Effects on apoB-100 secretion and bile acid synthesis by redirecting cholesterol efflux from HepG2 cells

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Abstract **This study determined the effects of apoA-I, HDL3, or hydroxy-β-cyclodextrin on apoB-100 secretion and bile acid synthesis by HepG2 cells. The principal observations were that: *1*) ApoB-100 secretion into the medium was significantly less after the addition of any of the three agents. *2*) Triglyceride mass was not significantly changed from control in the medium but was significantly, although modestly, reduced in the cells. *3*) Neither free cholesterol (FC) nor cholesteryl ester (CE) mass in the medium was changed; by contrast, CE mass was reduced within the cells although FC was not. *4*) Although the total mass of cholesterol in the medium was unaffected, the proportion associated with apoB-100 was reduced, whereas the proportion associated with the non-apoB-100 fraction was increased. *5*) There was also an unanticipated, but substantial, increase in bile acid synthesis induced by apoA-I, HDL3, or hydroxy-β-cyclodextrin, which was time and concentration dependent, and which was associated with marked increases in cholesterol 7α-hydroxylase activity. There were no significant changes in ACAT activity and only modest increases in HMG-CoA reductase activity. These findings support previous clinical observations that an elevated apoB-100 can accompany a low HDL cholesterol in normotriglyceridemic subjects. They also point to physiologically important, although still only partially understood, metabolic relationships amongst hepatic apoB-100 secretion, cholesterol efflux, and bile acid synthesis.**—Sniderman, A. D., Z. Zhang, J. Genest, and K. Cianflone. **Effects on apoB-100 secretion and bile acid synthesis by redirecting cholesterol efflux from HepG2 cells.** ***J. Lipid Res.*** **2003.** 44: **527–532.**

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Chylomicrons and VLDL transport triglyceride from the intestine and the liver to peripheral tissues, whereas HDL is an integral link in the complex pathway by which cholesterol returns from the periphery to the liver. Although they fulfill very different physiologic roles, clinically significant interactions occur between them. In particular, as a consequence of the exchange of core lipids between chylomicrons and VLDL on the one hand and HDL on the other, hypertriglyceridemia is often associated with low HDL cholesterol (HDL-C) (1). This relation is well known and well accepted. However, the fact that low HDL-C may be associated with a high plasma apoB-100 even when plasma triglycerides are normal is not widely known.

The first evidence of such an association, as well as an indication of the mechanism that might be responsible, came from Ginsberg et al., who demonstrated that LDL production rates could be markedly elevated even in normotriglyceridemic patients with low HDL-C (2). The study of familial dyslipoproteinemias by Genest et al. was the next to firmly document the association. In their cohorts with familial hypoalphalipoproteinemia, total and LDL-C, and triglyceride levels were normal, but plasma apoB-100 levels were increased to the same extent as in familial combined hyperlipidemia (3). Based on these reports, we surveyed the database of the Quebec Cardiovascular Study and compared two groups, one of which was normotriglyceridemic with a low HDL-C, while the other was normotriglyceridemic with a high HDL-C. ApoB-100 was significantly higher in the former than in the latter (4).

These observations suggested that there might be a metabolic link between HDL and the hepatic apoB-100 secretion rate. One of the most important determinants of the rate of apoB secretion is the sterol balance in the hepatocytes (5). That is, increased cholesteryl ester (CE) mass within the hepatocyte is associated with increased apoB secretion (6). Accordingly, it seemed possible that HDL, by removing cholesterol from the liver just as it removes it from other tissues, might affect apoB-100 secretion. Therefore, we undertook a series of experiments using HepG2 cells to test this hypothesis. The results indicate that apoB-100 secretion in vitro can be substantially modulated by maneuvers that alter hepatocyte sterol balance. However, the effects extend beyond this and in-

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Abbreviations: βCD, hydroxy-β-cyclodextrin; CE, cholesteryl ester; FC, free cholesterol; TC, total cholesterol.

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clude unanticipated findings, such as a concurrent substantial increase in bile acid formation.

