Abstract

There is evidence that the overproduction of apoB-100-containing lipoproteins by the liver is the underlying event in some forms of dyslipoproteinemia. This metabolic status is associated to an increased risk of developing premature coronary artery disease CAD. The conclusions from previous studies suggested that the availability to the hepatocytes of cholesterol that is readily esterified is an important determinant for VLDL and LDL secretion. In the present study, we set out to investigate the effect of the specific stimulation and inhibition of the rate-limiting enzyme of the cholesterol esterification, acyl-CoA:cholesterol acyltransferase (ACAT, E.C. 2.3.1.26), on the lipid and on the apoB-100 secretion rate from a human hepatoma cell line (HepG2). When the specific ACAT inhibitor FCE 27677 (10⁵ M) was added to the cultures, a decrease of the cellular cholesteryl ester content and at the same time a significant reduction of the neutral lipids and of the apoB-100 secretion rate were noticed. The stimulation of ACAT by 25-hydroxycholesterol (20 μg/ml) caused a 4-fold increase of the cellular cholesteryl ester content and a 2-fold increase of the lipoprotein secretion rate. FCE 27677 (10⁵ M to 10⁷ M) prevented the effects elicited by the oxysterol. On the contrary, lovastatin (10⁶ M) and gemfibrozil (10⁵ M) had no effect. The analysis of the lipid and of the apolipoprotein composition of the lipoproteins secreted in the medium revealed that ACAT inhibition had the dual effect of both decreasing the number of apoB-100-containing lipoproteins secreted as well as their cholesteryl ester load. Altogether, these data support the idea of a close relationship between ACAT activation, leading to increased cholesteryl ester availability, and apoB-100-containing lipoprotein secretion. It is speculated that ACAT inhibitors may prove useful for the treatment of human dyslipoproteinemias caused by the hepatic overproduction of apoB-100-containing lipoproteins.―Musanti, R., L. Giorgini, P. Lovisolo, A. Pirillo, A. Chiari, and G. Ghiselli. Inhibition of acyl-CoA:cholesterol acyltransferase decreases apolipoprotein B-100-containing lipoprotein secretion from HepG2 cells. J. Lipid Res. 1996: 37: 1-14.

Supplementary key words hepatic cells• ACAT• ACAT inhibitors• lipid synthesis• cholesteryl esters• apolipoprotein B-100• dyslipoproteinemia

Whereas much is known about the plasma metabolism of apolipoprotein B-100 (apoB-100), the mechanisms involved in the regulation of its synthesis are incompletely understood (1). ApoB-100 is the only form of apoB that is synthesized by the liver (2) and is an essential structural component of VLDL and LDL as it is required for the intracellular assembly and the secretion of these lipoproteins (3). The elevation of the concentration of LDL cholesterol as well as of apoB-100 is regarded as an important risk factor for coronary artery disease (CAD) (4), therefore the factors that control the rate of secretion of apoB-100-containing lipoproteins by the liver are of considerable importance.

The intracellular assembly of the apoB-100-containing lipoproteins proceeds through a series of steps compartmentalized at specific intracellular sites (5). Soon after translation, the nascent apolipoprotein is combined with phospholipids and becomes enriched with triglycerides and cholesteryl esters as it moves across the endoplasmic reticulum (6). Subsequently, the bulk of phospholipids and cholesterol is added at the Golgi apparatus (7). At the same time, apoB-100 undergoes a number of modifications including proteolytic

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase (E.C. 2.3.1.26); VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-y1)2,5-diphenyltetrazoiium bromide; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid.

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cleavage (8), glycosylation (9), and fatty acid acylation (10, 11) which appears necessary for the intracellular trafficking and the secretion of the nascent lipoprotein. At least under certain circumstances, the lipid supply appears to be the major determinant for the apolipoprotein synthesis and secretion. In this regard there is evidence that lipoprotein cholesterol may be of particular importance in promoting the synthesis of the apoB-100-containing lipoproteins. In fact, unlike fatty acids, whose flux to the liver mainly affects the lipoprotein triglyceride output (12-19), the availability to the hepatocyte of lipoprotein cholesterol, either LDL, beta-VLDL, or chylomicron remnants (20-23), has been shown to enhance the secretion of VLDL and LDL particles. Interestingly, this stimulating effect is not mimicked by non-lipoprotein cholesterol (23), apparently because it is not as good a substrate for esterification as the lipoprotein cholesterol internalized via a receptor-mediated mechanism (21, 23). In support of this idea, maneuvers to increase the cholesteryl ester content of HepG2 cells lead to stimulation of the d < 1.063 g/ml lipoprotein secretion (23, 24). Furthermore, in humans, increased apoB-100 production has been documented in patients with cholesteryl ester storage disease (25) and in those with betasitosterolemia (26). However, an unequivocal demonstration that cholesterol esterification rate in the hepatocyte directs the secretion of the apoB-100-containing lipoprotein has not yet been obtained. In this study, the effect of the stimulation and of the specific inhibition of acyl-CoA:cholesterol acyltransferase (E.C. 2.3.1.26, ACAT), the rate-limiting enzyme of cholesterol esterification, on the lipids and on the apoB-100 secretion rate by a human hepatoma cell line (HepG2) has been investigated. Results are consistent with the view that the activity of ACAT is a major determinant for the hepatic lipoprotein production.

MATERIALS AND METHODS

Materials

HepG2 human hepatoma cells were obtained from the ATCC cell repository (cat. no. HB 8065). Media, fetal calf serum, trypsin, and Na-pyruvate were purchased from Gibco. Inorganic salts were from Carlo Erba reagents. Gentamycin, PMSF, aprotonin, MT, SDS, and Trypan Blue were from Sigma. The calibrated BSA standard and a Lowry-based protein assay kit were obtained from Bio-Rad. Radiolabeled lipid precursors were from Amersham.

Cells

Cells were grown in Dulbecco's MEM supplemented with 1 mM sodium pyruvate (DMEM) and 10% FCS, in an humidified 5% CO₂ incubator at 37°C. The medium was routinely changed every 48 h. The cells were split at 1:3 ratio every 4 days when they had reached 80% confluence. For the experiments, cells were seeded into 35-mm tissue culture dishes or, alternatively, into 225-ml flasks, and used when they reached 70-80% confluence.

