cell protein dissolved in 0.1 N NaOH. LDL degradation products in the medium were measured after precipitation of remaining LDL with 10% trichloroacetic acid with 1 mg/ml bovine serum albumin as carrier protein, followed by precipitation of free iodine with 1.7% $AgNO_3$ as previously described (12).

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Protein and apoprotein quantitation

Cell protein was measured by the method of Bradford (17) using BSA as a standard. Apoprotein B and A-I concentrations in the medium were measured by sandwich enzyme-linked-immunoassay by the method of Young et al. (18) using a standard curve of 0.025-0.400 μg/ml of apoB. All results are the average of triplicate determinations for each experiment and are expressed per mg cell protein ± standard error of the mean (SEM). Significance was measured by paired Student's *T*-test between the test value and the control value. An individual control was run for each experiment.

RESULTS

Since the rate of triglyceride synthesis by HepG2 cells is known to be a function of the fatty acid concentration of the medium (6, 7), the object of the first series of experiments was to determine whether cholesteryl ester synthesis increased concurrently with increased triglyceride synthesis. This was tested in two ways: first, by increasing the concentration of oleate in the medium, and second, since ASP has previously been shown to be a potent stimulant of triglyceride synthesis in fibroblasts and adipocytes (13), by adding acylation stimulating protein (ASP) to the medium.

The effects of altering the concentration of oleate in the medium are shown in **Fig. 1**. Oleate concentration was increased up to 1 mM oleate complexed to BSA. Triglyceride synthesis was measured using [^{3}H]glycerol as radioactive tracer (avg. sp act = 440 dpm/pmol) while synthesis of cholesteryl ester was estimated from [^{3}H]acetate incorporation into cholesteryl ester (avg. sp act = 105 dpm/pmol). Fig. 1A demonstrates the changes observed in triglyceride and cholesteryl ester synthesis after a 24-h incubation. Synthesis of both increased significantly in a concentration-dependent manner ($P < 0.05$). At a concentration of 1 mM oleate, triglyceride synthesis, cholesteryl ester synthesis, and apoB secretion were still increasing. Nevertheless, the increase in triglyceride (almost 6-fold) was substantially greater than that observed for cholesteryl ester (almost 3-fold). In addition, secretion of both triglyceride and cholesteryl ester into the medium increased although the amount of both secreted was about one-tenth that synthesized intracellularly (data not shown). The effect of the concentration of oleate in the medium on the secretion of apoB and A-I is shown in Fig. 1B. ApoB secretion increased 2.5-fold ($P < 0.0125$) at 1 mM oleate whereas apoA-I secretion did not change. Thus, by increasing oleate concentration in the medium, directionally identical changes in intracellular synthesis and secretion of triglyceride and cholesteryl ester and secretion of apoB were observed.

Fig. 2 presents the time-dependence of the effect of fatty acid on the synthesis and secretion of lipoproteins in the presence and absence of oleate. The control medium contained 1% fatty acid free BSA while the other was supplemented with 825 μM oleate complexed to 1% BSA (molar ratio of 5:1). At all time points assayed, i.e., from 4 to 24 h, substantially more apoB was secreted into the oleate-enriched medium ($P < 0.01$). There was a wide range in the concentration of apoB measured in the fatty acid-supplemented

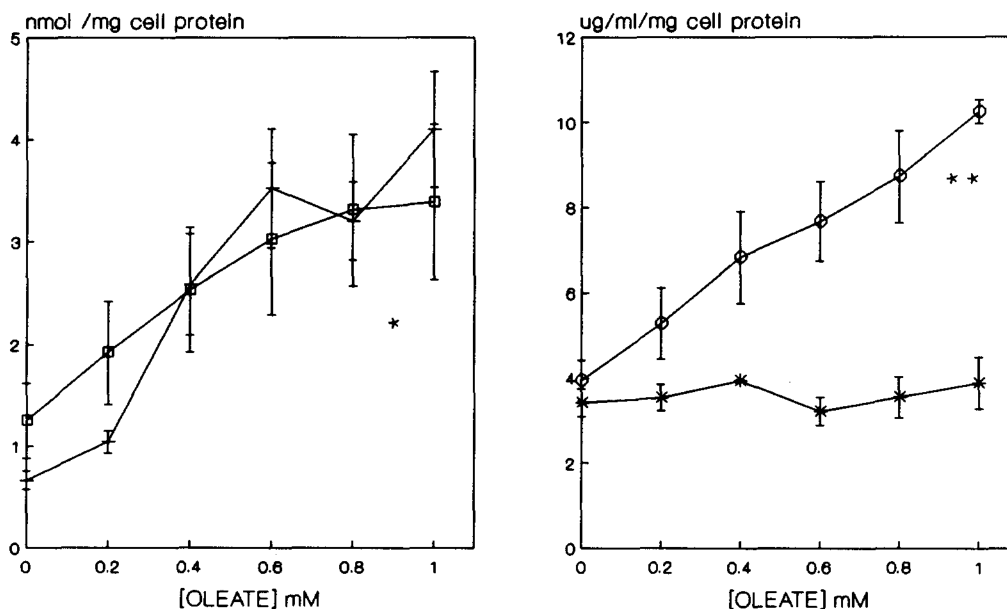

Fig. 1. Triglyceride and cholesteryl ester synthesis and apoB and apoA-I secretion. Cells were incubated in 0-1 mM oleate:BSA (molar ratio 5:1) for 24 h (n = 4 experiments). Panel A: [^{3}H]glycerol incorporation into cellular triglyceride (+) and [^{3}H]acetate incorporation into cellular cholesteryl ester (the latter × 10) (□) are expressed as nmol/mg cell protein ± SEM. Panel B: medium apoB (○) and apoA-I (*) are expressed as μg/ml per mg cell protein ± SEM. (★ $P < 0.05$; ★★ $P < 0.0125$).