METHODS

Tissue culture

HepG2 cells obtained from the American Tissue Culture Collection (Rockville, MD) were routinely grown in minimum essential medium supplemented with 10% fetal calf serum in 75 cm^2 flasks with 15 ml medium at 37°C with 5% CO_2. Flasks were subcultured every 7 days, with a split ratio of 1:3. The cells were dislodged from the culture flask with 0.25% trypsin in Ca^{2+} and Mg^{2+} free PBS with 0.02% EDTA for 5 min at 37°C. For experiments, cells were plated out at a density of 1.3×10^4 cells/cm^2 in 17 mm dishes (24-well plates) or 35 mm dishes (6 well plates).

Experimental conditions

ApoA-I was obtained from Academy Bio-Medical Co. (Houston, TX). HDL was isolated between density 1.063–1.21 g/ml by preparative ultracentrifugation (7). HDL3 was then prepared by isolation between 1.125–1.21 g/ml. Hydroxy-β-cyclodextrin (βCD) was purchased from the Aldrich Chemical Co.

Once confluent, HepG2 cells were washed three times with 0.1% BSA in PBS followed by cholesterol loading with 20 μg/ml cholesterol dispersed in ethanol (.02%) for 24 h (8). The medium was then removed and the cells were equilibrated for another 24 h in 0.1% BSA in DMEM. After changing the medium, the cells were incubated for 0 h, 6 h, or 24 h with additions to the medium as indicated. At the end of these periods, the medium was collected and the cells were washed three times with ice cold 0.1% BSA in PBS and the cells were dissolved in 0.1 N NaOH.

Lipid quantification

Total cholesterol was measured in the medium and the cells by the cholesterol enzymatic assay (Boehringer Mannheim #237574). Aliquots of cells and the medium were also extracted with chloroform-methanol (2:1, v/v) and the lipids separated by thin layer chromatography (Silica Gel G) using a solvent system consisting of hexane-ethyl ether-acetic acid (75:25:1, v/v/v). Lipid spots were identified by exposure to iodine vapor and compared with reference lipids. CE spots were scraped into tubes and CE determined by the method of Rudel and Morris (9). Cholesterol was determined by the same method using cholesterol as a standard. Triglyceride mass was measured by an enzymatic colorimetric assay (Boehringer Mannheim #701904). Non-apoB-100 cholesterol was obtained by precipitation of an aliquot from the medium with 4% phosphotungstic acid and 2.5 M magnesium chloride (10). After centrifugation at 4,000 rpm for 30 min, the supernatant was removed and cholesterol determined by TLC.

Measurement of LDL binding

The activity of the LDL pathway was estimated as previously described (11). Cells were incubated either under control conditions or after addition of any of the experimental interventions. After 24 h, the cells were placed on ice at 4°C and ^{125}I-LDL (SA 74 dpm/ng) in 0.1% BSA was added to the incubation medium. After 2 h, the medium was collected and the cells were washed three times with ice cold 0.1% BSA in PBS. Cell associated ^{125}I-LDL was determined by counting an aliquot of the cell protein dissolved in 0.1 N NaOH. Nonspecific ^{125}I-LDL cell association was measured in the presence of a 20-fold excess of LDL as described by Goldstein et al. (12). Specific cell associated ^{125}I-LDL was calculated as the difference between total and non-specific ^{125}I-LDLs.

Measurement of total protein synthesis and secretion

Cells were incubated for 24 h under control and each experimental condition in medium to which [^{3}H]leucine (14.7 dpm/ng) had been added. Following incubation, aliquots of the medium and the cells were applied to 20-mm filter paper circles. Proteins were precipitated by immersing the filter papers in 10% tricholoractetic acid for 30 min and then washing sequentially for 10 min each in 5% ice-cold, 5% boiling, and 5% ice-cold trichloroacetic acid. The filter strips were dried, and the protein was solubilized with Protosol (DuPont NEN) and counted in scintillation fluid in a scintillation counter.

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 represent the sum of both medium and cells.