Cell cytotoxicity

Cells in 35-mm dishes were washed twice with DMEM and placed in DMEM alone or in DMEM supplemented with 0.8 mM oleate bound to 2% BSA (BSA-oleate) prepared according to the method of van Harken, Dixon, and Heimberg (27). The drugs were added dissolved in ethanol and each well received 20 µl of the solution. Control wells received 20 µl of ethanol alone. After 24 h of incubation the cells were washed with phosphate-buffered saline, pH 7.4 (PBS) and trypsinized. After centrifugation the cells were resuspended in PBS and their viability was assessed by the Trypan blue (0.4%) dye exclusion method (28). Alternatively, the cells that had been incubated with the drugs were washed with PBS and incubated 4 h in DMEM containing 0.5 mg/ml MIT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The formazan dye formed was solubilized in a 10% SDS solution in 10 mM HCl, and the color was read at 550/690 nm (29). For the protein determination, cells that had been incubated 24 h with the drugs were washed in PBS and digested in 1 ml of NaOH 0.1 N for 2 h at room temperature. Proteins were quantified using a Lowry's assay kit.

In order to assess the effect of the drugs on the protein synthetic activity, the cells were pre-incubated 24 h in DMEM with or without BSA-oleate together with the compounds. The endogenous labeling of cell protein was performed by incubating the cells with methionine-deficient DMEM with or without BSA-oleate and with 10µCi/ml of [35S] methionine for 2 h. At the end of the incubation, the cells were washed with PBS, scraped off, recovered by centrifugation at 800 g
for 10 min, and resuspended in 100 µl of PBS. An aliquot of the cell suspension was used for the cell count on a Coulter counter Model ZM. After proper dilution to achieve the same cell number concentration in all the samples, an aliquot was processed in SDS electrophoresis sample buffer and subjected to electrophoresis on a 10% PAGE-SDS slab gel. The autoradiography of the radiolabeled proteins was performed by exposing the dried gel to a Kodak XAR2 film for 48 h. The relative intensity of the bands appearing on the film was read on a Shimadzu CS-9001PC TLC scanner set on transmission at 300 nm with automatic integration of the peaks.

In another set of experiments, after the incubation with the labeled precursor, the cells were homogenized by three freeze–thawing cycles and mixed with 9 volumes of 5% BSA solution. The proteins were precipitated in 12% TCA and, after careful washing of the pellet, the protein-associated radioactivity was read in a beta-counter using the precipitate from samples prepared with fresh medium containing 10 µCi of [35S]methionine to correct for the nonspecific associated radioactivity. The cell medium that had been set apart at the end of the incubation with the labeled precursor was instead mixed with gentamycin sulfate (0.005%), PMSF (1 mM), and aprotinin (200 kallikrein-inactivating units/ml), placed in dialysis tubing (cutoff 3,000 Da) and dialyzed for 24 h against 0.08% NaHCO3, 1 mM Tris-HCl, pH 7.4, buffer containing gentamycin and PMSF, with frequent change of the dialyzate until the counts of the radioactivity of the control samples had reached background levels. Half of each sample was lyophilized and the recovered material was subjected to electrophoresis over a 10% PAGE-SDS slab gel. The gels were processed for the autoradiography as described for the cell extracts except that the Kodak films were exposed for 14 days. The rest of the medium was mixed with 9 volumes of 5% BSA solution and the proteins were precipitated in 12% TCA and counted.

### Cellular lipid biosynthesis

Cells in 35-mm dishes were prepared by washing two times with DMEM. Each dish then received 2 ml DMEM supplemented with or without BSA-oleate plus the indicated concentration of the drugs in 20 µl of ethanol. Controls received ethanol alone. The labeled precursors (2 µCi/ml of [14C]acetate or 0.3 µCi/ml of [14C]oleate complexed with BSA, see ref. 27) were added 18 h later and after an additional 6 h of incubation the incubations were terminated. The cells were washed twice with PBS and the lipids were extracted in situ with 2 ml of hexane–isopropanol 3:2 (v/v) for 30 min at room tempera-

**Fig. 2.** Autoradiograph of [35S]methionine-labeled proteins from HepG2 cells (panel A), and the cell media (panel B). The cells were incubated 24 h in DMEM with or without 0.8 mM oleate/2% BSA in the presence of different concentrations and combinations of the drugs. The medium was then removed and the cells received 10 µCi/ml of [35S]methionine in methionine-deficient DMEM. After 2 h incubation, the cells were washed, recovered by scraping, dissolved in lysis buffer (1.25% SDS, 0.125% beta-mercaptoethanol, 1 mM PMSF in 0.125 M Tris-HCl buffer, pH 6.8), and subjected to electrophoresis over a 10% PAGE-SDS slab gel. In another set of experiments, the cell medium was dialyzed, lyophilized, and the resulting material was dissolved in the lysis buffer and applied to the slab gel. The autoradiography was performed by exposing the dried gel to a Kodak XAR2 film. The letters identify the following conditions: a through d, cells incubated in DMEM without BSA-oleate complex; e through h, cells incubated with BSA-oleate complex; a and e, FCE 27677 10^{-6} M; b and f, FCE 27677 10^{-6} M; c and g, FCE 27677 10^{-7} M; d and h, FCE 27677 10^{-9} M and 25-hydroxycholesterol 20 µg/ml.

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Fig. 3. Incorporation of $^{14}$C-oleate into cholesteryl esters (●), triglycerides (△), and phospholipids (○) after incubation of HepG2 cells with different concentrations of FCE 27677. Results were calculated as the percent of the incorporation values in untreated cells. Each points represent mean ± SD from four separate experiments with duplicate samples.

ture (30). The extract was collected by aspiration, evaporated under N$_2$, and the lipids were redissolved in hexane. Phospholipids, cholesterol, triglycerides, and cholesteryl esters were separated by thin-layer chromatography using precoated Silica Gel 60 F254 plastic sheets from Merck. The developing system used was composed of heptane–ethyl ether–acetic acid 90:30:1 (v/v/v). The lipids were visualized with 8% phosphomolybdic acid. The bands having a migration $R_f$ as that of the pure standards were cut out and counted. The cell debris that remained attached to the dishes after the lipid extraction was digested in 1 ml of 0.1 M NaOH and the cell proteins were measured as described.

