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J Clin Invest. 1987;**80**(6):1692-1697. <https://doi.org/10.1172/JCI113259>.

Research Article

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Suppression of Apolipoprotein B Production during Treatment of Cholesteryl Ester Storage Disease with Lovastatin

Implications for Regulation of Apolipoprotein B Synthesis

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Abstract

Cholesteryl ester storage disease (CESD) is characterized by the deficient activity of lysosomal cholesteryl ester (CE) hydrolase, accumulation of LDL-derived CE in lysosomes, and hyperlipidemia. We studied the kinetics of VLDL and LDL apolipoprotein B (apoB), using ¹²⁵I-VLDL and ¹³¹I-LDL, in a 9-yr-old female with CESD and elevated total cholesterol (TC) (271.0±4.4 mg/dl), triglyceride (TG) (150.0±7.8 mg/dl), and LDL cholesterol (184.7±3.4 mg/dl). These studies demonstrated a markedly elevated production rate (PR) of apoB, primarily in LDL, with normal fractional catabolism of apoB in VLDL and LDL. Urine mevalonate levels were elevated, indicative of increased synthesis of endogenous cholesterol. Treatment with lovastatin, a competitive inhibitor of hydroxymethylglutaryl coenzyme A reductase, resulted in significant reductions in TC (196.8±7.9 mg/dl), TG (100.8±20.6 mg/dl), and LDL cholesterol (102.0±10.9 mg/dl). Therapy reduced VLDL apoB PR (5.2 vs. 12.2 mg/kg per d pretreatment) and LDL apoB PR (12.7 vs. 24.2 mg/kg per d pretreatment). Urine mevalonate levels also decreased during therapy. These results indicate that, in CESD, the inability to release free cholesterol from lysosomal CE resulted in elevated synthesis of endogenous cholesterol and increased production of apoB-containing lipoproteins. Lovastatin reduced both the rate of cholesterol synthesis and the secretion of apoB-containing lipoproteins.

Introduction

Cholesteryl ester storage disease (CESD)¹ is an inherited disorder characterized by significant or total deficiency of the lysosomal enzyme, cholesteryl ester (CE) hydrolase (1). The more severe form of this disorder, Wolman's disease, is associated with failure to thrive and death in infancy (1-3). In contrast, subjects with CESD may be asymptomatic, and only

diagnosed when hepatosplenomegaly is noted (1, 3, 4). CESD patients commonly have elevated plasma levels of cholesterol, with a type IIA or IIB phenotype (1, 3, 4). In addition, elevated plasma concentrations of VLDL triglycerides and markedly reduced plasma levels of HDL cholesterol have been associated with the increased concentrations of LDL cholesterol (3, 4).

The mechanisms underlying the altered plasma lipid levels present in individuals with CESD is unknown. However, the combination of increased LDL and reduced HDL present in subjects with CESD suggests that these individuals might be at increased risk for the development of early vascular disease (4). Recently, we had the opportunity to investigate, in detail, the metabolism of plasma VLDL and LDL in a 9-yr-old female with CESD who presented with severe hypercholesterolemia, hypertriglyceridemia, and markedly reduced levels of HDL cholesterol. Because of the significant hepatosplenomegaly present in this patient, and based on the results of studies of the kinetics of apolipoprotein B (apoB) metabolism in this subject before treatment, we initiated a trial of therapy with lovastatin, the competitive inhibitor of hydroxymethylglutaryl coenzyme A (HMGCoA) reductase (5). Repeat studies of lipoprotein metabolism, carried out after 8 mo of lovastatin therapy, suggested that the secretion of apoB-containing lipoproteins by hepatocytes may be regulated by the rate of synthesis of cholesterol in those cells.