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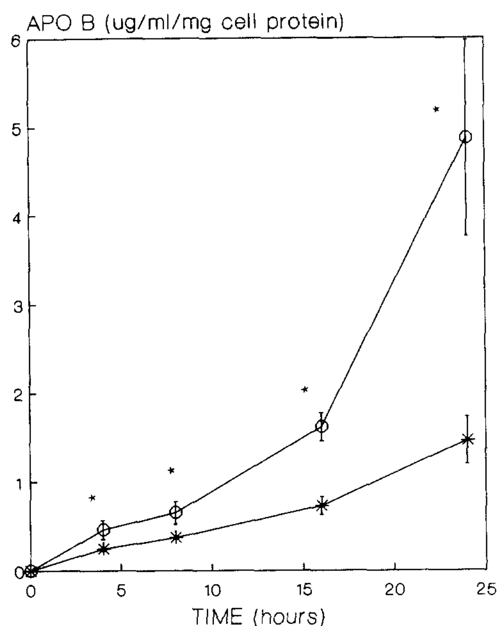

Fig. 2. ApoB secretion in oleate medium versus basal medium. Cells were incubated in 825 μM oleate:BSA (molar ratio 5:1) medium or 1% BSA alone (basal medium) for 4 to 24 h (n = 4 experiments). ApoB in oleate (○) versus basal (*) medium is expressed as average μg/ml per mg cell protein ± SEM. (★ $P < 0.01$ at all time points).

medium (avg. 4.8 ± 1.1 μg/ml per mg cell protein, range 2 to 7.5 μg/ml per mg cell protein) from one experiment to the next. However, in each experiment, the concentration of apoB in the fatty acid-supplemented medium was significantly higher than in the paired control dishes. Though not shown, triglyceride and cholesteryl ester synthesis and secretion into the medium also increased significantly over time in the oleate medium as compared to the control. By contrast, the rate at which apoA-I was secreted into the medium was unaltered by addition of oleate to the medium (data not shown).

Next the effect of ASP was investigated. In these experiments, ASP was added to the medium in concentrations from 20 to 50 μg/ml at a constant oleate concentration of 825 μM complexed to 1% BSA at a molar ratio of 5:1, and [^{14}C]oleate (avg. sp act = 5.9 dpm/pmol) incorporation into triglyceride and cholesteryl ester was measured. **Fig. 3A** demonstrates that ASP caused triglyceride synthesis to increase by 58% ($P < 0.0125$). Triglyceride secretion also increased (117%, $P < 0.05$). Note that essentially identical results were obtained using either [^{3}H]glycerol or [^{14}C]oleate as tracer to estimate triglyceride synthesis (data not shown). Cholesteryl ester synthesis and secretion also increased (108% and 70%, respectively, $P < 0.05$ at the highest concentration of ASP). Moreover, ASP provoked a substantial increase in the amount of apoB secreted into the medium; the amount secreted rose by 50%, $P < 0.0025$ (Fig. 3B). By contrast, no effect was observed on apoA-I release into the medium. The effect of ASP was in addition to the stimulatory effect of the oleate alone. Again therefore, concordance was demonstrated amongst the changes induced by ASP in that cholesteryl ester and triglyceride synthesis and secretion and apoB secretion all rose in parallel.