**Lipoprotein and albumin secretion**

Cells were grown in 225-cm$^2$ flasks. For the experiments, the cells were washed twice with PBS and received 15 ml of DMEM with or without BSA-oleate and the drugs dissolved in 150 μl of ethanol. Controls received ethanol alone. The incubations were terminated after 24 h. The medium was set apart for the separation of the lipoproteins, whereas the cell monolayers were washed twice with PBS and the cell lipids were extracted with hexane–isopropanol as described. The quantitation of the neutral lipid content of the samples was performed by high performance thin-layer chromatography (HPTLC) (31). The cell lipids were redissolved in hexane, applied onto a silica HPTLC plate, and separated by developing with hexane–heptane–diethyl ether–acetic acid 63:18.5:18.5:1 (v/v/v/v). At the end of the chromatographic run the HPTLC plates were dried at 110°C for 10 min, rinsed in 16 mM MnCl$_2$ solution in methanol–water–sulfuric acid 48:48:4 (v/v/v), and then placed at 110°C for 30 min. The color intensity of the lipid spots was read on the TLC scanner set on reflectance at 370 nm with automatic integration of the peaks. For the standards, dilutions of 99.9% pure lipids were applied to the HPTLC plates in a volume of 5 μl containing 200–3200 pg of tripalmitin, and 50–800 pg of cholesterol or of cholesteryl oleate.

The cell-conditioned medium was processed for the isolation of the lipoproteins. Part of the medium from the incubation performed in the absence of BSA-oleate was set apart for the quantitation of the albumin secreted. To prevent degradation, the preservatives gentamycin sulfate (0.005%) and PMSF (1 mM) were immediately added. After a centrifugation at 2000 rpm for 15 min at 4°C to remove small amounts of cells and debris, the lipoproteins were separated by ultracentrifugation on a Beckman 50.2 Ti rotor. All the density solutions used to adjust the flotation density of the lipoproteins (VLDL, d < 1.006 g/ml; LDL, d 1.019–1.063 g/ml; HDL, d 1.063–1.210 g/ml, or of the whole lipoprotein fraction, d < 1.210 g/ml) contained 1 mM Na$_2$EDTA. After the isolation, the lipoproteins were dialyzed overnight against PBS and concentrated by ultrafiltration on an Omegacell apparatus fitted with a filter with a cutoff
point of 8,000 Daltons. The concentrated lipoproteins were sterile-filtered, stored at -20°C, and analyzed within 48 h.

The agarose electrophoresis of the lipoproteins was performed on Paragon Lipo-Gel strips from Beckman using the Beckman electrophoretic apparatus and following the manufacturer’s direction for the electrophoresis and the lipid staining. Freshly isolated VLDL, LDL, and HDL from a normolipidemic human serum served as standard for the migration of the lipoproteins in the pre-beta, beta, and alpha positions.

The analysis of the lipoprotein apolipoprotein content was carried out by SDS-PAGE. Coomassie Blue G 250-stained apolipoprotein bands were identified based on their apparent molecular weight using molecular weight standards from Pharmacia or, alternatively, using purified human apoA-I, apoA-IV, and apoE.

An aliquot of the concentrated lipoprotein fraction was used for the quantitation of apoB-100 using a commercially available RIA kit from Pharmacia. For the calculations of the absolute apoB-100 mass in each sample, the standards used were those provided in lyophilized form by the manufacturer and purified human plasma LDL. The latter had been isolated by ultracentrifugation (d 1.02–1.05 g/ml) from pooled plasma from three normolipidemic healthy volunteers. This preparation gave a single beta-migrating band on agarose gel electrophoresis. Contamination by VLDL and HDL was excluded as these lipoprotein apolipoproteins were not evidenced by SDS-PAGE of the delipidated LDL preparation. In purified LDL, the ratio of the apoB-100 content assayed by RIA using the Pharmacia’s reference standard to the protein content measured by the Lowry method was 1.12 ± 0.06 (mean ± SD, n = 4). The sample apoB-100 concentrations determined by RIA were not corrected for this small discrepancy. The interassay CV of the assay was less than 8%, and the intraassay CV was less than 6%.

The lipoprotein neutral lipid masses were quantified by HPTLC as described for the cell lipids, except that the lipoprotein lipids were extracted according to Kates (32).

For the quantitation of the albumin secretion rate, the medium was concentrated by ultrafiltration and an aliquot was used to assess the concentration of albumin using a Pharmacia Human Albumin Kit and the kit’s own reference standard for the calculations. The interassay CV of the assay was less than 5%, and the intraassay CV was less than 3%.

**Statistical analysis**

The significance of the difference between the means of the values was assessed using the Student’s t-test.

**RESULTS**

**Pharmacology and cytotoxicity of the compounds**

FCE 27677 (N,N′-[2,6-bis(1-methylethyl)phenyl]-N,N′-[4R,5R]-2-(4-dimethylaminophenyl)-4,5-dimethyldioxo...
lan-2-yI]methylurea hydrochloride) is a novel potent ACAT inhibitor belonging to the class of the ketalic disubstituted ureas (see Fig. 1 for the chemical structural formula) that has been synthesized at Farmitalia Carlo Erba as part of a program of discovery and development of bioavailable ACAT inhibitors (33). The compound inhibited the microsomal enzyme from rabbit intestine, rabbit aorta, and monkey liver with IC50 of, respectively, 9.31 nM, 6.99 nM, and 71.7 nM. The IC50 for the inhibition of cholesterol esterification in macrophages (J774-A1 cells) stimulated by acetylated LDL was 30.2 nM. When administered to rats fed a 1% cholesterol-enriched diet, the drug prevented the increase of liver ACAT, FCE 27677 inhibited the enzyme with kinetic constants consistent with a non-competitive and reversible mechanism. FCE 27677 up to 10^4 M did not have a significant effect on the cytosolic cholesteryl esterase from rabbit intestine or on the plasmatic lecithin:cholesterol acyltransferase activity.