Methods

All studies were carried out while the patient was in the General Clinical Research Center (GCRC) at the Mount Sinai Medical Center. During the entire period of study, both as an outpatient and during the GCRC admissions, the patient consumed a diet consisting of 30% fat, 50% carbohydrate, and 20% protein. The diet had a ratio of polyunsaturated to saturated fat of 1.0 and included < 200 mg cholesterol per d. Percutaneous liver biopsies and measurements of lipoprotein metabolism were performed during each GCRC admission. After the first admission and after several months of diet therapy, a trial of therapy with gemfibrozil was undertaken. Because there was no change in plasma lipid levels over the next 3 mo, gemfibrozil was discontinued and therapy with lovastatin was initiated. The patient was followed closely for the next 6 mo and was then readmitted to the GCRC for repeat studies. All investigational tests were approved by the Institutional Review Board at the Mount Sinai Medical Center. Studies were performed with the consent of the patient and both her parents.

Measurements of the kinetics of apoB metabolism in VLDL, intermediate density lipoprotein (IDL) and LDL were carried out as previously described (6, 7). Briefly, VLDL and LDL were isolated from the patient's plasma by sequential ultracentrifugation and radiolabeled with ¹²⁵I and ¹³¹I, respectively. ¹²⁵I-VLDL was administered intravenously and blood samples were collected at timed intervals for 48 h. ¹³¹I-LDL was then administered and blood samples obtained during the next 2 wk. VLDL, IDL, and LDL were isolated from each blood

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Received for publication 5 March 1987 and in revised form 1 June 1987.

1. Abbreviations used in this paper: apoB, apolipoprotein B; CE, cholesteryl ester; CESD, CE storage disease; FCR, fractional catabolic rate; GCRC, General Clinic Research Center; HMGCoA, hydroxymethylglutaryl coenzyme A; IDL, intermediate-density lipoprotein; PR, production rate; TC, total cholesterol.

J. Clin. Invest.

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0021-9738/87/12/1692/06 \$2.00

Volume 80, December 1987, 1692-1697

sample by sequential ultracentrifugation, and apoB specific radioactivity was determined in each lipoprotein as previously described using 1,1',3,3'-tetramethyl urea (8). Steady-state levels of apoB were determined over the course of the study using a specific radioimmunoassay (9).

The apoB specific activity curves for each lipoprotein (Figs. 1 and 2) were analyzed with compartmental models to generate fractional catabolic rates (FCR) (10, 11). During the baseline study a fraction of the injected dose of ^{131}I -LDL infiltrated the subcutaneous tissue. Lacking a pure pulse injection of tracer (Fig. 2 A), the kinetics of ^{131}I -LDL apoB in that study were analyzed using a model that included both a pulse and an infusion of tracer (12). This model estimated the fraction of injected ^{131}I -LDL that infiltrated the subcutaneous tissues. Both VLDL studies and the second LDL study were carried out without difficulty. The FCRs for apoB in VLDL and LDL in each study were multiplied by the apoB mass in these lipoproteins to calculate the absolute turnover or production rate (PR) of apoB.

Plasma cholesterol and triglyceride levels were determined by enzymatic methods using an ABA-100 (Abbott Laboratories, Chicago, IL). HDL cholesterol was determined after precipitation of whole plasma with heparin-MnCl₂ (12). Urine concentrations of mevalonate were kindly measured in the laboratories of Dr. Thomas Parker and Dr. Roger Illingworth by the method developed by Parker et al. (13).

Results

The patient, who was referred to us for evaluation of short stature, was below the 10th percentile for height and weight, and had massive hepatosplenomegaly. She was otherwise in good health. The diagnosis of CESD was made by the demonstration of deficient lysosomal CE hydrolase by direct assay of both circulating leukocytes and cultured fibroblasts from the patient (14, 15). Cellulose acetate electrophoresis was also used to confirm the absence of the enzyme (15, 16). A percutaneous liver biopsy revealed massive accumulation of cholesteryl esters in lysosomes, as judged by Shultz stain and positive birefringence (1). In addition, there was significant hepatic fibrosis, indicative of early cirrhosis.