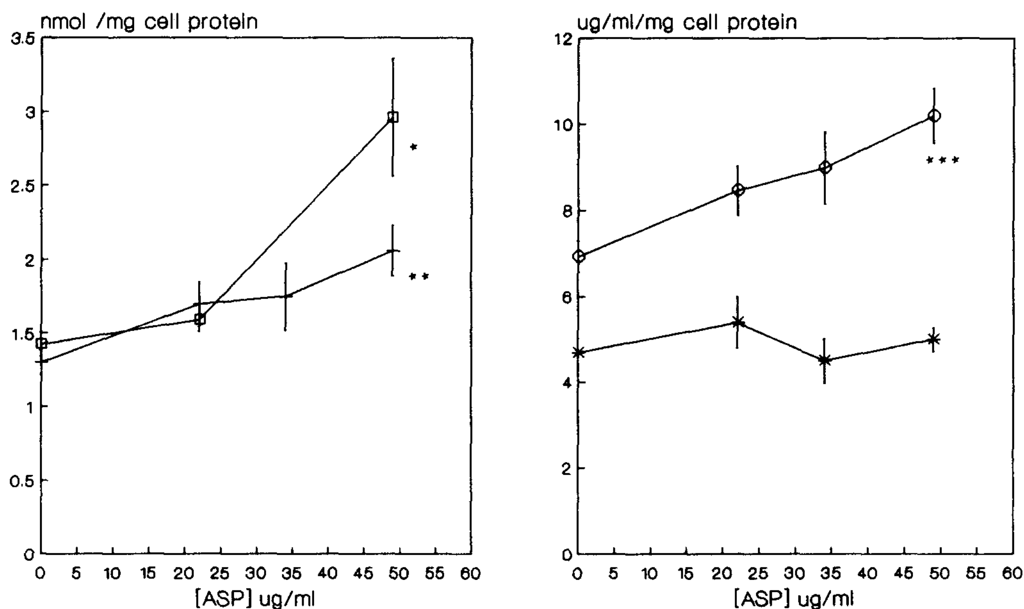

Fig. 3. Effect of ASP on intracellular lipid synthesis and apoprotein secretion. Cells were incubated with 0 to 50 μg/ml ASP in 825 μM [^{14}C]oleate:BSA (molar ratio 5:1) for 24 h (n = 10 experiments). Panel A: cellular [^{14}C]triglyceride (+) and cellular [^{14}C]cholesteryl ester (the latter × 40) (□) expressed as nmol/mg cell protein ± SEM. Panel B: medium apoB (○) and apoA-I (*) are expressed as μg/ml per mg cell protein ± SEM. (★ $P < 0.05$; ★★ $P < 0.0125$; ★★★ $P < 0.0025$).

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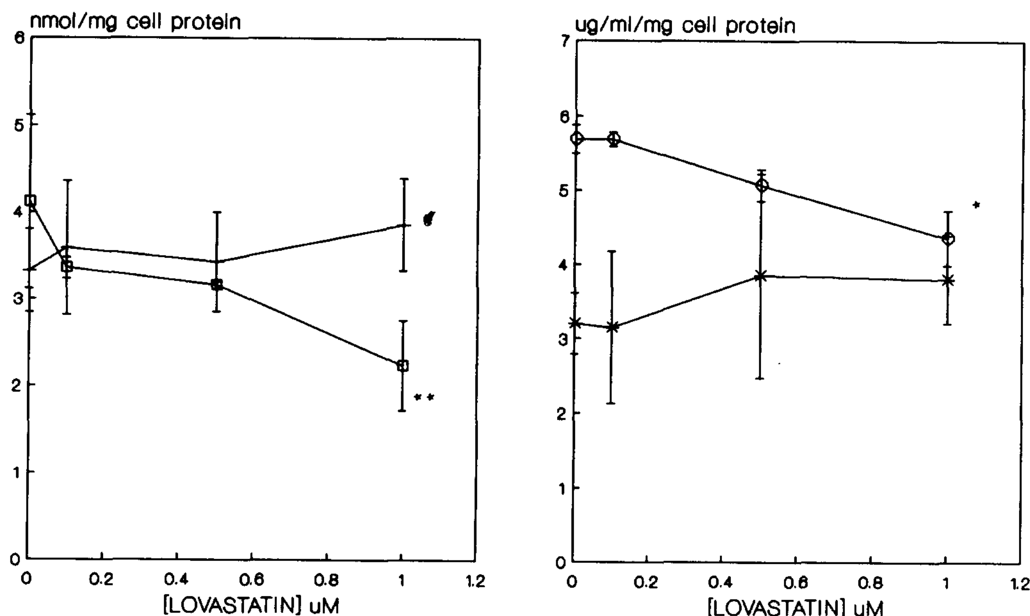

Fig. 4. Effect of lovastatin on intracellular lipid synthesis and apoprotein secretion. Cells were incubated with 0-1 μM lovastatin in 825 μM [^{14}C]oleate:BSA (molar ratio 5:1) for 24 h (n = 10 experiments). Panel A: cellular [^{14}C]triglyceride (+) and cellular [^{14}C]cholesteryl ester (the latter × 40) (□) expressed as nmol/mg cell protein ± SEM. Panel B: medium apoB (○) and apoA-I (*) are expressed as μg/ml per mg cell protein ± SEM. (★ $P < 0.05$; ★★ $P < 0.0025$).

In the next series of experiments, we attempted to dissociate the changes in triglyceride and cholesteryl ester synthesis to determine which, if either, was directly associated with changes in apoB secretion. Four agents were used: lovastatin, 2-bromooctanoate, progesterone, and 58-035. The goals were to inhibit cholesterol synthesis and so reduce cholesteryl ester synthesis (lovastatin) (19); to reduce triglyceride synthesis with little impact on cholesteryl ester synthesis (2-bromooctanoate) (20); or, finally, to inhibit cholesteryl ester synthesis (progesterone and 58-035) (21, 22). In all cases, the cells were incubated in 825 μM [^{14}C]oleate medium with the various additions for 24 h (avg. sp act = 5.9 dpm/pmol).