To exclude possible toxic effects of the ACAT inhibitor and of 25-hydroxycholesterol at the concentrations used in the experiments, the cytotoxicity of the compounds was carefully evaluated. The two agents were added alone or in combination up to concentrations of 10^5 M and 50 µg/ml, respectively. The experiments were performed either in the presence or the absence of the BSA-oleate complex. Whether or not the medium had been supplemented with the complexed fatty acid, the cytotoxicity of the compounds had the same threshold value. By the Trypan blue dye exclusion method, no statistical difference in the viability was found between the controls and the cells incubated with FCE 27677 up to 10^5 M. 25-Hydroxycholesterol alone or together with FCE 27677 up to 10^5 M did not affect the cell viability when tested up to a concentration of 50 µg/ml. However, when the cells were incubated with the oxysterol at 50 µg/ml alone or together with FCE 27677 at 10^5 M, the cell viability began to decrease (by 9% and 25%, respectively, both P < 0.05 vs. controls). When the cytotoxic effect of the drugs was assessed by monitoring the cell respiratory function by the MTT method, the decrease of the index was compounded at 5% (NS) when the highest concentration of the ACAT inhibitor was added, and at 8% (NS) when the oxysterol (50 µg/ml) was also present in the incubation. Finally, a change of the cell morphology (assessed by scores) and a decrease of the cell attachment to the plastic (assessed by measuring the protein content of the adhering cells) began to appear only when FCE 27677 and the oxysterol were added together at the highest tested concentration. In this case also the changes did not reach statistical significance.

To investigate whether the compounds had deleterious effects on the cell metabolism, their effect on the cell protein synthesis was evaluated by performing a series of [35S]methionine protein incorporation studies. The pattern of the radioactivity incorporation in the cell proteins and in the proteins secreted in the medium was assessed both by autoradiography of the proteins separated by SDS-PAGE as well as by measuring the radioactivity recovered in the TCA precipitate of the cells and the medium extracts. Representative autoradiograms from these experiments are illustrated in Fig. 2. The densitometric scanning of the autoradiographic films did not reveal differences in the relative intensity of the protein-associated radioactivity in any of the experimental conditions tested. The TCA precipitation studies further confirmed that [35S]methionine incorporation

TABLE 1. Effect of FCE 27677 on the cellular neutral lipid content and on the neutral lipid and apoB-100 secretion rates from HepG2 cells

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<td></td>
<td>Triglycerides</td>
<td>Cholesterol</td>
</tr>
<tr>
<td></td>
<td>µg/mg cell protein</td>
<td>µg/mg cell protein/24 h</td>
</tr>
<tr>
<td>Control</td>
<td>35.6 ± 8.6</td>
<td>14.6 ± 5.7</td>
</tr>
<tr>
<td>FCE27677</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
<td>42.2 ± 12.5</td>
<td>17.2 ± 5.5</td>
</tr>
<tr>
<td>1 µM</td>
<td>38.6 ± 6.9</td>
<td>15.3 ± 6.6</td>
</tr>
<tr>
<td>0.1 µM</td>
<td>40.8 ± 13.6</td>
<td>15.1 ± 6.3</td>
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</table>

HepG2 cells were incubated for 24 h in DMEM containing 0.8% oleate/2% BSA and with FCE 27677 at the indicated concentrations. At the end of the incubation, the cells and the medium lipids were extracted and quantitated by HPTLC. ApoB-100 concentration in the medium was assessed by RIA on concentrated medium samples. Values are given as mean ± SD from three experiments with duplicate samples.

*P < 0.05 versus control.
into proteins had not been affected by the drug treatments. The TCA-precipitable radioactivity from cells incubated in the presence or the absence of BSA-oleate averaged (n = 3) 215,234 ± 13,345 and 254,112 ± 24,002 cpm/mg cell protein, respectively. That from cells treated similarly except for the addition of FCE 27677 to concentrations of 25-hydroxycholesterol, did not differ by more than 8% (NS) from these values. Similar results were obtained when the TCA-precipitable radioactivity from the medium was examined. The medium TCA-precipitable radioactivity from control cells incubated with BSA-oleate and without were, respectively, 30,280 ± 4,999 and 42,020 ± 4,045 cpm/mg cell protein and the values from the drug-treated cells did not differ by more than 15% (NS) from these control values. Further support for the conclusion that the drugs did not affect the hepatocyte protein synthesis in an aspecific fashion came from the results in which their effect on the HepG2 secretory rate of albumin was investigated (see below).

**Effect on cell lipid biosynthesis**

Either labeled acetate or oleate was used as precursor to study the effect of FCE 27677 on the HepG2 lipid biosynthetic rate. Concentrations of the compound ranging between 10⁻⁸ and 10⁻⁵ M, were tested. In view of the protocol to be later adopted for the lipoprotein secretion experiments, the studies concerning the effects of the various agents on the lipid biosynthetic activity of HepG2 were carried out with incubations lasting 24 h. In order to minimize the metabolic recycling of the precursors, the labeled substrates were added during the last 6 h of incubation.

The results of the effect of the addition of different concentrations of FCE 27677 on the lipid biosynthesis from [¹⁴C]oleate are presented in Fig. 3. A significant reduction in the incorporation of the labeled precursor into cholesteryl esters occurred at drug concentrations as low as 10⁻⁶ M (23% reduction of the cholesterol esterification activity). The maximal inhibitory effect, in excess of 90%, was achieved with the drug at 10⁻⁷ M or at higher concentrations. Conversely, the incorporation of [¹⁴C]oleate into the phospholipid and the triglyceride fractions was minimally affected by the addition of the drugs to the cells. Similar results were obtained when [¹⁴C]acetate was used as precursor (Fig. 4). Again, whereas the incorporation of the precursor into cholesteryl esters was significantly affected by FCE 27677, the precursor incorporation into triglycerides and phospholipids was negligibly affected. The synthesis of cholesterol was suppressed by 47% when the enzyme inhibitor was added to the cells at 10⁻⁵ M.

**Effect on lipoprotein and albumin secretion**

Both in the presence or the absence of oleate during the incubation, the accumulation of lipoproteins in the medium was found to proceed at a constant rate up to 48 h (data not shown). There is consensus in the literature that reuptake by the HepG2 cells of the newly secreted lipoproteins is very small if present at all (20, 22–24). As our incubation conditions do not differ substantially from those adopted by these other authors, the assumption is also made in this study that the concentration of the lipoproteins present in the medium at the end of the incubation period solely reflects the secretory rate. The amount of apoB-100 secreted by HepG2 cells in our study is comparable to that reported

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**TABLE 2. Effect of FCE 27677 on the 25-hydroxycholesterol-mediated cell accumulation of cholesteryl esters and on the neutral lipids and apoB-100 secretion rates from HepG2 cells**

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<td>µg/mg cell protein</td>
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</tr>
<tr>
<td>Control</td>
<td>36.8 ± 4.1</td>
<td>15.4 ± 8.3</td>
</tr>
<tr>
<td>FCE 27677 (10⁻⁵ M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µg/ml + 20 µM</td>
<td>34.4 ± 3.1</td>
<td>12.8 ± 6.7</td>
</tr>
<tr>
<td>25-OH-cholesterol</td>
<td></td>
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<tr>
<td>20 µg/ml + 0.1 µM</td>
<td>37.8 ± 0.3</td>
<td>13.4 ± 5.2</td>
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</table>

HepG2 cells were incubated for 24 h in DMEM containing 0.8% oleate/2% BSA and with 25-hydroxycholesterol alone or together with FCE 27677 at the indicated concentrations. At the end of the incubation, the cells and the medium lipids were extracted and quantitated by HPTLC. ApoB-100 concentration in the medium was assessed by RIA on concentrated medium samples. Values are given as the mean ± SD from three experiments each with duplicate samples.

