Role of Cholesterol Ester Mass in Regulation of Secretion of ApoB100 Lipoprotein Particles by Hamster Hepatocytes and Effects of Statins on That Relationship

Zujun Zhang, Katherine Cianflone, Allan D. Sniderman

Abstract—Our understanding of the factors that regulate the secretion of apoB100 lipoproteins remains incomplete with considerable debate as to the role, if any, for cholesterol ester in this process. This study examines this issue in primary cultures of hamster hepatocytes, a species in which both cholesterol and apoB100 metabolism are very similar to man. Addition of oleate to medium increased the mass of triglyceride and cholesterol ester within the hepatocyte and also increased the secretion of triglycerides, cholesterol ester, and apoB100 into the medium. Next, the responses of hamster hepatocytes to addition of either an HMG-CoA reductase inhibitor (lovastatin) or an acyl-CoA cholesterol acyltransferase inhibitor (58-035) to the medium, with or without added oleate, were determined. Effects of either agent were only evident in the oleate-supplemented medium in which cholesterol ester mass had been increased above basal. If oleate was not added to the medium, neither agent reduced apoB100 secretion; equally important, over the 24-hour incubation, neither agent, at the concentration used, produced any detectable change in intracellular cholesterol ester mass. However, in contrast to the estimates of mass, which were unchanged, under the same conditions radioisotopic estimates of cholesterol ester synthesis were markedly reduced. Any conclusion as to the relation of cholesterol ester mass to apoB100 secretion would therefore depend on which of the 2 methods was used. Overall, the data indicate a close correlation between the mass of cholesterol ester within the hepatocyte and apoB100 secretion from it and they go far to explain previous apparently contradictory data as to this relation. More importantly, though, taken with other available data, they indicate that the primary response of the liver to increased delivery of lipid is increased secretion rather than decreased uptake. These results point, therefore, to a hierarchy of hepatic responses to increased flux of fatty acids and increased synthesis of cholesterol that in turn suggests a more dynamic model of cholesterol homeostasis in the liver than has been appreciated in the past. (Arterioscler Thromb Vasc Biol. 1999;19:743-752.)

Key Words: hepatocytes ■ lipoprotein ■ cholesterol ester

The rate at which the apoB100 lipoprotein particles are secreted by the liver is a major determinant of IDL and LDL particle number in plasma. Accordingly, it is a major determinant of atherogenic risk.1 Unfortunately, our understanding of the factors that regulate the rate of apoB100 secretion by the liver is recent and remains incomplete. In the case of apoB100 lipoprotein particles, variation in the rate at which they are secreted appears to be determined principally by variation in the proportion of newly synthesized apoB100 molecules, which are degraded compared with the proportion that are secreted, reflecting the complex coupling of multiple lipids to protein involved in the assembly and secretion of a lipoprotein particle.2

There are now considerable in vitro and in vivo data that point to a direct relation between the rate of cholesterol synthesis or mass of cholesterol ester within the hepatocyte and the secretion rate of hepatic apoB100 lipoproteins (for review see Reference 3). Nevertheless, not all studies agree. A number of in vitro studies, all done in HepG2 cells,4–8 have shown that reduced rates of cholesterol ester synthesis, induced by statins, as measured by radioisotopic techniques, did not result in a reduced rate of apoB100 secretion. This cell, unfortunately, has major limitations. The most important are that little of the neutral core lipid that is synthesized is secreted, and consequently few of the particles that are secreted resemble VLDL, most being considerably smaller with much less triglyceride in their core.9–11

Rat hepatocytes, the other most commonly used model, secrete apoB48 as well as apoB100,12,13 making extrapolations to humans difficult. By contrast, hamster hepatocytes, like human hepatocytes, secrete only apoB100.14,15 In addition, the total body cholesterol synthetic rates are comparable in humans and hamsters as is the level of acyl-CoA cholesterol acyltransferase (ACAT).16 Of note also, in primary cultures, no important differences between human and hamster hepatocytes with respect to cholesterol metabolism,17 lipoprotein synthesis and secretion,17 LDL binding and degradation,18 and bile acid synthesis19 have been observed.
Importantly, most of the apoB100 particles secreted by hamster hepatocytes are triglyceride-rich, and when triglyceride synthesis is increased by increased delivery of glucose or fatty acids, most triglycerides are exported in apoB100 particles rather than being retained within the cell.

The present studies were designed to examine further the relations between the masses of cholesterol and cholesterol ester and their rates of synthesis and the secretory rate of apoB100 lipoprotein particles by hamster hepatocytes. To do so, the rates of synthesis of cholesterol and cholesterol ester were manipulated by addition of either an HMG-CoA reductase inhibitor or an ACAT inhibitor, whereas apoB secretion was increased by addition of olate. The results indicate that under all circumstances there was a clear relation between the mass of cholesterol ester and the rate of secretion of apoB100 lipoprotein particles. This is apparent, however, only if mass is measured but is not evident if only radioisotopic estimates are used. Moreover, whether apoB100 secretion is reduced by a statin depends on the potency of the agent and the experimental conditions under which it is studied.