Table I presents the results of initial plasma lipid and enzyme diagnostic studies in the 9-yr-old female patient and her parents. The patient had total cholesterol (TC) (271.0 ± 4.4 mg/dl), total triglyceride (150.0 ± 7.8 mg/dl), and LDL cholesterol (184.7 ± 3.4 mg/dl) concentrations that were all above the 95th percentile for her age and sex (16). Her HDL cholesterol

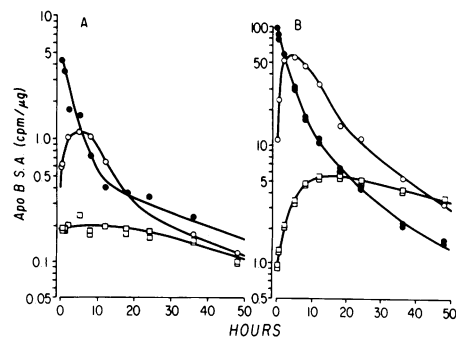


Figure 1. ApoB specific activity in VLDL (●), IDL (○), and LDL (□) after injection of ^{125}I -VLDL before (A) and during (B) lovastatin treatment. Data in both studies indicate complex precursor-product relationships between VLDL and LDL, with an unlabeled source (either IDL or LDL) of LDL apoB strongly suggested during the baseline study (A).

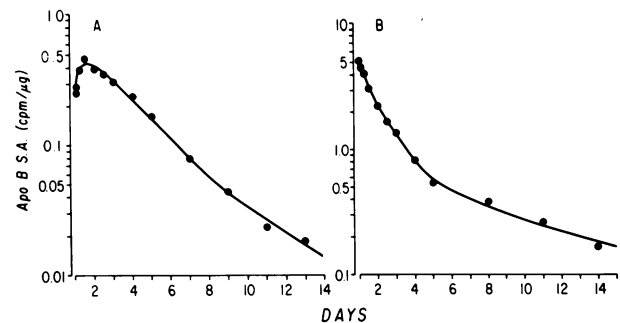


Figure 2. ApoB specific activity in LDL after injection of ^{131}I -LDL before (A) and during (B) lovastatin treatment. The initial rising portion of the LDL apoB specific activity curve in the study before treatment (A) was due to infiltration of some of the injected dose into the subcutaneous tissues around the venipuncture site. This infiltration resulted in an infusion of that portion of the dose into the circulation. The tracer injection during lovastatin therapy was solely intravenous and hence the specific activity curve has only decay components (B).

concentration (24.0 ± 4.6 mg/dl) was below the fifth percentile (16). Both parents were in excellent health and had normal plasma lipid and lipoprotein cholesterol concentrations.

At the time of the baseline studies, plasma levels of VLDL apoB (5.65 ± 0.64 mg/dl) were modestly elevated, whereas the FCR for apoB in VLDL (0.20 h^{-1}) was in the normal range for healthy adult males in our laboratory (7). The production rate for VLDL apoB was 12.2 mg/kg per d, a value within the range measured in normal adult males (7). However, this rate was higher than values we have measured in a 9-yr-old heterozygous female and a 10-yr-old homozygous female with familial hypercholesterolemia (unpublished results). LDL apoB concentrations (141.4 ± 25.4 mg/dl) were increased significantly above normal whereas the LDL apoB FCR (0.38 d^{-1}) was within the normal range (7). The combination of a normal FCR and an elevated concentration of LDL apoB was associated with a significantly increased LDL apoB PR (24.2 mg/kg per d) (7). Compartmental analysis of the apoB specific activity curves for VLDL, IDL, and LDL indicated that $\sim 88\%$ of the VLDL apoB was converted to LDL during the baseline study period. However, the production rates indicated that a significant proportion of apoB-containing lipoproteins was entering the plasma at densities > 1.006 . Finally, concomitant with the overproduction of apoB, urine mevalonate levels were elevated ($2.1 \mu\text{mol/d}$ vs. $1.2 \pm 0.18 \mu\text{mol/d}$ in normal subjects [13]), indicative of high rates of endogenous cholesterol synthesis in this patient.