*P < 0.05 versus controls.

*P < 0.05 versus 25-hydroxycholesterol only treated cells.
for rat hepatocytes (34), and by others (18, 23, 24, 35-37) for HepG2 cells. In the absence of oleate it averaged 1.68 µg/mg cell protein per 24 h, whereas in the presence of oleate the apoB-100 production averaged 2.34 µg/mg cell protein per 24 h. The secretion rates of triglycerides, cholesterol, and cholesteryl esters also compare favorably with those already reported (15, 18, 23, 24, 35-37) for HepG2 cells. In the absence of oleate it by 31%. At presence of oleate are presented in lipoprotein secretion by HepG2 cells incubated in the absence of oleate it averaged 1.68 pg/mg cell protein per 24 h, whereas in the presence of oleate the apoB-100 production averaged 2.34 pg/mg cell protein per 24 h. The secretion of triglycerides, cholesterol, and cholesteryl esters was higher associated cholesterol. The effect on the secretion of cholesteryl esters was higher (43%) and reached significance (P < 0.05). ApoB-100 secretion rate was decreased by 31%. At 10^6 M and at 10^7 M, FCE 27677 lost its effect on the HepG2 secretion of cholesterol, yet an inhibitory effect on the cholesteryl esters and apoB-100 output could still be evidenced. Concomitant with the changes in lipoprotein secretion rate, FCE 27677 at 10^5 M caused a 17% decrease of the cellular cholesteryl ester content (P < 0.05). Lower concentrations of the drug appeared to be ineffective in this regard. Unlike that of the sterols, the triglyceride secretion rate was not significantly affected by the drug at any of the concentrations tested.

Another series of experiments in the presence of oleate was carried out with the purpose of investigating the effect of ACAT inhibition on cells in which the enzyme had been activated by 25-hydroxycholesterol (20 µg/ml) (40). Compared to the control cells, those that had been exposed to the oxysterol had a 4-fold increase of the cellular cholesteryl ester content (see Table 2). By contrast, the intracellular cholesterol and triglyceride concentrations were only modestly affected by the treatment with the oxysterol. Concomitant to the enhancement in the cholesterol esterification rate, both the cell output of cholesteryl esters and of apoB-100 increased markedly. FCE 27677 at 10^7 M to 10^5 M counteracted the effect of the oxysterol. The drug prevented the increase of the cholesteryl ester cellular content over that of the controls at all the concentrations tested. At the same time, the lipoprotein lipid output in the medium was either drastically reduced or normalized. In particular, compared to the values of the control cells, when the drug was added at 10^5 M, the secretion rate of the lipids was decreased by 68% and that of apoB-100 by 89%.

The effect of gemfibrozil and lovastatin, two drugs acting on lipid metabolism through mechanisms different than an ACAT inhibitor, was also evaluated for

**TABLE 3. Effect of various agents on the 25-hydroxycholesterol-mediated cell accumulation of cholesteryl esters and on neutral lipid and apoB-100 secretion rates from HepG2 cells**

| Intracellular Lipozids | Secretion Rates in Medium | | |
|------------------------|----------------------------|----------------------------|
|                        | µg/mg cell protein | µg/mg cell protein/24 h | µg/mg cell protein | µg/mg cell protein/24 h |
| Control                | 40.7 ± 4.5          | 14.5 ± 0.5               | 7.4 ± 1.2         | 2.32 ± 0.56          | 1.13 ± 0.23               | 0.50 ± 0.12               | 2.65 ± 0.60               |
| 25-OH-cholesterol      | 30.3 ± 3.3^a        | 9.6 ± 0.5^a              | 16.2 ± 2.2^a      | 3.95 ± 1.22^a        | 0.90 ± 0.11^a            | 1.48 ± 0.22^a            | 5.94 ± 1.28^a            |
| 20 µg/ml               | FCE 27677 10 µm     | 40.8 ± 5.4               | 13.6 ± 1.5        | 4.0 ± 0.6^a          | 1.17 ± 0.20^a           | 0.79 ± 0.11^a            | 0.20 ± 0.03^a            | 1.30 ± 0.25^a            |
| 25-OH-cholesterol + FCE 27677 20 µg/ml + 1 µm | 39.5 ± 6.0^a        | 11.8 ± 1.0^a             | 3.5 ± 1.1^a       | 2.1 ± 0.26^a         | 0.61 ± 0.25^a           | 0.25 ± 0.09^a           | 3.00 ± 1.01^a            |
| Gemfibrozil 10 µm      | 42.5 ± 3.7          | 13.5 ± 2.2               | 6.9 ± 0.2         | 1.67 ± 0.42^a        | 0.88 ± 0.22^a           | 0.31 ± 0.09^a           | 2.16 ± 0.81^a            |
| 25-OH-cholesterol + gemfibrozil 20 µg/ml + 1 µm | 33.7 ± 3.0^a        | 8.4 ± 0.8^a              | 16.9 ± 2.3^a      | 3.58 ± 1.02^a        | 0.71 ± 0.12^a           | 1.33 ± 0.21^a           | 6.04 ± 1.25^a            |
| Lovastatin 10 µm       | 51.6 ± 6.2^a        | 15.4 ± 1.2               | 3.3 ± 0.8^a       | 1.81 ± 0.55^a        | 0.83 ± 0.15^a           | 0.25 ± 0.12^a           | 1.91 ± 0.09^a            |
| 25-OH-cholesterol + lovastatin 20 µg/ml + 1 µm | 29.8 ± 3.1^a        | 10.3 ± 2.1^a             | 15.2 ± 2.1^a      | 4.53 ± 1.11^a        | 1.01 ± 0.08             | 1.50 ± 0.33^a           | 6.77 ± 2.01^a            |

HepG2 cells were incubated for 24 h in DMEM containing 0.8% oleate/2% BSA and with 25-hydroxycholesterol, FCE 27677, gemfibrozil, or lovastatin, or alternatively, the oxysterol plus the different drugs. At the end of the incubation, the cells and medium lipids were extracted and quantitated by HPTLC. ApoB-100 concentration in the medium was assessed by RIA on concentrated medium samples. Values are given as mean ± SD from three experiments each with duplicate samples.