Protein and apoB-100 quantification

Cell protein was measured by the method of Bradford using BSA as a standard (13). ApoB-100 was determined by an in-house competitive immunoassay using a rabbit polyclonal antihuman apoB-100 antibody as previously described (14).

Measurements of enzyme activities

HMG-CoA reductase activity was determined in cell homogenates as described by Erickson and Fielding (15) while ACAT and cholesterol 7α-hydroxylase activities were measured as per Huff et al. (16) and Hitoshi et al. (17), respectively.

Statistics

Each experimental point is the average of at least 15 observations except for bile acids, which were an average of at least three. Each result is expressed as mean ± SEM. The significance of experimental changes was determined by one-way ANOVA using the modified Bonferroni correction factor for multiple analyses. A P value of <0.05 was taken as significant.

RESULTS

The effects of the three interventions-apoA-I, βCD, or HDL3 on apoB-100 concentration in the medium after 24 h incubation are shown in **Fig. 1**. Each reduced apoB-100 concentration in the medium by at least half. There was, however, no change in either total protein secretion by

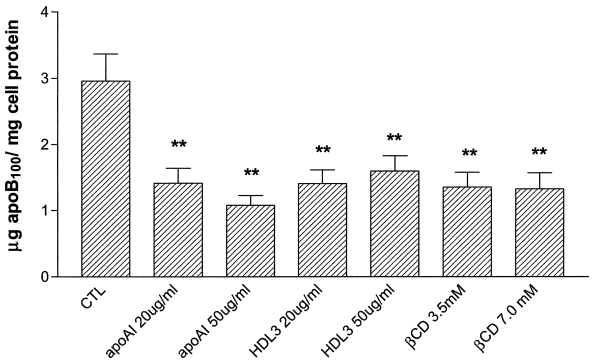

Fig. 1. Concentration of apolipoprotein B-100 (apoB-100) in the medium 24 h after addition of indicated concentrations of apoA-I, HDL3, or hydroxy-β-cyclodextrin (βCD) to the medium. Values are means ± SEM. ** $P < 0.005$–0.001.

the cells (data not shown) or in the activity of the LDL pathway (data not shown). Taken together, therefore, these data establish that addition of any of three agents known to increase cholesterol efflux from cells by a non-apoB-100 mediated route resulted in significant specific reduction in apoB-100 secretion by HepG2 cells. These same effects were evident in HepG2 cells that were not cholesterol loaded (data not shown). The absolute reduction in apoB-100 concentration was less pronounced however, because, as anticipated from previous work (5), the basal secretion rates of apoB-100 were also less.

The effect of these interventions on triglyceride concentration in the cells and the media and these data are shown in **Fig. 2**. There was no change in triglyceride concentration in the medium, whereas there was approximately a significant 25% reduction in triglyceride mass within the cells. Characteristically for HepG2 cells, the mass of triglyceride in the medium is much less than the cells consistent with their known inability to export triglycerides.

The changes in free cholesterol (FC) and CE mass in the cells and the medium are shown in **Fig. 3A** and 3B respectively. Overall, there was no significant change in FC mass in either the medium or the cell with any of the three interventions. With regard to CE, there were no significant changes in the medium. However, with all except one of the interventions, there were significant reductions in CE mass within the cells.

The consistent and substantial reductions in apoB-100 secretion in the face of unchanged total cholesterol mass