Based on the patient's clinical status and the results of the metabolic studies indicating overproduction of both endogenous cholesterol and apoB, a trial of lovastatin therapy was initiated with 5 mg/d. During the next 8 mo, plasma levels of TC, triglyceride, and LDL cholesterol gradually decreased as the dose of lovastatin was raised to a final level of 20 mg twice daily (Table II). Plasma HDL cholesterol concentrations were variable but increased slightly overall. At the time of the repeat kinetic studies, the patient had been receiving 20 mg of lovastatin twice daily for 6 wk, and the means of eight determinations of plasma concentrations of TC (196.8 ± 7.9 mg/dl), triglyceride (100.8 ± 20.6 mg/dl), and LDL cholesterol (102.0 ± 10.9 mg/dl) during the 2-wk study period were signifi-

Table I. Biochemical Features

Subject	Age	TC*	TG*	LDLC*	HDLC*	CEH [‡]
	yr	mg/dl	mg/dl	mg/dl	mg/dl	cpm/mg
Patient	9	271.0±4.4	150.0±7.8	184.7±3.4	24.0±4.6	200
Mother	32	192	84	113	62	3,400
Father	34	111	85	51	43	4,460
Normal range [§]		131–197	32–126	68–140	36–73	3,910–6,260

* TC, total cholesterol; TG, triglycerides; LDLC, low-density lipoprotein cholesterol; HDLC, high-density lipoprotein cholesterol. [‡] CEH = cholesteryl ester hydrolase activity measured using cholesteryl ¹⁴C-oleate as substrate (14). Values are in cpm/mg leukocyte protein. [§] Normal range (5–95%) for plasma lipids are presented only for the patient. Both parents had values within the normal range for sex and age (15).

cantly reduced compared with the baseline values (Fig. 3). Neither the ratio of triglyceride to apoB in VLDL (13.2 vs. 11.8; pretreatment vs. post-therapy), nor the ratio of cholesterol to apoB in LDL (1.53 vs. 1.69) were significantly altered by lovastatin treatment. Clinically, the patient did well, with some increase in weight and height and no evidence of any adverse effects of treatment. The size of her liver and spleen were stable during the treatment period, as assessed by liver-spleen scans, and histologic examination of a repeat liver biopsy indicated no significant change in the degree of CE accumulation. Direct measurements of hepatic CE or apoB were not performed. During the course of therapy, urinary mevalonate levels fell in parallel with increases in the dose of lovastatin. At the time of the repeat kinetic studies, 24-h urinary mevalonate excretion rates were 0.27 and 0.20 μ mol/d, commensurate with the reduction achieved in LDL cholesterol (13).

Treatment also resulted in significant reductions in plasma VLDL apoB concentrations (2.18 ± 0.44 mg/dl) (Fig. 4). The FCR for VLDL apoB did not change from the baseline value (0.22 h^{-1}). However, the VLDL apoB PR decreased to 5.2 mg/kg per d during lovastatin therapy. LDL apoB levels (53.2 ± 5.7 mg/dl) fell dramatically during treatment as well (Fig. 5). The FCR of LDL apoB (0.53 d^{-1}) increased by $\sim 40\%$

whereas the LDL apoB PR fell $\sim 50\%$ to 12.7 mg/kg per d. The rate of conversion of VLDL apoB to LDL was unchanged from that determined during baseline, and there still appeared to be significant apoB entering the circulation independent of VLDL secretion.

Discussion

Elevated levels of LDLC have been uniformly associated with CESD (1, 3, 4). This disorder results from the deficiency of lysosomal CE hydrolase activity (1). As a result, LDL CEs delivered to cells via the LDL receptor pathway remain trapped inside lysosomes. This, in turn, leads to inadequate delivery of free cholesterol to a cytosolic pool(s) that appears to regulate the synthesis of endogenous cholesterol by cells. Studies by Goldstein et al. (17) with fibroblasts from a patient with CESD demonstrated reduced ability of LDL-derived CE to suppress HMGCoA reductase in those cells. These results would suggest that the rate of endogenous cholesterol synthesis would be elevated in cells from patients with CESD.