The data, therefore, appear to reconcile differences in interpretation reached in previous work. Even more importantly, they point to a hierarchy of mechanisms to modulate sterol balance in the hepatocyte, with increased secretion under these circumstances playing the primary role. Finally, the data explain why HMG-CoA reductase inhibitors work so effectively in humans with increased secretion of hepatic cholesterol, whereas apoB secretion is reduced by a statin.

Methods

Materials and Supplies

All reagents used were obtained from Fisher Scientific and Sigma unless otherwise indicated. Tissue culture medium, FCS, and other tissue culture supplies were obtained from Gibco. Hamster plasma was obtained from BioProducts for Science. [1-14C]Oleic acid (specific activity 50 mCi/mmol), [3H]acetate (specific activity = 2.5 Ci/mmole), and [3H]mevalonate (specific activity = 30 Ci/mmole) were obtained from DuPont-NEN. Olate was complexed to BSA as described by Van Harken et al. The specific inhibitor of ACAT (58-035) was obtained from Sandoz Pharmaceuticals, and lovastatin and simvastatin, both HMG-CoA reductase inhibitors, were obtained from Merck Frosst.

Isolation and Culture of Hamster Hepatocytes

Male Syrian golden hamsters weighing 100 to 120 g were used for hepatocyte isolation. Under appropriate anesthesia, the abdomen was opened and the liver was perfused with 10 mmol/L HEPES in Ca2+- and Mg2+-free Hanks' buffer at 37°C at 4 mL/min for 20 minutes. The liver was then perfused with 0.025% collagenase A (Sigma Chemicals) dissolved in Hanks' buffer containing Ca2+ and Mg2+ at 37°C, 4 mL/min for 20 minutes. The liver was removed and placed in a sterile Petri dish with 20 mL of digestion solution for 15 minutes (0.1% collagenase A, 0.05% hyaluronidase [Boehringer Mannheim], and 0.01% DNase [Sigma] dissolved in DMEM/F12 media) at 37°C. After digestion, cells were filtered through sterile cotton gauze, washed 2 times (by centrifugation) with DMEM/F12 containing 20% FCS and penicillin G/streptomycin. Cells were then resuspended in culture medium (20% FCS, 10 μg/mL insulin, penicillin G/streptomycin in DMEM medium). Cells were then plated out at a concentration of 2×10^6 cells/mL (1 mL/35-mm well) into tissue culture dishes that were coated with a solution of 3 μg/mL of collagen type IV (Becton Dickinson Labware) and incubated overnight to allow cells to attach.

Experimental Conditions

Before the experiments were performed, cells were preincubated for 4 hours at 37°C in 5% CO2 in serum-free medium supplemented with 1% BSA. Cells were then changed to fresh serum-free medium supplemented with 1% BSA and the indicated additions and incubated at 37°C overnight. At the end of the incubation period, the cells were placed on ice and the medium removed and set aside for analysis. The cells were washed 3 times with 1 mL ice-cold PBS. The intracellular lipids were extracted twice with 1 mL heptane-isopropanol (3:2 vol/vol) at room temperature for 30 minutes, and the extracts were pooled. Cell protein was solubilized in 1 mL of 0.1 N NaOH and quantified by the method of Bradford using a commercial assay (BioRad) with BSA as a standard.

Determination of Intracellular and Secreted Lipids

Cell lipid extracts were concentrated and separated by thin-layer chromatography (Silica Gel G) using a solvent system consisting of hexane-ethyl ether-acetic acid (75:25:1 vol/vol/vol). Lipid spots were identified by exposure to iodine vapor and compared with reference lipids (Sigma). Triglyceride spots were scraped into tubes, and the lipid was extracted by adding 0.5 mL isopropanol/mass; mass was determined by the method of Neri and Frings using a standard curve of 0 to 52 μg/mL triolein. Cholesterol ester spots were similarly scraped into tubes and cholesterol ester determined by the method of Rudel and Morris using a standard curve of 0 to 14 μg/mL cholesterol olate. Cholesterol was determined in the same way using cholesterol as a standard. Conditioned medium was extracted with 3 volumes isopropanol-heptane (1:1 vol/vol) and the remainder extracted again with isopropanol-heptane (4:1 vol/vol) and 0.05% KOH. The lipid extract was then analyzed as above for medium cholesterol ester, triglyceride, and cholesterol mass. Bile acids (cholate plus deoxycholate) were measured in conditioned medium with 6% trichloroacetic acid with 1 mg/mL BSA as carrier protein, followed by precipitation of free iodine with 1.7% AgNO3.

Determination of Intracellular and Secreted Bile Acids

Bile acids (cholate plus deoxycholate) were measured in concentrated medium and cell homogenates using a commercial colorimetric enzyme assay (Sigma). Total bile acids represents the sum of both medium and cells.