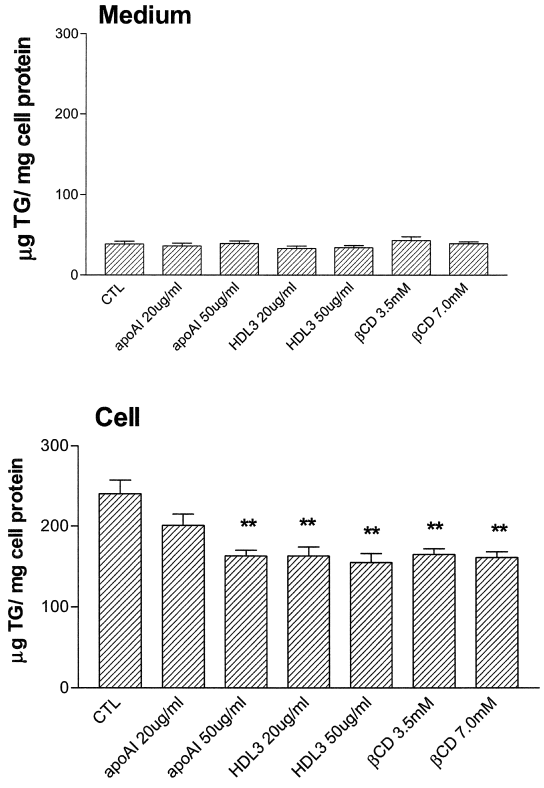

Fig. 2. Triglyceride mass in medium or cells 24 h after addition of indicated concentrations of apoA-I, HDL3, or βCD to the medium.

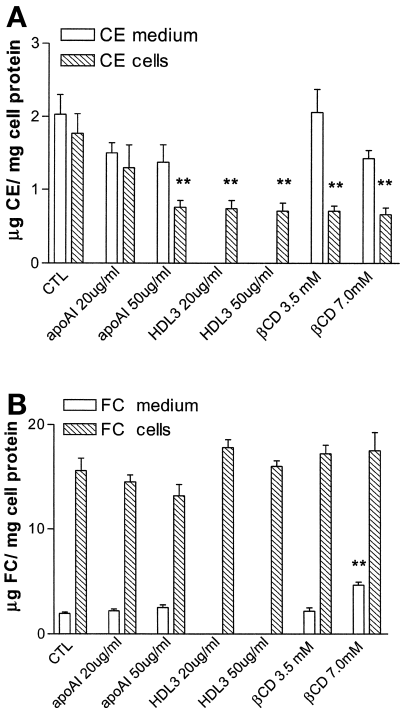

Fig. 3. Cholesteryl ester (CE) mass in cells and medium (A) 24 h after addition of indicated concentrations of apoA-I, HDL3, or βCD to the medium. Free cholesterol (FC) mass in cells and medium (B) 24 h after addition of indicated concentrations of apoA-I, HDL3, or βCD to the medium. Note no value is listed for CE or FC for the medium when HDL3 is added to the medium. ** $P <$ 0.005–0.001.

in the medium suggested that the pattern of cholesterol secretion might have changed. Accordingly, the relative amounts of cholesterol in the apoB-100 and non-apoB-100 lipoprotein fractions were determined. These results are shown in **Fig. 4**. Note that the majority of the cholesterol in the control media was in the apoB-100 fraction, whereas with both concentrations of apoA-I and of βCD at both time points, except for one instance, more choles-

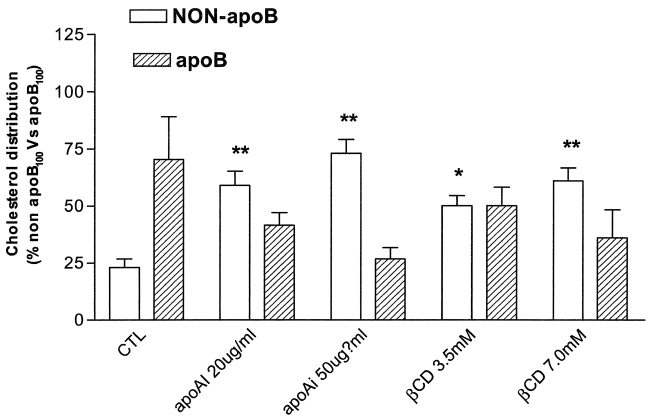

Fig. 4. Percent distribution of cholesterol in the medium in non-apoB-100 fraction (open bar) and apoB-100 fraction (hatched bar) 24 h after addition of indicated concentrations of apoA-I or βCD. CTL, control. * $P <$ 0.05–0.01; ** $P <$ 0.005–0.001.