In view of the defects in cellular cholesterol metabolism just outlined, it seemed likely that overproduction of endogenous cholesterol in the liver of patients with CESD might play a role in the pathogenesis of the hypercholesterolemia observed in these individuals. Diminished clearance of plasma LDLC by CE-loaded hepatocytes might also be involved in the etiology of the hypercholesterolemia. Because apoB is necessary for initial hepatic secretion of VLDL, is essentially the sole apolipoprotein in LDL, and is the ligand for LDL clearance via hepatic apoB, E receptors, the rates of synthesis and catabolism of VLDL and LDL apoB were investigated in a subject with CESD. The baseline studies not only supported the possibility that endogenous cholesterol synthesis was elevated in this patient, but they provided direct evidence that the production and secretion of apoB-containing lipoproteins from the liver

Table II. Effect of Lovastatin Therapy

Date	Dose	TC	TG	LDLC*	HDLC
4/9/85	No treatment	305	213	234	28
4/10/85	No treatment	322	219	248	30
4/14/85	5 mg twice daily	322	301	236	26
5/5/85	5 mg twice daily	277	166	194	30
5/12/85	5 mg a.m./10 mg p.m.	251	158	191	28
5/26/85	5 mg a.m./10 mg p.m.	275	151	209	36
6/9/85	10 mg twice daily	256	158	192	36
6/30/85	10 mg twice daily	247	155	156	40
7/14/85	10 mg a.m./15 mg p.m.	235	115	174	38
7/28/85	10 mg a.m./15 mg p.m.	233	125	174	34
8/12/85	15 mg twice daily	206	115	149	34
11/10/85	20 mg twice daily	186	101	128	38

* Calculated according to the methods used in the Lipid Research Clinics Program (12).

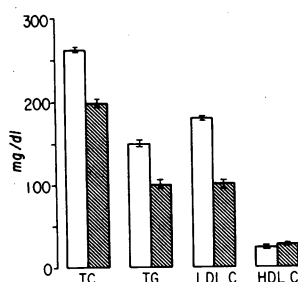


Figure 3. Effect of lovastatin (20 mg twice daily) on plasma lipid levels. Total cholesterol (TC), triglycerides (TG), and LDL cholesterol (LDL C) were all reduced during lovastatin treatment (hatched bars) compared with pretreatment baseline levels (open bars). HDL cholesterol (HDL C) increased slightly during therapy.

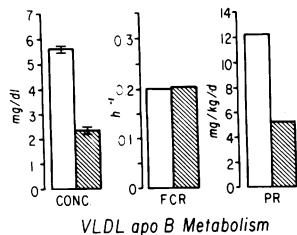


Figure 4. Effect of lovastatin (20 mg twice daily) on plasma VLDL apoB metabolism. During lovastatin treatment (hatched bars) there was a significant reduction in plasma VLDL apoB concentration compared with the baseline level (open bars). This was associated with a marked reduction in

VLDL apoB production (PR) and no change in the fractional catabolic rate (FCR) of VLDL apoB.

were increased as well. Finally, the kinetic studies carried out while the patient was receiving lovastatin demonstrated that inhibition of endogenous cholesterol synthesis by this drug was paralleled by concomitant reductions in apoB production in both VLDL and LDL.

During the baseline studies, measurements of urinary mevalonate levels indicated that endogenous cholesterol synthesis was increased in our patient. Parker et al. (13) demonstrated a strong correlation between plasma and urinary mevalonate levels and cholesterol biosynthesis as determined by sterol balance measurements. These correlations persisted during short- and long-term perturbations of cholesterol production (13). The urinary levels in our CESD patient were two to three times greater than normal, a situation compatible with the proposed pathophysiologic consequence of lysosomal CE hydrolase deficiency. Measurements of plasma mevalonate supported these urinary data (results not shown).

Evidence for increased synthesis of endogenous cholesterol in this CESD patient was associated with an increased rate of secretion into plasma of apoB-containing lipoproteins. Although VLDL apoB production was not elevated in comparison with the rates we reported for normal adults males (7), the PR measured in our patient with CESD was ~40% greater than those observed in two other females of similar age who were homozygous and heterozygous, respectively, for familial hypercholesterolemia (unpublished results). A more clear abnormality, however, was the observation that the LDL apoB PR in the CESD patient was significantly greater than that measured in normal adults by several investigators (7, 18, 19). Indeed, the rate of 24.2 mg/kg per d for LDL apoB production was similar to rates reported for patients with homozygous familial hypercholesterolemia (20–22). Finally, this patient appeared to have an LDL apoB production rate that was twice the rate of apoB entry into plasma as VLDL.