Determination of Hamster ApoB100 and ApoA1

An indirect competitive ELISA was used to quantify the apoB100 secreted by the hamster hepatocytes. To do so, hamster LDL was first isolated by ultracentrifugation (d = 1.019 to 1.063 g/mL) and then separated by 3% to 15% polyacrylamide gradient SDS-PAGE gel electrophoresis under denaturing conditions as described by Laemmli. The gel was cleaned with Coomassie blue, and the band corresponding to apoB100 was excised, emulsified with Freund's adjuvant, and injected subcutaneously 6 times at 3-week intervals to generate polyclonal antibody. Microtiter plates were coated overnight with 3 μg/mL LDL protein in PBS (pH = 7.2) at 4°C and then blocked with 1.5% BSA in PBS for 2 hours at room temperature. Standards (range, 0.031 to 4 μg/mL) of LDL-derived apoB100 were prepared. Rabbit anti-hamster apoB100 polyclonal antibody (150 μL of antibody diluted appropriately in PBS containing 0.5 mL/L Tween 20) was added to each sample (standards and hamster media) and incubated overnight. Then 100 μL of each sample was added in triplicate to the microtiter plate for 2 hours at 37°C. After this
incubation, plates were washed, and 100 μL anti-rabbit IgG conjugated to horseradish peroxidase (diluted in 4% polyethylene glycol, 0.5 mL/L Tween 20 in PBS) was added to each well and incubated at 37°C for 30 minutes. Plates were again washed, and a colorimetric reaction was initiated by adding 100 μL of 1 mg/mL α-phenylenediamine dihydrochloride in 0.018% H$_2$O$_2$ in 100 mmol/L sodium citrate, pH 5.0, with 0.5 mL/L Tween 20. The color reaction was stopped with 50 μL of 4 N H$_2$SO$_4$, and the plate was read spectrophotometrically at 490 nm. A log-log plot of apoB100 concentration (μg/mL) versus absorbance at 490 nm was constructed, and linear least squares analysis of the data was performed.

To obtain hamster apoA1, HDL was isolated from hamster plasma by density gradient ultracentrifugation. This was then electrophoresed on a 10% SDS-polyacrylamide gel according to the method of Laemml,$^{25}$ and the band corresponding to apoA1 cut out, emulsified, and injected subcutaneously in a rabbit to generate a rabbit antibody. Antigen (3 mg/mL) was coated overnight at 4°C, and the plate was blocked for 2 hours with 250 μL of 1.5% BSA. Plates were washed in 0.9% saline 5 to 6 times between each step. Standards (0.03 to 1.25 μg/mL), blanks, controls, and samples were diluted appropriately in 0.05% (vol/vol) Tween in PBS, and 150 μL was added to 150 μL of diluted primary antibody and incubated overnight at 4°C. The remaining steps of the apoA1 assay are identical to those of the apoB100 assay.

**Lipoprotein Fractionation From Hamster Medium**

Medium from hamster hepatocytes was fractionated by sequential density ultracentrifugation as described previously.$^{20}$ Hamster cell medium was layered under a salt solution of density d 1.006 g/mL and centrifuged at 100,000g for 18 hours, and the top 2 mL was collected as VLDL. The remaining solution was adjusted to d 1.019 g/mL and recentrifuged for 20 hours at 100,000g. The top 2 mL was collected as IDL. The remaining solution was adjusted to d 1.063 g/mL and recentrifuged for 200 hours (20 μL). Finally, the remaining solution was adjusted to d 1.21 g/mL and recentrifuged (100 μL, 20 hours), and the top was collected as HDL. ApoB100, cholesterol ester mass, and triglyceride mass were measured on each fraction (VLDL, IDL, LDL, HDL, and the remaining infranatant fluid) as described above.

**Statistics**

Results are expressed as mean±SEM. Significance was determined by two-tailed t test where significance was set at P<0.05 and NS = not significant.

**Results**

The effect of oleate on the secretion of apoB100, cholesterol ester and triglyceride by hamster hepatocytes is shown in Figure 1. In these studies, the cells were incubated with or without 0.8 mmol/L oleate complexed to albumin, and the masses of apoB100, cholesterol ester, and triglyceride in the medium were determined at 3, 6, and 24 hours. Oleate caused a substantial increase in all 3 components at all 3 times. There was, however, no change in the cholesterol ester to apoB100 ratio (1.1±0.3 control [CTL] versus 0.8±0.1 oleate; P=NS) or the triglyceride to apoB100 ratio (18±5 CTL versus 11.3±2.6 oleate; P=NS) during the course of the experiment. Thus, increased delivery of oleate to hamster hepatocytes caused increased accumulation of apoB100 lipoproteins without evident change in their composition. This increase, however, was not linear, being most pronounced in the first 6 hours of the incubation, a finding that may reflect depletion of fatty acid in the medium.

Next, the effect of oleate on the density distribution of the apoB100 lipoproteins in the medium was determined. As illustrated in Figure 2 (top), even in the absence of oleate, most of the apoB100 in the medium (56±10%) is recovered in the VLDL-IDL density range. Oleate causes this increase still further. Thus with addition of oleate, the amount of VLDL apoB100 increased by 79% (P<0.025). At the same time, there was an increase in apoB100 mass in the IDL density range (d=1.006 to 1019 g/mL, 116% increase; P<0.0125) without any change occurring in the amount of apoB100 in the LDL density range (d=1.019 to 1.063 g/mL). Less than 2% of the apoB100 was detected in the higher density range (d=1.063 to 1.21 g/mL). Overall, there was both absolutely and proportionately more VLDL apoB100 secreted as a consequence of addition of oleate to the medium. With respect to cholesterol ester, the mass in VLDL increased as did that in IDL and HDL density range with little change in LDL cholesterol ester mass (Figure 2,
Triglyceride, however, increased significantly only in VLDL and LDL (Figure 2, bottom).