The results obtained when lovastatin, an HMG-CoA reductase inhibitor, was added to cells incubated in 825 μM [^{14}C]oleate medium are shown in **Fig. 4.** Over this range of concentrations, no effect on triglyceride synthesis was observed; however a dramatic decline in cholesteryl ester synthesis occurred (45% decrease at 1 μM lovastatin, $P < 0.0025$) a change that was associated with a significant reduction (25%, $P < 0.05$) in apoB secretion into the medium. Again there was no effect on apoA-I secretion. In this instance, therefore, intracellular triglyceride and cholesteryl ester synthesis were dissociated and the changes in apoB were directionally similar to those of cholesteryl ester and not triglyceride. In contrast, in basal medium unsupplemented with oleate, lovastatin had no effect on apoB secretion: 2.1 ± 2 basal versus 2.4 ± 3 μg/ml per mg cell protein with 1 μM lovastatin (*P*, not significant).

2-Bromooctanoate has been reported to inhibit triglyceride synthesis without effect on synthesis of other lipid classes (20). However, under the present experimental conditions, incubation of the HepG2 cells with 0.8 μM 2-bromooctanoate resulted in simultaneous decreases in intracellular triglyceride (− 18%) and cholesteryl ester (− 53%) with parallel changes in apoB concentration in the medium (− 31%).

Progesterone has been reported to inhibit cholesteryl ester synthesis and not triglyceride synthesis in fibroblasts (21). The results obtained when progesterone (30 μM) was added to the oleate medium are shown in **Table 1.** Parallel decreases were observed in all three parameters of primary interest: intracellular triglyceride, cholesteryl ester, and secreted apoB. Thus the addition of 2-bromooctanoate and progesterone produced parallel, and simultaneous, decreases in triglyceride and cholesteryl ester synthesis and apoB secretion.

TABLE 1. Effect of progesterone on apoB secretion

	Progesterone	
	Without	With
TG	4.00 (1.00)	2.68 (0.25)
CE	0.063 (0.021)	0.041 (0.004)
ApoB	5.15 (0.08)	3.83 (0.23)
ApoA-I	3.40 (0.70)	3.5 (0.20)

Cells were incubated without and with 30 μM progesterone in 825 μM [^{14}C]oleate:BSA (molar ratio 5:1) for 24 h (n = 5 experiments). Cellular [^{14}C]triglyceride and [^{14}C]cholesteryl ester are expressed as nmol/mg cell protein (± SEM) and medium apoB and apoA-I concentrations are expressed as μg/ml per mg cell protein (± SEM).

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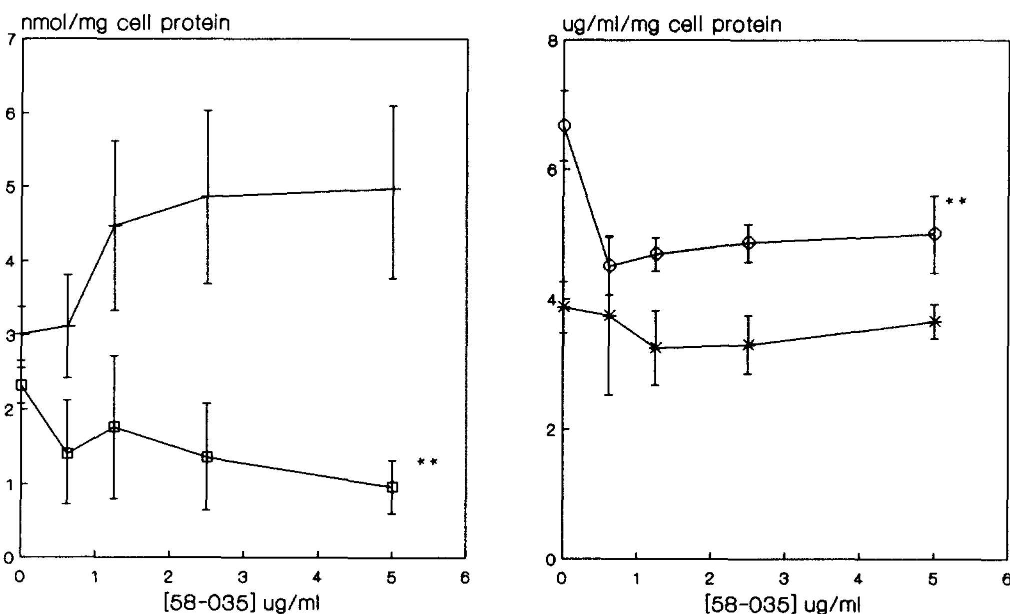

Fig. 5. Effect of compound 58-035 on intracellular lipid synthesis and apoprotein secretion. Cells were incubated with 0-5 μg/ml compound 58-035 in 825 μM [^{14}C]oleate:BSA (molar ratio 5:1) for 24 h (n = 4 experiments). Panel A: cellular [^{14}C]triglyceride (+) and cellular [^{14}C]cholesteryl ester (the latter × 40) (□) are expressed as nmol/mg cell protein ± SEM. Panel B: medium apoB (○) and apoA-I (*) are expressed as μg/ml per mg cell protein ± SEM. (★★ $P < 0.005$).