Two caveats concerning these results must be addressed.

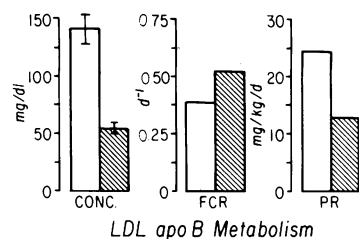


Figure 5. Effect of lovastatin (20 mg twice daily) on plasma LDL apoB metabolism. During lovastatin treatment (hatched bars) there was a significant reduction in plasma LDL apoB concentration compared with the baseline

level (open bars). This was associated with both a marked reduction in LDL apoB production (PR) and an increase in the fractional catabolic rate (FCR) of LDL apoB.

First, we had technical difficulties with the injection of ¹³¹I-LDL in this young, small patient during the baseline study, and a fraction of the tracer infiltrated into the subcutaneous tissue of her forearm. As described in Methods, we analyzed the resultant ¹³¹I-LDL apoB specific activity data (Fig. 2 A) with a model that included both a pulse injection and an infusion of the tracer. The FCR of 0.38 d⁻¹ was estimated with this model. If we either (a) ignored the rising portion of the specific activity curve and fit the remaining data with a single exponential, or (b) ignored the rising portion of the specific activity curve and fit the remaining data with a biexponential curve using the injected dose to estimate an initial specific activity, we generated FCRs of 0.30 and 0.40 d⁻¹, respectively. The calculated production rates of LDL apoB (using the two latter FCR values) were 19.1 and 25.4 mg/kg per d. Hence, all of the three estimated values (including 24.2 mg/kg per d) for LDL apoB PR were significantly elevated in this patient before treatment.

Second, we certainly acknowledge that there is controversy concerning the possible entry of apoB containing lipoproteins into plasma, independent of VLDL production (23). In the present study, underestimation of the rate of flux of VLDL or of the conversion of VLDL to LDL could have made it appear that apoB was entering plasma as IDL or LDL. We sampled very frequently during the first hours after injection of radio-labeled VLDL, and used a complex model, which includes a cascade scheme describing the progressive delipidation of nascent VLDL (10), to analyze the VLDL apoB specific activity data. Hence we believe that it is unlikely that we excluded a fraction of VLDL that was rapidly turning over from our analysis. In addition, our estimates of VLDL conversion to LDL were high during both study periods. Erroneous estimates of apoB flux, both in VLDL and LDL, could also derive from altered composition of these lipoproteins before therapy. Determination of the lipid/protein ratios in both VLDL and LDL suggested that these lipoproteins were of normal composition before treatment. Posttherapy studies did not reveal significant changes in composition. Thus, without ignoring the complexities of analysis of lipoprotein kinetic data, we believe that the present results demonstrate that total apoB production (measured VLDL production plus LDL production minus VLDL apoB converted to LDL) was significantly elevated in this patient before treatment.

Repeat studies of the kinetics of VLDL and LDL apoB metabolism carried during therapy, when endogenous cholesterol synthesis as estimated from urinary mevalonate levels was reduced, clearly demonstrated a reduction in the total flux of apoB-containing lipoproteins, particularly LDL. Kovanen et al. (24) first reported a reduction in LDL flux during administration of lovastatin to dogs. No measurements of VLDL apoB production were made in that study, and so it was not possible to differentiate between increased VLDL removal from plasma without conversion to LDL, and reduced production of VLDL or total apoB by the liver. Lovastatin treatment in normal rabbits resulted in a significant decrease in LDL apoB production without effecting production of VLDL apoB (25). Huff et al. (26) observed that the combination of cholestyramine and lovastatin inhibited LDL apoB production in miniature pigs, also without affecting VLDL flux. In these latter two studies, large proportions of the LDL fluxes at baseline appeared to be independent of VLDL production, and it was likely that the VLDL-independent LDL fluxes were signif-