The impact of varying the concentration of oleate in the medium on the secretion of apoB100 and apoA1 was then examined. The data in Figure 3 (left) illustrate that a stepwise increase in the concentration of oleate in the medium had no effect on the accumulation of apoA1 in the medium whereas the concentration of apoB100 increased progressively, from an initial level of $6.0 \pm 1.15 \mu g/mg$ cell protein to a final value of $15.2 \pm 2.8 \mu g/mg$ cell protein, a $252\pm37\%$ increase overall. As shown in Figure 3 (top right), the total mass of cholesterol ester—that is, the mass in the cells and the medium—increased progressively as the oleate concentration in the medium was increased, as did also the total mass of triglyceride in the cells and medium (Figure 3, bottom right). Addition of oleate to the medium therefore resulted in substantial increase in secretion of apoB100 as well as marked increases in the synthesis and secretion of both cholesterol ester and triglyceride.

Having characterized the responses of the hamster hepatocytes to increased delivery of oleate, the next objective was to examine the effects of agents that alter the synthesis of cholesterol and cholesterol ester on these processes. To do so, an HMG-CoA reductase inhibitor, lovastatin, was added to the medium, with or without additional oleate (0.8 mmol/L). Similarly, the effects of an ACAT inhibitor, 58-035, were studied under basal and oleate stimulated conditions. The masses of cholesterol and cholesterol ester were determined in both the cells and the medium, and the concentration of apoB100 was determined in the medium.

The results are shown in Table 1. In the absence of oleate in the medium, the mass of apoB100 did not change significantly with addition of either inhibitor to the medium, a finding that might lead to the conclusion that the secretion of apoB100 was unrelated to either cholesterol or cholesterol ester. The determination of cholesterol and cholesterol ester...
masses in the cells and the medium make it evident, however, that their masses were also unchanged. Thus, under basal conditions, neither the HMG-CoA reductase inhibitor, lovastatin, nor the ACAT inhibitor, 58-035, at the concentrations used altered hepatocyte cholesterol or cholesterol ester homeostasis in terms of mass. Accordingly, these experimental conditions do not allow the hypothesis that apoB100 secretion might relate to one or other of these variables to be either confirmed or refuted.

Very different results were obtained when apoB100 secretion was studied after addition of oleate to the medium. As demonstrated previously, oleate caused apoB100 concentration in the medium and cholesteryl ester mass in the hepatocytes and in the medium to increase substantially (Table 1). As well, the proportion of cholesterol ester to free cholesterol in the medium increased also. Addition of the ACAT inhibitor did not affect the mass of free cholesterol in the cells or the medium (Table 1). However, the mass of cholesteryl ester was significantly reduced within the cells to the level present under basal conditions. The mass of apoB100 and the mass of cholesteryl ester in the medium were also both significantly reduced below the levels produced by oleate stimulation. Thus, in effect, the ACAT inhibitor restored basal conditions with respect to cellular cholesterol ester mass and apoB100 secretion.

Although acting at a different site in the cholesterol-cholesterol ester synthetic cycle, much the same results were obtained with addition of the HMG-CoA reductase inhibitor to the medium. Again, the mass of cholesteryl ester within the cell was restored to basal conditions, but the mass of free cholesterol remained unchanged from basal. ApoB100 in the medium was reduced to basal levels as were the amounts of both free cholesterol and cholesteryl ester in the medium (Table 1). Neither inhibitor significantly affected total triglyceride mass (data not shown).

If there was no difference produced by the experimental interventions in cholesterol breakdown (see below), differences in the total mass of cholesterol and cholesteryl ester in the cells and medium under the different conditions must represent differences in the amount of cholesterol synthesized by the cell during this period. Under basal conditions, this did not change with addition of either the HMG-CoA reductase inhibitor or the ACAT inhibitor to the medium. With oleate stimulation, however, the mass of total cholesterol, and therefore cholesterol synthesis, increased markedly. Both lovastatin and 58-035 reduced cholesterol synthesis to that present under basal conditions.

Although the cholesterol ester to apoB100 ratio in the medium was the same under both control and oleate-stimulated conditions (data not shown), the triglyceride to apoB100 ratio increased significantly when apoB100 secretion was reduced by either the ACAT inhibitor or the HMG-CoA reductase inhibitor (69 ± 19 [oleate alone] versus 101 ± 22 [58-035 + oleate], P < 0.05; versus 135 ± 25 [lovastatin + oleate], P < 0.05). Thus both agents resulted in the secretion of fewer particles, but those that were secreted were enriched in triglyceride.

Next, the observation period during which the cells were incubated with and without lovastatin (5 μmol/L) was extended to 48 hours. In the oleate-stimulated cells, apoB100 secretion (Table 2) and cholesterol ester mass (not shown) were less at all times with addition of lovastatin to the medium. By contrast, in the control cells, there was evidence of an inhibitor effect only at 36 and 48 hours, indicating that decreases in cholesterol ester and apoB100 are only seen after sustained enzyme inhibition is achieved.