^P < 0.05 drugs or drugs plus 25-hydroxycholesterol-treated cells versus controls.

^P < 0.05 drugs plus 25-hydroxycholesterol-treated cells versus 25-hydroxycholesterol-treated cells.
comparative purposes. In preliminary experiments (data not shown), both drugs had a weak effect on the cholesteryl ester synthetic rate of HepG2 cells. A reduction of at least 25% of the incorporation of [14C]oleate in the cell cholesteryl ester fraction was achieved at concentrations approaching 10^{-5} M, whereas at a higher concentration both drugs had cytotoxic effects. Gemfibrozil, however, had a significant effect on the triglyceride synthesis starting at 10^{-5} M, whereas at a higher concentration both drugs had cytotoxic effects. Lovastatin significantly decreased the intracellular concentration of cholesteryl esters and significantly increased that of triglycerides. Gemfibrozil and lovastatin were tested at 10^{-5} M when added alone or at 10^{-6} M when added in the presence of 25-hydroxycholesterol (20 μg/ml) (Table 3). Lovastatin significantly decreased the intracellular concentration of cholesteryl esters and significantly increased that of triglycerides. Gemfibrozil had no detectable effect on the intracellular lipid concentration. Both drugs, however, decreased the neutral lipid secretion rates, although only lovastatin affected the apoB-100 secretion. Neither gemfibrozil nor lovastatin counteracted the effects elicited by 25-hydroxycholesterol. Thus, in contrast to what was seen with FCE 27677, the cholesteryl ester content and the lipid and the apoB-100 output of HepG2 cells incubated with the oxysterol and lovastatin or gemfibrozil remained elevated.

To investigate the effect of the inhibition of cholesterol esterification on the composition and on the distribution of the lipoproteins secreted by the hepatoma cells, the cell-conditioned medium was subjected to ultracentrifugation, and the isolated lipoproteins were analyzed for their neutral lipid and apolipoprotein content (Table 4). As previously reported (36, 37, 41), HepG2 cells secreted only a small amount of VLDL. This was confirmed by performing an agarose gel electrophoresis analysis of the d < 1.210 g/ml fraction of the cell medium which showed no pre-beta migrating material. FCE 27677 (10^{-5} M) reduced the LDL lipid output with minor effect on the HDL fraction. On the other hand, the incubation of the HepG2 cells with 25-hydroxycholesterol (20 μg/ml), enhanced both the LDL and the HDL output largely due to an absolute increase, compared to the untreated cells, of the cholesteryl esters. Addition of FCE 27677 to cells incubated with the oxysterol reduced the lipoprotein production. Computationally VLDL, LDL, and HDL isolated from the medium were similar to the lipoproteins secreted by HepG2 cells in the presence of the ACAT inhibitor alone. ApoB-100 appeared to be secreted only in LDL. No apoB-100 immunoreactive material could be detected by RIA in VLDL or HDL. However, a trace amount of high molecular weight material, which may be apoB-100, was detected when the lipoproteins were analyzed by SDS-PAGE. As VLDL and LDL contain a single apoB-100 polypeptide chain (42), the decrease of apoB-100 secretion by HepG2 cells after treatment with FCE 27677 suggests the view that incubation of the cells with FCE 27677 had the effect of decreasing the number of the lipoprotein particles secreted.

To assess the specificity of ACAT inhibition on apoB-100 and lipoprotein production, the effect of FCE 27677 on the unstimulated and the 25-hydroxycholesterol-stimulated HepG2 cells was investigated by simultaneously measuring apoB-100 and albumin production by the cells. To enable the measurement of the albumin

### Table 4: Effect of FCE 27677 and of 25-hydroxycholesterol on the neutral lipid and on the apoB-100 secretion rates into VLDL, LDL, and HDL fractions

<table>
<thead>
<tr>
<th></th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trig</td>
<td>Chol</td>
<td>Chol.E</td>
</tr>
<tr>
<td>Control</td>
<td>0.20</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>25,OH-cholesterol 20 μg/ml</td>
<td>0.20</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>FCE 27677 10 μM</td>
<td>0.07</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>25,OH-cholesterol + FCE 27677 20 μg/ml + 1 μM</td>
<td>0.14</td>
<td>0.02</td>
<td>0.06</td>
</tr>
</tbody>
</table>

HepG2 cells were incubated for 24 h in DMEM containing 0.8% oleate/2% BSA with 25-hydroxycholesterol alone, or FCE 27677 alone, or finally with a combination of the two agents. At the end of the incubation, the medium from three 225-cm² culture flasks was pooled, concentrated by ultrafiltration, and subjected to sequential ultracentrifugation for the separation of the lipoprotein fractions. After isolation, the lipoprotein lipids were quantitated by HPTLC and apoB-100 in LDL was quantitated by RIA. ApoB-100 could not be detected in VLDL or HDL by RIA. Values are given as mean from two experiments.
secreted, the incubations were performed with medium without BSA and olate. The results from these experiments are presented in Table 5. FCE 27677 significantly decreased the cholesteryl ester content and also prevented the increase promoted by 25-hydroxycholesterol in a dose-dependent fashion. As in the previous experiments carried out in the presence of the BSA-oleate complex, FCE 27677 potently counteracted the increase in the secretion of apoB-100 mediated by 25-hydroxycholesterol. At the same time, under no circumstances was a change in the albumin secretion rate observed.

DISCUSSION

The mechanisms involved in the regulation of the synthesis and secretion of apoB-100-containing particles by the liver remain poorly understood (1). A variety of systems has been used to address this question, including perfused rat (12), pig (43), and monkey liver (44), and cultured rat hepatocytes (15, 34, 38, 39); however, because there are major differences among species, a human model is desirable. The human hepatoblastoma-derived cell line, HepG2, has been found to retain many liver-specific functions and secretes normal liver proteins, including lipoprotein apolipoproteins (21, 36, 37, 41). Therefore, HepG2 cells were chosen to address the question of the role of cholesterol esterification in lipoprotein secretion by liver-derived cells.