Finally, the effect of addition of a specific inhibitor of acyl CoA:cholesterol acyltransferase (ACAT), compound 58-035 (22), to the oleate medium was determined. These results are shown in **Fig. 5**. Over the concentration range of 58-035 examined (i.e., from 0 to 5 μg/ml), cholesteryl ester synthesis and apoB secretion both declined, a decline that occurred in the face of a net increase in intracellular triglyceride synthesis. The maximal decline in cholesteryl ester synthesis was 59% ($P < 0.005$), in apoB secretion was 25% ($P < 0.005$), while the net increase in triglyceride synthesis was 65% (P, NS).

To validate the estimates of lipid synthesis derived from the radioactive tracers, the absolute mass of the relevant lipids (cholesteryl ester and triglyceride) was also measured by HPTLC under the same experimental conditions. As shown in **Table 2**, the directional changes in mass of triglyceride and cholesteryl ester are identical to the changes in synthesis of triglyceride and cholesteryl ester determined by radioactive tracer.

In **Fig. 6**, the changes in apoB secretion are compared to the changes in triglyceride and cholesteryl ester synthesis in the presence of 825 μM oleate medium with the various additions (Fig. 3-5 and Table 1). There is no significant correlation between triglyceride synthesis and apoB concentration ($r = 0.137$ P, NS). By contrast, a strong correlation between cholesteryl ester synthesis and apoB secretion exists ($r = 0.928$, $P < 0.0005$).

The decrease in medium apoB concentration observed in the experiments with lovastatin and 58-035 could, of course, be due to increased uptake of secreted apoB by the HepG2 cells rather than decreased secretion. Conversely, the increase in apoB concentration in the medium sup-

TABLE 2. Effect of media additions on triglyceride and cholesteryl ester mass

	TG		CE	
Addition	Measured[a]	Change	Measured[a]	Change
1% BSA basal	4.25 (0.59)		0.77 (0.18)	
+ 825 μM Oleate	16.13 (1.17)	+ 279%	0.95 (0.24)	+ 23%
+ 825 μM Oleate + 1 μM lovastatin	13.61 (0.86)	− 15%	0.57 (0.14)	− 40%
+ 825 μM Oleate + 5 μg/ml 58-035	13.67 (0.70)	− 15%	0.46 (0.14)	− 51%

Cells were incubated for 24 h in the presence of the indicated additions. Triglyceride and cholesteryl ester mass were measured by HPTLC as described in Methods.
[a]nmol/mg cell protein (± SEM).

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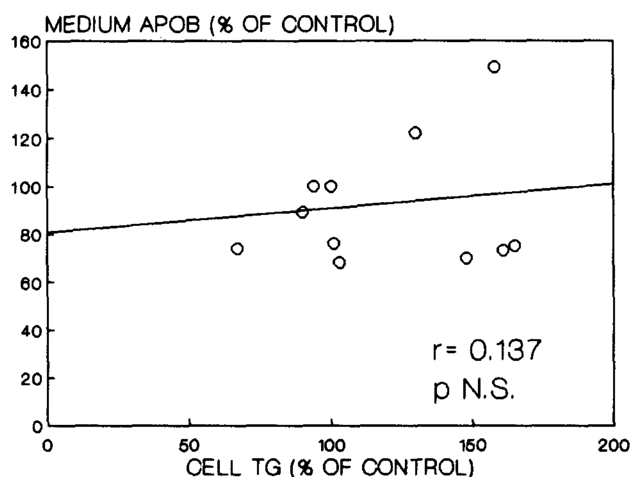


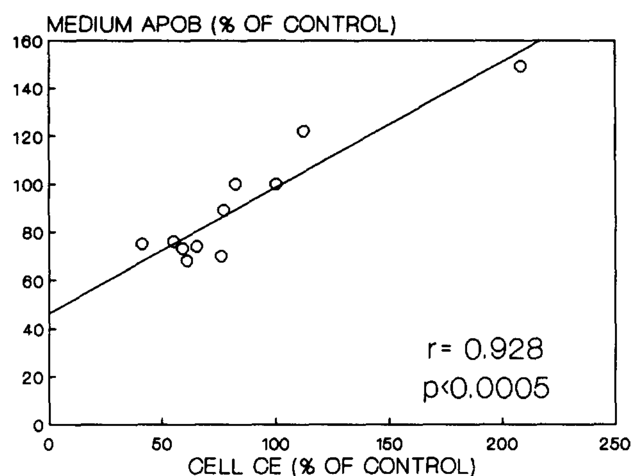
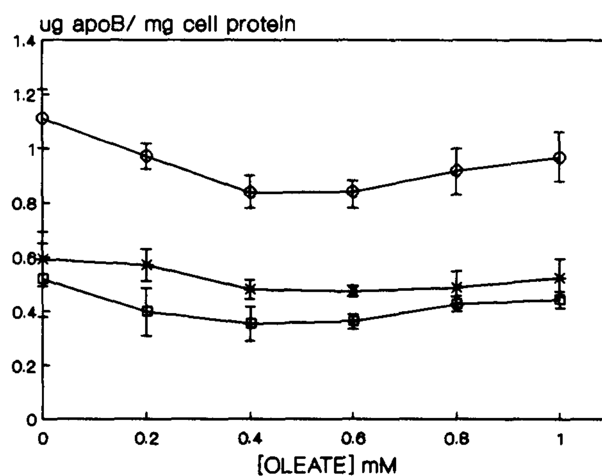


Fig. 6. Correlation of medium apoB with intracellular triglyceride and cholesteryl ester. The data from Figs. 3-5 and Table 1 were integrated. Each value is expressed as a percentage of the control value which is taken as 100%. Upper panel: % change in medium apoB versus % change in intracellular triglyceride (r = 0.137; not significant). Lower panel: % change in medium apoB concentration versus % change in intracellular cholesteryl ester (r = 0.928; P < 0.0005).

plemented with oleate or ASP could be the result of a decrease in LDL removal from the medium. LDL could be removed either via the receptor specific pathway or via nonspecific association with the HepG2 cells. For this reason, the removal of LDL by HepG2 cells under these experimental conditions was investigated in two ways.