**Table 1.** Effect of Fatty Acid, Lovastatin, and 58-035 on Hepatocyte Sterol Mass and ApoB100 Secretion

<table>
<thead>
<tr>
<th></th>
<th>Cells</th>
<th>Medium</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>FC</td>
<td>CE</td>
<td>Total</td>
</tr>
<tr>
<td>CTL</td>
<td>3.68 ± 0.39</td>
<td>1.40 ± 0.33</td>
<td>5.08 ± 0.24</td>
</tr>
<tr>
<td>CTL + 58-035</td>
<td>5.10 ± 0.42</td>
<td>1.67 ± 0.31</td>
<td>6.67 ± 0.35</td>
</tr>
<tr>
<td>CTL + Lov</td>
<td>4.05 ± 0.32</td>
<td>1.02 ± 0.24</td>
<td>5.07 ± 0.22</td>
</tr>
<tr>
<td>FA</td>
<td>4.67 ± 0.49</td>
<td>2.37 ± 0.31*</td>
<td>7.04 ± 0.49</td>
</tr>
<tr>
<td>FA + 58-035</td>
<td>4.40 ± 0.46</td>
<td>1.24 ± 0.30†</td>
<td>5.64 ± 0.49</td>
</tr>
<tr>
<td>FA + Lov</td>
<td>4.65 ± 0.40</td>
<td>1.06 ± 0.10‡</td>
<td>5.70 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>2.73 ± 0.34</td>
<td>1.85 ± 0.35</td>
<td>4.58 ± 0.25</td>
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<td>1.66 ± 0.54</td>
<td>4.96 ± 0.42</td>
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<td>2.94 ± 0.25</td>
<td>1.73 ± 0.40</td>
<td>4.67 ± 0.77</td>
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<td>3.82 ± 0.45</td>
<td>5.80 ± 1.7*</td>
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<td></td>
<td>3.12 ± 0.41</td>
<td>1.95 ± 0.68‡</td>
<td>5.07 ± 1.6</td>
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<td></td>
<td>2.75 ± 0.42</td>
<td>2.87 ± 1.60‡</td>
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<td>3.25 ± 0.96</td>
<td>9.66 ± 2.2</td>
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<td></td>
<td>8.40 ± 1.33</td>
<td>3.33 ± 1.17</td>
<td>11.72 ± 2.5</td>
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<tr>
<td></td>
<td>6.99 ± 2.75</td>
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<tr>
<td></td>
<td>8.49 ± 1.87</td>
<td>8.17 ± 16.61</td>
<td>13.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>7.52 ± 3.19</td>
<td>10.47 ± 4.1</td>
<td>4.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>7.40 ± 3.88</td>
<td>11.31 ± 5.1</td>
<td>5.1 ± 1.7</td>
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</table>

Primary hamster hepatocytes were incubated for 18 hours with or without 0.8 mmol/L oleate (FA), with or without 5.0 μmol/L ACAT inhibitor, Sandoz compound 58-035, and with or without 5.0 μmol/L lovastatin (Lov). The masses of free cholesterol (FC), cholesteryl ester (CE), and total (FC + CE) were determined in the cells and the medium and as described in Methods and are expressed as microgram per milligram cell protein. ApoB100 was determined in the medium and expressed as microgram per milligram cell protein. Results are expressed as the average for 12 dishes in 3 experiments. *P < 0.05 vs CTL and †P < 0.05, ‡P < 0.01 FA.

**Table 2.** Effect of Lovastatin on ApoB100 Secretion During Long-Term Incubation of Hamster Hepatocytes

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Basal</th>
<th>Olate</th>
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<tr>
<td></td>
<td>−</td>
<td>+</td>
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<tr>
<td>3 (n = 9)</td>
<td>5.5 ± 0.8</td>
<td>4.6 ± 1.0</td>
</tr>
<tr>
<td>(−17%)</td>
<td>(−42%)</td>
<td></td>
</tr>
<tr>
<td>6 (n = 8)</td>
<td>5.5 ± 1.2</td>
<td>4.7 ± 1.5</td>
</tr>
<tr>
<td>(−15%)</td>
<td>(−22%)</td>
<td>0.05</td>
</tr>
<tr>
<td>24 (n = 8)</td>
<td>5.0 ± 0.5</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>(NC)</td>
<td>(−35%)</td>
<td>0.01</td>
</tr>
<tr>
<td>36 (n = 8)</td>
<td>3.1 ± 0.6</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>(−42%)</td>
<td>(−46%)</td>
<td>0.05</td>
</tr>
<tr>
<td>48 (n = 8)</td>
<td>5.9 ± 1.5</td>
<td>3.3 ± 1.0</td>
</tr>
<tr>
<td>(−44%)</td>
<td>(−31%)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Primary hamster hepatocytes were incubated for varying periods up to 48 hours in the presence (+) or absence (−) of oleate (0.8 mmol/L) and the presence (+) or absence (−) of lovastatin (5 mmol/L). ApoB100 concentration in the medium is determined as described in the Methods and expressed as microgram per milligram cell protein with the percent change in parentheses. Results are expressed as the mean ± SEM for the indicated number of experiments (n) where NC indicates no change.
The effects of another HMG-CoA reductase inhibitor, simvastatin, were also examined. As shown in Figure 4, after 24 hours of incubation simvastatin reduced apoB100 concentration in the medium under both basal and oleate-supplemented conditions. This effect was achieved at low doses of the inhibitor (0.5 μmol/L). Simvastatin is, therefore, a more potent agent than lovastatin. Of note, simvastatin inhibited both the basal level of apoB100 secretion (14% to 21% inhibition, P<0.02 by ANOVA) and that in oleate-treated hepatocytes (21% to 67% inhibition, P<0.001 by ANOVA).