icantly diminished by lovastatin therapy. Finally, Grundy and Vega (27) reported that lovastatin therapy induced a dose-related reduction in LDL apoB production in subjects with moderate hypercholesterolemia. No VLDL apoB studies were reported by these investigators. In sum, these reports support our findings that LDL apoB flux can be significantly reduced by administration of lovastatin and suggest (25, 26) that a component of this effect acts directly upon secretion of apoB-containing lipoproteins.

Although VLDL is the major apoB-containing lipoprotein produced by the liver, previous studies have not demonstrated a strong link between triglyceride synthesis and apoB production (6, 28). The possibility raised by the present studies, that the concentration of cholesterol within some regulatory pool in hepatocytes might regulate either the synthesis of apoB or its packaging into lipoproteins, is attractive if one considers the primary role of hepatic lipoproteins to be the transport vehicle for endogenously synthesized cholesterol rather than for triglyceride. Therapy with bile acid sequestrants, which also results in increased activity of HMGCoA reductase and elevated rates of hepatic cholesterol synthesis, appears to cause an increase in VLDL production (29). The preliminary report by Monge et al. (30) provides further support for this proposal. These investigators demonstrated that, in vitro, incubation of HepG2 cells with lipoprotein-deficient serum, a condition that would result in increased synthesis of cholesterol by these cells, induced a 60% increase in apoB mRNA and an eightfold increase in apoB concentration in the media.

We were faced with important practical implications of our therapeutic approach when we chose lovastatin to treat this individual. Because lovastatin is thought to increase hepatic LDL receptor activity, a key component of the clinical efficacy of our therapeutic approach was that total apoB flux (and therefore cholesterol flux) would be diminished, and that plasma VLDL and LDL levels would not be reduced simply as the result of upregulation of hepatic LDL receptors by lovastatin. If only the latter occurred, reduced plasma cholesterol concentrations might actually indicate accelerated lysosomal accumulation of cholesteryl ester. Our kinetic data indicated that although the LDL apoB FCR did increase during therapy, the net effect of treatment was a reduction in the absolute flux of lipoproteins. Hence, the absolute delivery of cholesteryl ester to hepatic lysosomes was reduced by lovastatin therapy. We would caution, however, against the general use of this drug in subjects with CESD unless similar kinetic studies, or more direct measurements of hepatic cholesteryl ester content before and after therapy, are available.

In conclusion, total apoB production and endogenous cholesterol synthesis were both increased in a patient with CESD, a disease in which regulation of endogenous cholesterol synthesis by delivery of lipoprotein cholesterol to hepatic cytosolic pools is defective (17). Increased secretion of apoB-containing lipoproteins from the liver resulted in hyperlipidemia which, in turn, probably accelerated the accumulation of lipoprotein-derived CE in hepatic lysosomes. Therapy with lovastatin resulted in diminished cholesterol synthesis and reduced rates of apoB production in both VLDL and LDL. Plasma levels of these lipoproteins decreased and the patient's clinical status appeared to stabilize. These results support the proposal that the rate of endogenous cholesterol synthesis and/or the concentration of free cholesterol in a specific regulatory pool within the hepatocyte plays a role in the regulation

of apoB production. Direct confirmation of this hypothesis, with measurements of apoB mRNA in hepatocytes with and without exposure to lovastatin, is awaited. This study also suggests that lovastatin therapy may be effective in subjects with hypercholesterolemia associated with elevated rates of apoB production, even if LDL receptor function is normal.

Acknowledgments

This work was supported by the following grants: United States Public Health Service HL-23077, HL-36000, AM-34045, and RR-71. Dr. Ginsberg is the recipient of a National Institutes of Health Research Career Development Award (HL-00949). Dr. Short is the recipient of a National Institutes of Health Postdoctoral Fellowship in Medical Genetics (5-T32 HD-07105).

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