First, the effect of oleate concentration on LDL uptake and degradation was investigated in the presence of two concentrations of ^{125}I-labeled LDL apoB in the medium. Concentrations of 1 μg/ml and 5 μg/ml were chosen since they represented the average amount of apoB present in the medium after 24 h of incubation in the absence and in the presence of 825 μM oleate (1.84 ± 0.38 and 5.37 ± 1.20 μg/ml per mg cell protein, respectively). As shown in **Fig. 7**, the overall amount of ^{125}I-labeled LDL apoB removed from the medium did not change by more than 20% at all oleate concentrations up to 1 mM with either 1 μg/ml or 5 μg/ml of medium ^{125}I-labeled LDL apoB. This was the case for both cell-associated LDL apoB and the degraded apoB in the medium.

The same agents used to examine modifications in apoB production were also examined for an effect on ^{125}I-labeled LDL removal. ASP, lovastatin, progesterone, bromooctanoate, and 58-035 were added to the cells in the presence of 825 μM oleate and the amount of ^{125}I-labeled LDL that was cell-associated or degraded was measured after a 24-h incubation. Lovastatin increased the LDL apoB uptake and degradation slightly (18%, not significant) whereas none of the other additions had any effect on ^{125}I-labeled LDL apoB uptake and degradation (**Fig. 8**). About 50% of the ^{125}I-labeled LDL catabolized was present as degradation



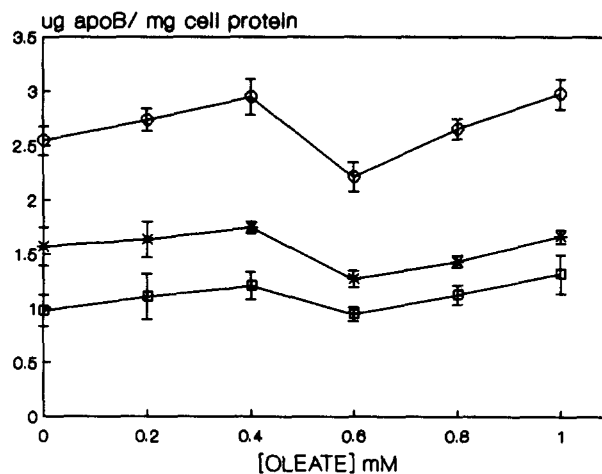


Fig. 7. Effect of oleate on ^{125}I-labeled LDL degradation. Cells were incubated with 1 μg/ml (upper panel) or 5 μg/ml (lower panel) of ^{125}I-labeled LDL apoB with increasing concentrations of oleate:BSA (molar ratio 5:1) for 24 h (n = 4 experiments). Cell-associated (*), degraded (□), and total (○) ^{125}I-labeled LDL B are expressed as μg/mg cell protein ± SEM, where total LDL apoB removed is the sum of cell-associated and apoB degradation products in the medium.

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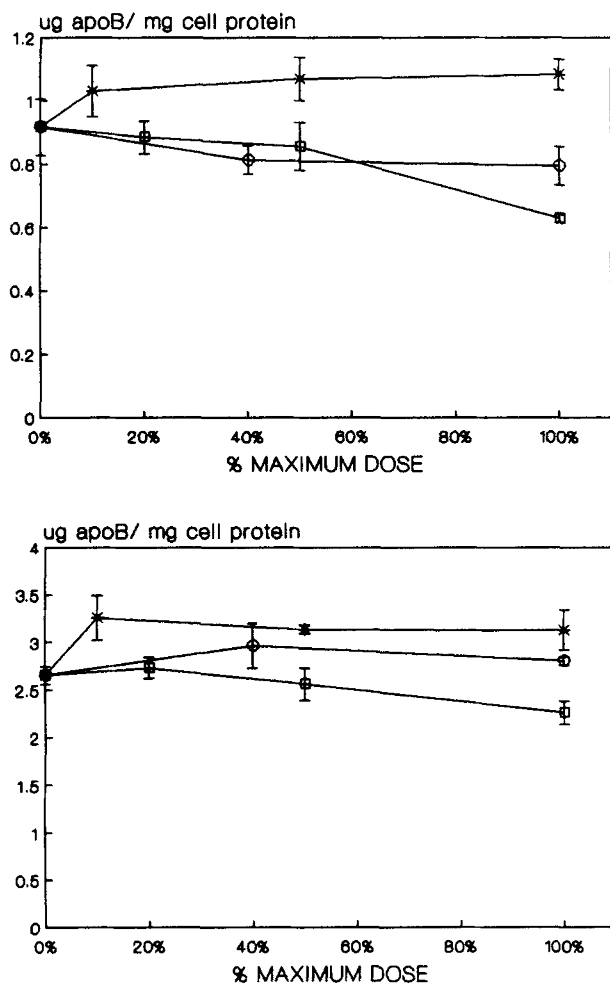