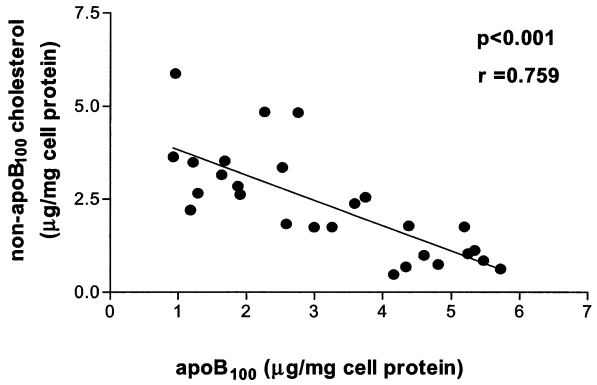

Fig. 5. The concentration of non-apoB-100 cholesterol in the medium is plotted against the concentration of apoB-100 in the medium 24 h after addition of concentrations of apoA-I or βCD, which were shown.

terol was present in the non-apoB-100 fraction. The difference from control was statistically significant in every instance. Moreover, as shown in **Fig. 5**, there was a significant inverse linear relation between apoB-100 concentration and non-apoB-100 associated cholesterol. Thus, it appears to be principally the route, rather than the amount of cholesterol leaving the HepG2 cells, which is affected by addition of apoA-I and βCD to the medium.

To complete our assessment of sterol balance, we examined the effects of these maneuvers on bile acid synthesis and, concurrently, apoB-100 secretion. The results for apoA-I are shown in **Fig. 6**. They demonstrate a concentration dependent, inverse relation between the amount of apoA-I added to the medium and the apoB-100 concentration in the medium. By contrast, there is a strong, positive relation between the amount of apoA-I added to the medium and bile acid concentration indicating an increase in bile acid synthesis in response to the addition of apoA-I to the medium. Exactly the same relations were found for βCD. As anticipated from previous work (4), addition of either LDL or cholesterol phospholipid vesicles to the medium caused apoB-100 concentration in the medium to increase (data not shown). Bile acid synthesis also increased significantly in both cases (data not shown).

The net effect of addition of apoA-I or βCD on total sterol balance is shown in **Table 1**. When bile acids as well as FC and CE in both cells and media are taken into account, only the addition of 7.0 mM βCD results in any significant increase in total sterol. As noted above, both apoA-I and βCD cause bile acid synthesis to increase.

The final series of experiments examined the effects of apoA-I and βCD on the activity of the three enzymes that play key roles in the regulation of hepatic sterol balance. These results are shown in **Table 2**. After addition of apoA-I to the medium, there was a modest (35%) but significant increase in the activity of HMG-CoA reductase, but not in ACAT activity. By contrast, with addition of βCD, there was a 230% increase in HMG-CoA reductase activity, again without a significant change in ACAT activity. The changes in cholesterol 7α-hydroxlase were more

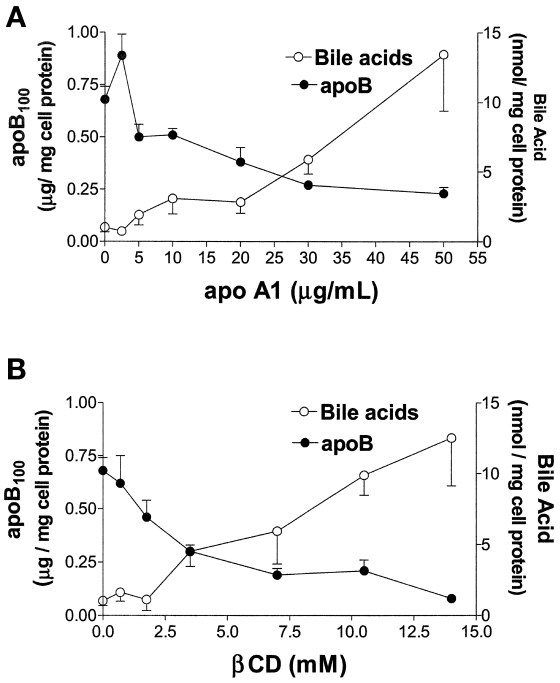

Fig. 6. The concentration of apoB-100 (closed circles) and bile acid (open circles) in the medium and cells 24 h after addition of apoA-I (A) and βCD (B).

dramatic. Addition of apoA-I induced a 465% ($P < 0.005$) increase in cholesterol 7α-hydroxylase activity, whereas addition of βCD induced a 640% increase ($P < 0.005$). The increase in enzyme activity is, of course, concordant with the increase in bile acid synthesis that was observed.