The time course of simvastatin on apoB100 secretion was then examined. As shown in Figure 5, at all times from 3 to 48 hours, simvastatin inhibited the oleate-induced stimulation of apoB100 by −18% to −46%, P<0.05. Cholesterol ester mass was reduced by simvastatin at all times with oleate stimulation as well (data not shown). Moreover, in contrast to lovastatin, under basal conditions at all times after 3 hours, basal secretion of apoB100 was reduced by −9% to −44% by simvastatin. Cholesterol ester mass was reduced by simvastatin at all times under basal conditions as well (data not shown). Therefore, under basal conditions, as with lovastatin, it takes longer for the effect of simvastatin on apoB100 secretion to be evident compared with the oleate-stimulated cells. Nevertheless, simvastatin was clearly more potent than lovastatin.

In many studies, the effects of various interventions on synthesis and mass of lipids are inferred from radioisotopic data, the masses of both cholesterol and cholesterol ester in the cells and the medium should have decreased substantially, but according to the mass measurements, neither did. Depending, therefore, on which technique is used, apoB100 secretion would either correlate well, or not at all, with cholesterol and cholesterol ester metabolism.

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In many studies, the effects of various interventions on synthesis and mass of lipids are inferred from radioisotopic results. In the experiments just reported, the effects of both the ACAT inhibitor and the HMG-CoA reductase inhibitor on the rates of lipid synthesis as deduced by radioisotopic techniques were also determined. These data are presented in Tables 3 and 4. With respect to the ACAT inhibitor, under basal conditions, there was an apparent decrease of approximately 40% in cholesterol ester synthesis using either meva-
LDL receptors. The same result has been obtained in mice in which LDL receptor activity had been genetically deleted and in Watanabe Hereditable Hyperlipidemic rabbits. There is, therefore, a wide array of in vitro and in vivo observations that establish that statins markedly reduce hepatic apoB100 secretion and that this effect may be a principal therapeutic mechanism of this class of agents.

The present data add to that evidence. Importantly, they also help explain why a number of in vitro studies have not demonstrated this effect. We have shown that the statin effect in vitro depends on the potency and concentration of the statin. Simvastatin reduced apoB100 secretion at all concentrations and under both basal and oleate-stimulated conditions. By contrast, lovastatin reduced apoB100 secretion under basal condition only with more prolonged incubation times or if oleate had been added to the medium.

The data demonstrate that as cholesterol ester mass increased because of increased delivery of fatty acids to the liver, apoB100 secretion increased, and that when cholesterol ester mass was returned to normal by either a statin or an ACAT inhibitor, apoB100 secretion returned to basal levels. On the other hand, under basal conditions, apoB100 secretion was not reduced unless cholesterol ester mass was also. The data demonstrate, therefore, a clear and consistent relationship between cholesterol ester mass and apoB100 secretion.

Different interpretations were reached in previous studies in which changes in cholesterol ester mass were inferred from radioisotopic methods and marked

### Discussion

Statins markedly reduce plasma LDL, and upregulation of hepatic LDL receptors has been widely thought to be principally responsible for this effect. More recent studies, however, point to statins also reducing VLDL secretion with a consequent decrease in LDL production. Both atorvastatin and simvastatin will lower LDL in patients with homozygous familial hypercholesterolemia. HMG-CoA reductase inhibitors will, therefore, reduce LDL levels in the absence of LDL receptors. The same result has been obtained in mice in which LDL receptor activity had been genetically deleted and in Watanabe Hereditable Hyperlipidemic rabbits. There is, therefore, a wide array of in vitro and in vivo observations that establish that statins markedly reduce hepatic apoB100 secretion and that this effect may be a principal therapeutic mechanism of this class of agents.

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### Table 3. Effect of ACAT Inhibitor 58-035 on Intracellular Cholesterol Ester, Cholesterol, and Triglyceride Synthesis as Estimated by Radioisotopic Tracers

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Basal</th>
<th>+0.8 mmol/L Oleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE synthesis</td>
<td>Olate</td>
<td>–44 ± 9%; P&lt;0.0005</td>
</tr>
<tr>
<td></td>
<td>Mevalonate</td>
<td>–39 ± 4%; P&lt;0.0125</td>
</tr>
<tr>
<td>CHOL synthesis</td>
<td>Acetate or mevalonate</td>
<td>6 ± 14%; NS</td>
</tr>
<tr>
<td>TG synthesis</td>
<td>Olate</td>
<td>25 ± 18%; NS</td>
</tr>
</tbody>
</table>

Primary hamster hepatocytes were incubated 24 hours with or without 0.8 mmol/L oleate and 5 μmol/L HMG-CoA reductase inhibitor (Lovastatin). Radiolabeled tracers ([14C]oleate and [3H]acetate or [3H]mevalonate) were added during the incubation period. Incorporation of radiolabeled tracers into lipids was determined as described in Methods to measure cholesterol ester (CE) synthesis, cholesterol (CHOL) synthesis, and triglyceride (TG) synthesis. Results are expressed as percent inhibition of results obtained with versus without inhibitor ± SEM for n=12 dishes in 3 experiments.