Fig. 8. Effect of lovastatin, 58-035, and ASP on ^{125}I-labeled LDL degradation. Cells were incubated with 1 μg/ml (upper panel) or 5 μg/ml (lower panel) of ^{125}I-labeled LDL apoB with 825 μM oleate:BSA (molar ratio 5:1) for 24 h (n = 4 experiments). Lovastatin (*) maximum concentration 1 μM, 58-035 (☐) maximum concentration 5 μg/ml, and ASP (○) maximum concentration 50 μg/ml were added in increasing concentrations. Results are expressed as total LDL catabolized in μg/mg cell protein ± SEM where total LDL apoB removed is the sum of cell-associated and apoB degradation products in the medium. Data for bromooctanoate and progesterone not shown (see text).

products in the medium while the remaining was present as ^{125}I-labeled cell-associated LDL apoB. Similarly, there was no effect when these were examined individually. It should be noted that the maximal decreases in apoB mass accumulation in the medium with the lipid inhibitors were substantially greater than the total amount of ^{125}I-labeled LDL apoB catabolized during the same time period.

DISCUSSION

The mechanisms that regulate the synthesis and secretion of apoB particles by the liver have so far remained obscure. In studies of HepG2 cells from which apoB secretion increased after a fatty acid challenge (5-9), no corresponding change in apoB mRNA levels that might have mediated this increase was demonstrated, suggesting that post-transcriptional control existed (8, 9). The present study was designed to test one specific model of such control, namely that synthesis of cholesteryl ester in the rough endoplasmic reticulum is a key event coordinating the secretion of apoB in response to increased triglyceride synthesis in the smooth endoplasmic reticulum.

The rate of triglyceride synthesis in the smooth endoplasmic reticulum is a function of the rate of delivery of the key substrates, fatty acids, and glycerol-3-phosphate, and the activity of the series of enzymes involved in its synthesis. The rate of cholesteryl ester synthesis in the rough endoplasmic reticulum is, in turn, a function of the concentration of substrates, free cholesterol, and fatty acid, and the activity of the enzyme, ACAT. Moreover, it should also be noted that apoB is itself acylated (23, 24), and therefore, this process as well could be affected by the level of the fatty acid precursor pool.

It was anticipated that increased influx of fatty acid into the cell would result in increased synthesis of both triglyceride and cholesteryl ester. Our experimental results demonstrate this to be the case. As extracellular oleate concentration was increased, intracellular triglyceride synthesis and cholesteryl ester synthesis increased pari passus. At the same time, secretion of apoB increased as well. As noted previously (7, 8), although there was considerable variability from one experiment to the next, apoB concentration from the cells incubated in the presence of oleate always increased significantly as compared to the concurrent control within each experiment. Furthermore, incubation of the cells with ASP, a plasma protein that markedly enhances triglyceride synthesis in adipose tissue and fibroblasts (13), also enhanced triglyceride and cholesteryl ester synthesis in the HepG2 cells, and in so doing, resulted in the same outcome, increased apoB concentration. Thus the first element of the hypothesis, namely that increased fatty acid entry into the hepatocyte would result in concurrent increases in cholesteryl ester and triglyceride synthesis and apoB accumulation in the medium, was validated.

The amount of apoB in the medium, however, represents the balance between cellular secretion and removal from the medium. An increase in medium apoB could be due either to an increased production or decreased removal. In these experiments, there was no effect on overall ^{125}I-labeled LDL apoB uptake and catabolism at a high concentration of LDL apoB (5 μg/ml) and only a marginal effect at a lower concentration of LDL apoB (1 μg/ml). These results are similar to previous findings with pulse chase studies (7). Moreover, Fuki et al. (25) demonstrated a decrease in ^{125}I-labeled LDL binding with increasing oleate concentration, but the decrease in binding was not sufficient to account for the oleate-induced increase in apoB in the medium.

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Altered catabolism cannot account, therefore, for the degree to which the apoB increased in the medium. Moreover, under all subsequent experimental conditions, where there was a change in apoB concentration (that is with the addition of ASP, lovastatin, or 58-035 to an oleate-containing medium), there was no effect on ^{125}I-labeled LDL apoB removal from the medium. Finally, it must be noted that these estimates of catabolism were based on LDL isolated from normal human plasma. Since it has been recently reported that there is little or no interaction of newly secreted apoB-containing lipoproteins with HepG2 cells, the influence of catabolism on apoB concentration in the medium might be even less than is suggested by our results (25, 26).