DISCUSSION

The present studies demonstrate that apoB-100 secretion by HepG2 cells is substantially reduced by addition of HDL3, apoA-I, or βCD. HDL3 and apoA-I act physiologically to remove cholesterol from cells, whereas βCD has been shown to be an effective vehicle by which to transfer cholesterol to cells, to remove cholesterol from cells, and to shift cholesterol within cell components (18–20). All three agents produced substantial reductions in the concentration of apoB-100 in the medium. Given there was no change in the activity of the LDL pathway, changes in secretion must be responsible. It is important to note that overall protein synthesis and secretion were unaffected.

The mass of triglyceride was unchanged in the medium and modestly, but significantly, reduced within the cells. The distribution of triglyceride in the medium was not determined. Given that there has been no evidence previously that any pathway other than apoB-100 might be involved, we assume that triglyceride-enriched apoB-100 particles were secreted. This phenomenon has been directly demonstrated in the past. Thus, increased fatty acid flux to HepG2 cells results in increased hepatic triglyceride synthesis and section, increased CE synthesis and secretion, and increased secretion of apoB-100 particles of nor-

TABLE 1. Effect of apoA-I and βCD in total sterol mass

	CTL	ApoA-I (20 μg/ml)	ApoA-I (50 μg/ml)	βCD (3.5 mM)	βCD (7.0 mM)
TC (cells + medium) (nmol/mg cell p.)	53.8 ± .03	48.9 ± .01	40.8 ± .02	54.4 ± .01	60.5 ± .02
Bile acids (cells + medium) (nmol/mg cell p.)	1.0 ± .06	2.8 ± 1.4	13.4 ± 7.0	4.5 ± 1.1	5.9 ± 4.0
Total sterol (nmol/mg cell p.)	54.8 ± .6	51.8 ± 1.4	54.2 ± 7.1	58.9 ± 1.1	66.4 ± 4.0[a]

[a] $P < 0.05$ versus control.

mal composition. By contrast, increased delivery of glucose results in increased triglyceride synthesis and secretion, unchanged CE mass and secretion, and production, therefore, of triglyceride-enriched apoB-100 particles.

The mass of FC was unchanged in both the cells and the medium. Of importance, as apoB-100 secretion was reduced, so also was the mass of CE mass was reduced in the HepG2 cells, consistent with multiple previous observations from many laboratories in many systems. In the present context, the novel finding was the reciprocal relationship between the mass of cholesterol present in the non-apoB-100 fraction in the medium versus that in the apoB-100 fraction. Since the mass of cholesterol in the medium was unchanged, it appeared that increased removal of cholesterol from the cell via apoA-I, HDL3, or BCD results in diminished secretion within apoB-100 lipoproteins. This further strengthens the concept that a physiologic role of the apoB-100 lipoprotein secretory system is to maintain sterol balance within the hepatocyte (4). Indeed, once bile acids are taken into account, overall sterol balance was virtually undisturbed.

Our results appear to be at variance with those reported by Peluso and Dixon (18), who noted that whereas βCD had no effect on apoB synthesis by HepG2 cells, apoB secretion increased with 0.5% βCD. This increase, however, was not concentration dependent. No obvious explanation for the difference in results is apparent, although as we have previously noted, very different methods to measure apoB have been used (6).

We also noted a significant increase in HMG-CoA reductase activity induced by βCD. Again, this finding differs from that observed by Peluso and Dixon (18). Nevertheless, given that cholesterol was displaced from the plasma membrane by βCD, this, in turn, should induce transfer of cholesterol from the endoplasmic reticulum to the plasma membrane (21). Cholesterol synthesis would then be expected to increase in order to restore the cholesterol content in the endoplasmic reticulum.