The results of a number of previous studies indicate that the enhanced secretory rates of lipids in response to fatty acid provision are not always paralleled by changes of the same magnitude in the secretion of all the apolipoproteins (12–19). Regarding apoB-100 in particular, it has been suggested (20) that free fatty acids alone may not be an adequate signal to induce its secretion and that a more complex set of events must precede the induction of the synthesis. In addition to free fatty acids, cholesterol appears to be necessary to stimulate an increase in apolipoprotein and in lipoprotein secretion. Dashti (23) found that the rate of apoB-100 net accumulation in the medium of HepG2 cells shows a highly significant positive correlation with the cellular concentration of cholesteryl esters. Others (24) have suggested more specifically that cholesteryl ester synthesis might be a critical element regulating apoB-100 production by the hepatocytes. It is noteworthy that the intracellular site of this reaction, mediated by the ACAT enzyme, is different from that of the other lipid synthetic pathways. It occurs in the rough instead of the smooth endoplasmic reticulum, i.e., at the same site of the apolipoprotein synthesis and their initial assembly with lipids (45). On speculative grounds, the coordination of cholesterol esterification and of apoB-100 synthesis in the liver may be thought of as a coordinated pathway for the maintenance of the cellular cholesterol homeostasis because one reaction is concerned with the biotransformation of the potentially harmful excess cholesterol to the biologically inactive cholesteryl esters, and the other with the cellular disposition of a major cholesterol catabolite.

### Table 5. Effect of FCE 27677 on the 25-hydroxycholesterol-mediated accumulation of cholesteryl esters and on the neutral lipid, apoB-100, and albumin secretion rates from HepG2 cells

<table>
<thead>
<tr>
<th>Intraacellular Lipids</th>
<th>Secrecion Rates in Medium</th>
<th>μg/mg cell protein</th>
<th>μg/mg cell protein/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Triglycerides</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>29.7 ± 1.8</td>
<td>15.1 ± 2.0</td>
</tr>
<tr>
<td>25, OH-cholesterol + FCE 27677</td>
<td>none + 10 μM</td>
<td>30.0 ± 1.9</td>
<td>14.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>none + 1 μM</td>
<td>32.3 ± 4.8</td>
<td>14.7 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>none + 0.1 μM</td>
<td>28.0 ± 4.0</td>
<td>13.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>20 μg/ml + none</td>
<td>27.0 ± 3.2</td>
<td>10.6 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>20 μg/ml + 10 μM</td>
<td>32.4 ± 5.6</td>
<td>19.8 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>20 μg/ml + 1 μM</td>
<td>29.1 ± 3.3</td>
<td>12.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>20 μg/ml + 0.1 μM</td>
<td>26.1 ± 4.0</td>
<td>11.7 ± 2.1</td>
</tr>
</tbody>
</table>

HepG2 cells were incubated for 24 h in DMEM without BSA-oleate together with 25-hydroxycholesterol alone or in addition to FCE 27677. At the end of the incubation, the cells and medium lipids were extracted and quantitated by HPTLC. The concentration in the medium of apoB-100 and of albumin was assessed by RIA on concentrated medium samples. Each value is the mean ± SD from three experiments except for the control group, whose values are from six experiments. Each experiment was performed with duplicate samples.

*P < 0.05 versus control.

*P < 0.05 25-hydroxycholesterol plus FCE 27677 versus 25-hydroxycholesterol only treated cells.
The idea of coordination between cholesteryl ester synthesis and apoB-100 secretion, although intriguing, still lacks probating experimental evidences. For example (23), although the stimulation of cholesterol esterification by 25-hydroxycholesterol is accompanied by a concomitant increase of apoB-100 secretion in the medium of HepG2 cells, it should be kept in mind that this oxysterol has a number of other biological effects directly concerned with the metabolism of cholesterol (40). Therefore, the hypothesis that the effect of 25-hydroxycholesterol on apoB-100 secretion is solely due to the effect it has on cholesterol esterification must be considered with caution. Also, the evidence that progesterone (23) counteracts the effect of 25-hydroxycholesterol on cholesteryl ester synthesis and at the same time abolishes the increase of apoB-100 secretion cannot be regarded as conclusive as this hormone has a number of other effects on the lipid metabolism beside the postulated direct inhibition of ACAT (46). Undoubtedly the use of a specific ACAT inhibitor can provide distinct advantages. By using a first-generation ACAT inhibitor, Sandoz 58-035, Pullinger et al. (18) and Gianfalone et al. (24) have obtained contrasting results. Whereas the first group found a slight enhancement of apoB-100 secretion from HepG2 cells, the second group reported a decrease. In addition, both have found that whereas Sandoz 58-035 decreased the HepG2 cholesteryl ester content, at the same time it substantially increased that of triglycerides, an important confounding effect if the specific role of cholesteryl ester synthesis on lipoprotein production is to be investigated. More recently, Wu et al. (47), using the same synthetic inhibitor, have concluded that cholesteryl esters do not affect the assembly and secretion of apoB-100-containing lipoproteins from HepG2 cells. In their studies, neither the Sandoz inhibitor nor the stimulation of cholesterol esterification by incubating the cells with oleate or VLDL, by transfecting the cells with HMG-CoA reductase cDNA, or by incubation with sphingomyelinsase had an effect on the secretion of apoB-100. However, the attempts to change the HepG2 cholesteryl ester mass had limited effect compared to that achieved, for example in this study, with 25-hydroxycholesterol. This is a crucial point as it is possible that apoB-100 secretion from HepG2 cells is increased only when the cellular cholesteryl ester concentration exceeds a threshold value (see below). Furthermore, the Sandoz compound could not totally prevent the effects elicited on the cell cholesteryl ester content by the different treatments. In a subsequent paper (48) the same authors have concluded that VLDL-mediated stimulation of apoB-100 secretion from HepG2 cells occurs primarily by supplying fatty acids for triglyceride synthesis. The concomitant effect of VLDL on the cellular cholesteryl ester content was again considered of no impact on the apoB-100 synthetic rate. A key result from that study was that Triacsin D, a bacterial inhibitor of the triglyceride synthesis acting via a competitive inhibition of fatty acyl-CoA synthase, significantly inhibited the VLDL-induced stimulation of apoB-100 secretion. On the other hand, partial inhibition by Sandoz 58-035 of cholesteryl ester formation after VLDL incubation did not decrease apoB-100 secretion. As cholesteryl ester synthesis requires CoA-activated fatty acids, it is, however, surprising that Triacsin D did not at the same time inhibit the VLDL-mediated raise of HepG2 esterified cholesterol. By the same token, an effect of this compound on the phospholipid synthesis would be expected. Finally, the fact that the compound only partially affected the HepG2 triglyceride synthetic rate, even when added at relatively high concentrations (12.5 μM), casts doubt regarding its specificity. Unfortunately, no result is provided to address this important point. Nor are data cited regarding the cytotoxicity of Triacsin D.