The second series of experiments were undertaken to attempt to dissociate the effects of triglyceride and cholesteryl ester on apoB secretion. The results obtained with progesterone and 2-bromooctanoate were disappointing in that, contrary to previous reports, neither agent was selective in its inhibition of lipid synthesis with regard to triglyceride and cholesteryl ester. Progesterone, which has been shown to inhibit cholesteryl ester synthesis but not triglyceride synthesis in fibroblasts (21), inhibited both in our HepG2 cells. Similarly, 2-bromooctanoate has been reported to inhibit triglyceride synthesis selectively in primary rat hepatocytes (20), but again, this did not appear to be the case in the HepG2 cells. The reasons for these discrepancies, though, are not apparent to us. With both agents, synthesis of triglyceride and cholesteryl ester decreased, as also did secretion of apoB, again a pattern of coordinate response.

On the other hand, the results obtained with the specific ACAT inhibitor, 58-035, and the HMG-CoA reductase inhibitor, lovastatin, appear to strongly support the hypothesis that cholesteryl ester synthesis might be a critical element regulating apoB secretion by hepatocytes. The data demonstrate that, in the presence of oleate, lovastatin reduced apoB secretion, diminished cholesteryl ester synthesis, but did not affect triglyceride formation. However, these effects of lovastatin on apoB secretion were not seen in the absence of oleate, results that are consistent with those of Pullinger et al. (8) who also examined the effect of lovastatin on apoB secretion in HepG2 cells and found no change using a medium that did not contain oleate.

Our results indicate that lovastatin decreases apoB secretion from oleate-supplemented HepG2 cells. These in vitro observations appear to complement the in vivo observations of Arad, Ramakrishnan, and Ginsberg (27) since, in their study, it was shown that in patients with combined hyperlipidemia treated with lovastatin, apoB production decreased with no effect on LDL apoB fractional catabolic rate. Of greater interest, with regard to mechanism, was the observation that in the face of a fatty acid challenge in the presence of 58-035, triglyceride synthesis increased but cholesteryl ester synthesis decreased. Now, with directionally opposite changes in intracellular synthesis of the two nonpolar lipids, apoB secretion diminished in parallel with the decrease in intracellular cholesteryl ester synthesis. In this regard, it should be noted that the importance of cholesteryl ester in the secretion of lipoprotein triglyceride has also been noted in intestinally derived CaCo-2 cells, although in these studies apoB levels in the medium were not determined (22).

The principal limitation of this study—the use of HepG2 cells as surrogates for normal human hepatocytes—must, of course, be emphasized. Although HepG2 cells retain many of the biologic capacities of the normal hepatocyte such as the capacity to synthesize lipoproteins (5-9), they are transformed cells, and premature generalization to the normal cell in situ must be avoided. In addition, only apoB secretion into the medium and not apoB synthesis was measured. Moreover apoB mRNA was not quantitated, although, to date, in all studies of apoB synthesis, whether of apoB-100 synthesis in the liver or of apoB-48 synthesis in the intestine, no evidence has yet appeared pointing to transcriptional regulation (8, 9, 28). On the contrary, all such studies have been interpreted as consistent with post-transcriptional regulation of apoB synthesis.

It is a biologic truism, though nonetheless vital, that cells strictly regulate their intracellular free cholesterol content. Free cholesterol can be inserted within phospholipid bilayers but the capacity to do so is limited. Excess cholesterol within the cell is esterified to cholesteryl ester, and as a consequence, because of its extreme nonpolarity, the product can no longer remain within the bilayer (29). In the case of the hepatocyte, cholesteryl esterification takes place in the rough endoplasmic reticulum as does synthesis of apoB (10). It seems possible, therefore, that cholesteryl ester, as it is synthesized in the bilayer, and then extruded from it, might be inserted between the newly synthesized apoB in the membrane and the membrane of the rough endoplasmic reticulum, detaching the apoB and initiating its passage through the cell, first to the smooth endoplasmic reticulum where triglyceride is added, and then, eventually, to the Golgi apparatus, after which it is finally secreted from the cell. Such a model is, we believe, consistent with the experimental observations presented in this study. It is also consistent with previous morphologic observations (30) and the observations made by Borchardt and Davis (31) that suggest that movement of apoB out of the endoplasmic reticulum is the rate-limiting step in secretion of lipoprotein.

The fact that cholesteryl ester synthesis within the liver is quantitatively much less than triglyceride synthesis in no way speaks against the potential validity of the model. Moreover the model outlines a series of mechanisms that might underlie the increased apoB secretion noted in a variety of disorders. For example, significant reduction in ASP receptors in peripheral cells has been documented in a substantial proportion of patients with hyperapobetalipoproteinemia (12). This reduced capacity to form triglyceride in peripheral cells might then lead to increased influx of fatty acids to

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the liver and increased apoB secretion by the route shown. Indeed the reduction in apoB levels due to decreased production of hepatic apoB particles observed after lovastatin therapy in patients with combined hyperlipidemia (27) and hyperapobetalipoproteinemia (32) might result from the mechanisms deduced from this study. Similarly the increased apoB secretion that has been documented in cholesteryl ester storage disease (33) and betasitosterolemia (34), as well as the increased plasma apoB levels documented in cholestanolosis (35), might relate to increased synthesis of the relevant sterol ester within the liver. Much beyond this, the present data suggest a model that intertwines lipid and protein secretion within the cell, a connection which itself is of note, so far as it may account for control of the secretion of hepatic apoB lipoproteins.

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