### Table 4. Effect of an HMG-CoA Reductase Inhibitor, Lovastatin, on Intracellular Cholesterol Ester, Cholesterol, and Triglyceride Synthesis as Estimated by Radioisotopic Tracers

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Basal</th>
<th>+0.8 mmol/L Oleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE synthesis</td>
<td>Olate</td>
<td>–28 ± 16%; P&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Mevalonate</td>
<td>–76 ± 5%; P&lt;0.0025</td>
</tr>
<tr>
<td>CHOL synthesis</td>
<td>Acetate or mevalonate</td>
<td>–67 ± 1%; P&lt;0.0025</td>
</tr>
<tr>
<td>TG synthesis</td>
<td>Olate</td>
<td>–2 ± 13%; NS</td>
</tr>
</tbody>
</table>

Primary hamster hepatocytes were incubated overnight with or without 0.8 mmol/L oleate ± 5 μmol/L HMG-CoA reductase inhibitor (Lovastatin). Radiolabeled tracers ([14C]oleate and [3H]acetate or [3H]mevalonate) were added during the incubation period. Incorporation of radiolabeled tracers into lipids was determined as described in Methods to measure CE, CHOL, and TG synthesis. Results are expressed as percent inhibition of results obtained with versus without inhibitor ± SEM for n=12 dishes in 3 experiments.
Table 5. Effect of Lovastatin on 125I-LDL Cell Association and Degradation

<table>
<thead>
<tr>
<th></th>
<th>125I-LDL Cell Associated (ng/mg cell protein)</th>
<th>125I-LDL Degraded (ng/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (basal)</td>
<td>304±16</td>
<td>2203±98</td>
</tr>
<tr>
<td>+ Lovastatin</td>
<td>392±19</td>
<td>1868±148</td>
</tr>
<tr>
<td>Fatty Acid</td>
<td>362±28</td>
<td>2871±80</td>
</tr>
<tr>
<td>+ Lovastatin</td>
<td>327±7</td>
<td>1867±63</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td>P&lt;0.02</td>
</tr>
</tbody>
</table>

Concentrations used are as follows: 125I-LDL: 7.7 μg/mL; oleate: BSA, 0.8 mmol/L; and lovastatin, 5 μmol/L. 125I-LDL cell association and degradation was measured over 24 hours at 37°C. Results are expressed as mean±SEM for n=6 dishes. Groups were compared by 1-way ANOVA, where * indicates P<0.05 for differences within groups.

Effects on apoB100 secretion were noted with very short incubation times with oleate.\(^4\)\(^-\)\(^8\) In this study, we have demonstrated that inferences from radioisotopic studies were not quantitatively valid with respect to changes in mass. These discrepancies likely arise for 2 principal reasons. The first is caused by unrecognized dilution of isotope in precursor pools, which are not estimated in these types of experiments. Second, as demonstrated in this study, it requires time to deplete the mass of cholesterol ester in the hepatocyte, and until this occurs, no change in apoB100 secretion would be anticipated. It is critical to note that, with one exception,\(^8\) all the negative studies relating to the relationship of sterol balance and apoB100 secretion showed that addition of either an HMG-CoA reductase inhibitor or an ACAT inhibitor to the medium reduced the rate of synthesis of cholesterol or cholesterol ester as estimated by radioisotopic studies in the hepatocyte, but, over the short time of the experiment, did not change the mass of cholesterol ester within it,\(^4\)\(^-\)\(^7\) as was indeed specifically noted in two of these studies.\(^5\)\(^-\)\(^6\) The data, therefore, of the previous negative studies and the present positive study are in accord.

The mass of cholesterol ester available to couple with apoB100 appears important in determining the proportion of newly synthesized apoB100 molecules that are degraded as opposed to the proportion that are secreted. Increasing the delivery of cholesterol to the liver via LDL has been shown by Tanaka et al.\(^10\) to increase the secretion efficiency of apoB100. By contrast, addition of atorvastatin has recently been shown to reduce secretion efficiency by impacting on the translocation of newly synthesized apoB100 molecules.\(^31\)

It is possible that the immediate effects of oleate on apoB100 secretion that have been reported\(^5\)\(^-\)\(^6\)\(^,\)\(^8\) may be produced by an entirely different mechanism than the longer term effects, which we suggest are caused by cholesterol ester. In the first instance, the onset and arrest of the early oleate effect is so rapid, within 30 minutes for both, for example, that no change in cholesterol ester mass is possible in this time frame. Second, the longer term effects of oleate on apoB100 secretion are much more modest than the shorter term ones but in line with differences from normal in humans with increased secretion of hepatic apoB100 lipoproteins.\(^3\) By contrast, short-term increases, which range from 3- to 18-fold, in apoB100 secretion have been noted in vitro studies,\(^6\)\(^-\)\(^8\) a value much beyond any we have noted in 24- to 48-hour incubations. By the same token, the degree of reduction of apoB100 secretion with administration of statins documented in this and other in vitro studies corresponds closely to the degree of reduction of LDL observed clinically.\(^3\)