An unanticipated observation was the major increase in bile acid synthesis due to increased cholesterol 7α-hydroxylase activity, raising the issue as to whether there are mechanistic links between bile acid metabolism and apoB-100 secretion. To be sure, addition of HDL to hepatocytes has previously been shown to produce an increase bile acid synthesis. However, these experiments have used total HDL to deliver cholesterol to the hepatocytes (5). In the present case, we have used HDL3 to remove it. Moreover, two other agents, βCD and apoA-I, were also used to remove rather than deliver cholesterol to the HepG2 cells, and these produced the same effect on bile acid synthesis as HDL3. With regard to apoB-100 metabolism, in CHO cells expressing apoB53, but not MTP, cholesterol 7α-hydroxylase protected against conjugation of the translocation arrested apoB53 with ubiquitin, and therefore reduced proteosome mediated degradation (22). On this basis, Davis has suggested that there is a direct relation between cholesterol 7α-hydroxylase activity and apoB-100 secretion and that this explains the increased hepatic triglyceride secretion in familial hypertriglyceridemia (23). This seems unlikely because, although bile acid synthesis and cholesterol 7α-hydroxylase are increased in familial hypertriglyceridemia, characteristically apoB-100 secretion is not (24).

Indeed, the data in this study demonstrate the opposite relation, namely that there is an inverse relation between bile acid synthesis and apoB-100 secretion. Our data, however, are consistent with the observations of Nanjee et al. (25), who demonstrated that infusions into humans of apoA-I/phosphatidylcholine discs reduced plasma apoB-100 concentrations and with those of Eriksson et al. (26), who demonstrated significant increases in excretion of bile acids and neutral sterols after infusions of recombinant pro-apoA-I. Inhibition of ACAT activity has been shown to increase bile acid synthesis (27). However, this could not explain the present results, since ACAT activity was not increased by the interventions.

By contrast, there is considerable evidence relating hepatic CE mass and apoB-100 secretion (6). The data from this study are consistent with this model although there was, in fact, also a modest but significant decrease in intracellular triglyceride mass induced as well.

TABLE 2. Enzyme activity in HepG2 cells

	CTL	ApoA-I (50 mg/ml)	βCD
HMG-CoA reductase activity (nmol/mg.P.min)	22.9 ± 0.6	31.0 ± 2.3[a]	52.4 ± 1.3[a]
Acyl-CoA:cholesterol acyltransferase activity (pmol/mg.P.min)	13.5 ± 1	11.3 ± 0.5	17.4 ± 1.8
Cholesterol 7α-hydroxylase activity (pmol/mg.P.min)	21.1 ± 4.4	98.5 ± 8.5[b]	135.5 ± 19.3[b]

[a] $P < 0.05$–0.01.
[b] $P < 0.005$–0.001.

Our study has important limitations. First, HepG2 cells are transformed hepatocytes and their metabolic responses may not faithfully mimic those of normal hepatocytes. Second, it shares the limitation common to all of its type, namely the assumption that short term in vitro responses to acute experimental interventions mirror long term in vivo relationships with less dramatic differences. In that regard, it is reassuring that the present observations are consistent with the clinical observations that stimulated the study, namely the association between low HDL-C and elevated plasma apoB-100 observed in some instances of familial hypoalphalipoproteinemia (3), the fact that apoB-100 can be elevated in normotriglyceridemic subjects with low HDL-C (4), and most particularly, with the kinetic studies of Ginsberg and his colleagues who demonstrated increased LDL apoB synthesis in normotriglyceridemic subjects with low HDL-C (2). More importantly, the observations in the present study also point to physiologically important, but unfortunately still only partially understood, metabolic relationships amongst apoB-100 secretion, cholesterol efflux and bile acid synthesis by hepatoyctes, relationships which are critical to explicate if we are to understand in full the determinants of the concentration of the apoB-100 lipoproteins in plasma.

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