In the work presented, the link between cholesteryl ester formation and the secretion of apoB-100-containing lipoproteins has been reassessed by investigating the effect of a novel highly specific ACAT inhibitor on HepG2 cells. Without affecting the synthetic rate of cholesterol, triglycerides and phospholipids, the drug in unstimulated HepG2 cells decreased the rate of cholesteryl ester formation. This led to the specific decrease of the cellular cholesteryl ester content. At the same time, cholesteryl ester and apoB-100 cell output were decreased. The same effect was observed when HepG2 cholesteryl ester synthesis was stimulated by 25-hydroxycholesterol. The oxysterol significantly increased the HepG2 content of cholesteryl esters with little effect on the concentration of the other neutral lipids. At the same time, the secretion rates of apoB-100 and of cholesteryl esters were increased. The addition of FCE 27677 to the medium completely prevented the effects elicited by the oxysterol. At 10^{-5} M, the ACAT inhibitor greatly decreased the secretion of lipoproteins affecting the HepG2 triglyceride synthetic rate, even when added at relatively high concentrations (12.5 μM), casts doubt regarding its specificity. Altogether, these data strongly support the view that there is a causal link between cholesterol esterification, cellular cholesteryl ester content, and the rate of assembly and secretion of the apoB-100-containing lipoproteins. Pullinger et al. (18) have presented results showing that in HepG2 cells, the expression of the apoB-100 gene is constitutive and that co- or post-translational mechanisms are responsible for the short-term regulation of apoB-100 secretion. The additional evidence that there is no apparent correlation between apoB-100 mRNA level and this apolipoprotein synthesis (18, 19) further supports this idea. The post-translational modifications may be needed for the intracellular translocation of the nascent apoB-100 protein.
lipoproteins containing apoB-100 for secretion (3). Both morphological and immunochemical imaging data suggest that movement of apoB-100 out of the endoplasmic reticulum is rate-limiting for the secretion of lipoprotein (1). Our data support the idea that availability of cholesteryl esters at the rough endoplasmic reticulum is also a rate-limiting factor.

The mechanism of activation by 25-hydroxycholesterol of ACAT likely involves the binding of the oxysterol to the enzyme at the same site as that of cholesteral as it has been shown that at cholesterol concentrations below saturation, the oxygenated sterol has a stimulatory effect, whereas if ACAT is saturated, it has no effect (40). It is interesting that FCE 27677 could still prevent the effects of 25-hydroxycholesterol at concentrations (10^-7 M) that were ineffective when the compound was added alone (see Tables 1 and 2). Previous studies have shown that FCE 27677 inhibits ACAT through a non-competitive mechanism (33), and a direct interaction of the drug with the enzyme has been postulated. The apparent higher sensitivity to FCE 27677 inhibition when ACAT is activated may be interpreted on the basis of the idea that a conformational change accompanies the activation of this enzyme (49). The structural rearrangement may allow a better accessibility to the enzyme of both the substrates as well as the inhibitors. In a physiological setting, the ability of ACAT to modulate its activity by conformational changes may warrant a rapid adjustment of the hepatic cholesteryl ester synthetic rate and of the lipoprotein secretion to a metabolic challenge without the need of further enzyme synthesis. This conceptualization fits in well with the idea that the main role of ACAT is the prevention of the buildup of the potentially toxic unesterified cholesterol. If this is the case, then suppression of the ACAT activity below the normal level is likely to have little effect on the cholesteryl ester content as the turnover rate of this lipid is already very low. On the other hand, under challenging conditions, the ACAT activity may rapidly increase as a large mass of cholesterol enters the esterification process and inhibition of the enzyme will have the greatest effect both on cholesteryl ester formation and, in turn, on the stimulated apoB-100 secretion.

In this study, in order to extend the observations on the effects of the cellular cholesteryl ester synthetic block on apoB-100 production, the cells were incubated with two hypolipidemic drugs with mechanism of action different from that of FCE 27677. Whereas gemfibrozil had no effect on the intracellular neutral lipid content, lovastatin significantly decreased the cholesteryl ester fraction but, at the same time, significantly increased the triglyceride fraction. Both drugs when added alone decreased the neutral lipid secretion, but only the HMG-CoA reductase inhibitor could decrease the secretion of apoB-100. However, when ACAT activity was stimulated with 25-hydroxycholesterol, neither gemfibrozil nor lovastatin was active. It has been proposed that neosynthesized cholesterol is a preferred substrate for ACAT (45). Conceivably, the decrease in cholesteryl ester content observed when the HepG2 cells are incubated with lovastatin alone is due to the potent suppressing activity this drug has on the cholesteryl biosynthesis and the observed inhibition of the apoB-100 secretion may be secondary to this. On the other hand, when ACAT is activated, only the specific inhibition of the enzyme can prevent the ensuing intracellular cholesteryl ester accumulation and the increase of apoB-100 secretion. Clearly, this has important implications regarding the unique therapeutic usefulness of the ACAT inhibitors. Another potentially relevant clinical feature of ACAT inhibition is suggested by the results showing that FCE 27677 treatment virtually normalized the hepatocyte secretion of d < 1.063 g/ml lipoprotein particles. It is known that the risk of premature CAD is, to a large extent, a function of the number of the circulating LDL particles. This number is dictated by the balance between the rate of synthesis and catabolism. Whereas the pathogenesis of lipoprotein disorders linked to impaired catabolism is now well understood and is, in most cases, pharmaco logically manageable, the metabolic dysfunction(s) leading to apoB-100-containing lipoprotein overproduction remains poorly controllable. The incidence in the population at large of these forms of dyslipoproteinemia has yet to be established, but they may be common in normolipidemic subjects who later developed premature CAD (50). In this population at high risk of cardiovascular mortality, ACAT inhibitors may therefore prove to be a clinically effective type of intervention.

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