Direct Correlation Between Cholesterol Synthesis and Hepatic Secretion of Apolipoprotein B-100 in Normolipidemic Subjects

G.F. Watts, R. Naoumova, M.H. Cummings, A.M. Umpleby, B.M. Slavin, P.H. Sönksen, and G.R. Thompson

The regulation of apolipoprotein B-100 (apo B) metabolism in man is not fully understood. *In vitro* studies suggest a key role for the hepatic availability of cholesterol substrate. We therefore examined whether there was a direct association between plasma mevalonic acid (MVA) concentration (an index of *in vivo* cholesterol synthesis) and hepatic secretion of very–low-density lipoprotein (VLDL) apo B in eight normolipidemic, healthy adult subjects. Hepatic secretion of VLDL apo B was estimated by endogenous labeling of apo B with an 8-hour primed, constant infusion of 1-^{13}C-leucine. Isotopic enrichment of VLDL apo B was measured by gas chromatography–mass spectrometry (GCMS), from which the fractional secretion rate (FSR) was derived by a modified monoexponential function. Plasma concentration of MVA was measured by gas chromatography–electron-capture mass spectrometry in blood samples taken at 9 AM. The absolute secretion rate (ASR) of VLDL apo B (mean ± SD) was 9.7 ± 2.6 mg/kg/d, and MVA concentration was 5.0 ± 2.5 ng/mL. There was a highly significant positive correlation between ASR of VLDL apoB and plasma MVA (r = .88, P = .004), which persisted after adjusting for apo E phenotype. The findings suggest that *in vivo* cholesterol synthesis is a determinant of hepatic secretion of apo B in normolipidemic subjects.

APOLIPOPROTEIN B-100 (apo B) is the major structural protein of the proatherogenic plasma lipoproteins.[1] Apo B is synthesized almost exclusively in the liver, and its hepatic secretion is an important determinant of both plasma lipid levels and risk of atherosclerosis.[1-3] The respective roles of cholesterol ester and triglyceride in regulating apo B secretion have recently been reviewed.[4] Results of experimental studies suggest that the availability of intracellular cholesterol plays a key role in regulating apo B secretion.[5-8] However, the opposite conclusion was reached in more recent studies with Hep G2 cells, which showed that triglyceride synthesis was the major determinant.[9] The availability of free fatty acids may also regulate apo B secretion by controlling the rate of both cholesterol and triglyceride synthesis.[4,6] A perfused-rat liver preparation suggested that cholesterol ester competes with triglyceride for the lipid core of very–low-density lipoprotein (VLDL) particles and that cholesterol feeding can stimulate VLDL secretion.[10] Furthermore, in a patient with cholesterol ester storage disease, pharmacologic inhibition of cholesterol synthesis decreased VLDL apo B secretion.[11] However, there is as yet no evidence that under physiologic conditions rates of cholesterol synthesis and of hepatic secretion of apo B are closely coupled in man.

The principal apo B-containing lipoprotein secreted by the liver is VLDL. Hepatic secretion of VLDL apo B may be studied directly by endogenously labeling of apo B with a stable isotope and sequential measurement of isotopic enrichment using gas chromatography–mass spectrometry (GCMS).[12] This technique overcomes many of the potential drawbacks of conventional radiokinetic methods, including those associated with exogenous labeling of apo B.[12,13] GCMS may also be used to measure the plasma concentration of mevalonic acid (MVA),[14] which reflects activity of the enzyme 3-hydroxy-3-methyl glutaryl coenzyme A (HMG CoA) and therefore the rate of *in vivo* cholesterol synthesis.[15] In the present study, we have used these novel techniques to examine whether there is a direct correlation between rate of hepatic secretion of VLDL apo B and rate of cholesterol synthesis in normolipidemic subjects.