Notwithstanding their potent effect on apoB100 secretion, clinically statins produce a more modest reduction in plasma triglyceride than they do on plasma apoB100 or total or LDL cholesterol. The observation in this study that addition of the statin to the medium resulted in the secretion of fewer apoB100 particles, which were, however, enriched in triglyceride, may explain this apparent paradox and is yet another example that hepatic triglyceride secretion and apoB100 secretion are not synonymous. Certainly, the tight linkage between increased delivery of fatty acids to the liver and increased synthesis of cholesterol and cholesterol ester within it provides a clear physiological rationale for the use of statins in the many patients with overproduction of hepatic apoB100 lipoproteins caused by increased delivery of fatty acids to the liver. In this regard, the demonstration of a highly significant correlation between cholesterol synthesis, as reflected by plasma mevalonic acid, and VLDL apoB100 secretion in both normals\(^32\)\(^,\)\(^33\) and patients with secondary hypertriglyceridemia\(^34\)\(^,\)\(^35\) is, we believe, strongly supportive of the present results.

Finally, the present data are difficult to reconcile with the model that the LDL pathway is the only important determinant of the concentration of LDL in plasma, and indeed they are difficult to reconcile with the generally accepted model of regulation of sterol balance within the hepatocyte. In this instance, sterol balance was perturbed by increasing fatty acid delivery to the liver. A positive effect of fatty acids on cholesterol biosynthesis was previously observed by Goh and Heimberg\(^16\) and related to an increase in HMG-CoA reductase activity.\(^37\) In this study, the effect was dramatic, with addition of oleate to the medium causing the total cholesterol

![Figure 6. Effect of lovastatin on bile acid production in hamster hepatocytes. Hepatocytes were incubated under basal conditions or with 0.8 mmol/L oleate supplemented with lovastatin (L; 5 μmol/L). Medium and cells were collected at the indicated times, and bile acids were measured in both cells and medium. Results are expressed as mean±SEM nanomoles total (medium plus cells) bile acids (cholate plus deoxycholate) per milligram cell protein for n=6 to 9 dishes in 3 experiments where * indicates P<0.025 vs basal; #, P<0.025 vs oleate supplementation.](http://atvb.ahajournals.org/)

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mass to double by increasing cholesterol synthesis. However, in the face of this marked increase in cholesterol synthesis, the activity of the LDL pathway was not downregulated as would be predicted by the LDL receptor paradigm.38

The explanation for this divergence would appear to be as follows: Increased delivery of fatty acids to the liver results in increased formation of cholesterol ester via the ACAT reaction, which leads in turn to increased secretion of cholesterol and cholesterol ester from the cell. To maintain the free cholesterol content of the cell, cholesterol synthesis must, therefore, increase. But the increased cholesterol synthesis, in effect, only reconstitutes the free cholesterol content of the hepatocyte. Thus, in the hepatocyte, in contrast to the fibroblast, an increase in cholesterol ester mass within the cell and an increase in cholesterol ester and free cholesterol mass secreted from the cell serve as safety valves to maintain hepatocyte membrane free cholesterol content within the narrow limits necessary for normal cell function without the automatic necessity for downregulation of the activity of the LDL pathway.

In our study, cholesterol flux through the hepatocyte was increased by increasing synthesis, but other investigators have shown increased cholesterol flux by increasing cholesterol delivery. Tanaka et al30 as well as Havekes and colleagues have shown that increased uptake of LDL by either rabbit hepatocytes or HepG2 cells, respectively, also do not alter LDL receptor activity. Cholesterol ester mass increases as does apoB100 secretion without free cholesterol being affected. Thus, once again, the total flux of cholesterol through the hepatocyte increased without any expansion of the mass of free cholesterol within it.

On the other hand, Havekes et al showed that addition of both LDL and an ACAT inhibitor to the incubation medium resulted in an increase in free cholesterol and a substantial decrease in LDL receptor activity in HepG2 cells. Taken together, we suggest the data are consistent with the hypothesis that apoB100 secretion is related to the cholesterol ester mass in the hepatocyte whereas LDL receptor activity is related to the free cholesterol content in the regulatory pool. Because cholesterol ester mass and apoB100 secretion are so tightly linked in the hepatocyte, increased cholesterol synthesis or increased cholesterol delivery will not automatically result in downregulation of the LDL pathway so long as the mass of free cholesterol within the hepatocyte remains constant. These experimental findings point, therefore, to a hierarchy of adaptations to altered sterol balance by the liver with, in general, alterations in secretion occurring before changes in clearance. If valid, they suggest that the LDL receptor paradigm, which was developed in the fibroblast and represents a sample model of static cellular sterol equilibrium, needs to be reinterpreted in the context of the hepatocyte, a complex model of dynamic sterol equilibrium.

Acknowledgments

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References


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