SUBJECTS AND METHODS

Subjects and Clinical Protocol

Eight normolipidemic, healthy subjects recruited from hospital and university employees were studied (Table 1). Plasma lipid measurements were performed after a 12-hour fast using conventional enzymatic methods. High-density lipoprotein cholesterol level was estimated after precipitation of apo B–containing lipoproteins by a heparin-manganese method; low-density lipoprotein (LDL) cholesterol level was calculated by the Friedewald equation. ApoE phenotypes were determined using an isoelectric focusing method.[16] All subjects were consuming ad libitum diets, which were assessed using a 24-hour dietary recall analyzed by a dietitian. None of the subjects had diabetes mellitus, proteinuria, and other secondary causes of hyperlipidemia, or were taking drugs known to influence lipoprotein metabolism. None reported a family history of hyperlipidemia or premature coronary artery disease.

After a 12-hour fast, 1-^{13}C-leucine (99.5 atom % excess) was administered by primed (1 mg/kg), constant (1 mg/kg/h) intravenous infusion for 8 hours via a plastic cannula placed in a superficial vein of the left antecubital fossa. Subjects were allowed only water and were studied in the recumbent position on a metabolic ward. Venous blood was collected half-hourly from the contralateral arm to measure isotopic enrichment of apo B and a-ketoisocaproic acid ([a-KIC] the deamination product of leucine used to estimate precursor pool enrichment).[17] A 9 AM fasting sample was also obtained to measure plasma MVA concentration.[15]

From the Department of Medicine, University of Western Australia, Perth, Western Australia; Departments of Endocrinology and Chemical Pathology, United Medical and Dental Schools of Guys and St Thomas' Hospital, St Thomas' Campus, London; and The Lipoprotein Team, Medical Research Council Clinical Sciences Centre, Hammersmith Hospital, London, UK.

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Address reprint requests to G.F. Watts, MD, Department of Medicine, University of Western Australia, Royal Perth Hospital, Box X2213 GPO, Perth, WA 6001.

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Table 1. Clinical and Biochemical Characteristics of the Subjects

Subject No.	Sex/Age (yr)	Weight (kg)	Body Mass Index (kg/m²)	Cholesterol (mmol/L)	Triglyceride (mmol/L)	LDL Cholesterol (mmol/L)	HDL Cholesterol (mmol/L)	Apo E Phenotype
1	M/42	74	23.9	5.0	1.4	3.5	0.9	E3/E3
2	M/32	76	25.1	4.0	0.9	2.2	1.4	E2/E2
3	M/37	72	23.2	4.8	0.6	3.1	1.4	E2/E3
4	M/48	86	27.7	4.7	1.0	3.2	1.0	E4/E4
5	M/49	72	21.5	5.3	0.9	3.6	1.3	E3/E3
6	F/28	66	23.4	4.1	0.4	2.4	1.5	E3/E3
7	F/50	59	19.9	4.7	1.0	2.5	1.7	E3/E3
8	F/59	57	22.5	6.2	0.8	3.8	2.0	E3/E3
Mean ± SD	43.1 ± 10.1	70.2 ± 9.5	23.4 ± 2.2	4.8 ± 0.6	0.9 ± 0.3	3.0 ± 0.6	1.4 ± 0.3	

Abbreviation: HDL, high-density lipoprotein.

Laboratory Methods

The laboratory methods are fully detailed elsewhere[18] and are summarized in the following paragraphs.

Isolation and measurement of isotopic enrichment of VLDL apo B. VLDL apo B was isolated by preparative ultracentrifugation of whole plasma at a solution density of 1.006 kg/L and 147,000 × *g* for 16 hours. Apo B was precipitated by the tetramethylurea method, which, as in other studies, in our hands is highly specific for apo B.[19-21] The precipitate was then delipidated with ether/ethanol solution (1:3 vol/vol) and then hydrolyzed with 6 mol/L hydrochloric acid. After reconstitution in 50% acetic acid, the amino acids were eluted by cation-exchange chromatography using 3 mol/L ammonia and then transformed into the bis-*tert*-butyldimethylsilyl derivative using acetonitrile and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)- trifluoroacetamide. Samples were then reconstituted in decane for GCMS analysis. Isotopic enrichment was determined by selected ion monitoring of derivatized samples at *m/z* 303 and 302 with a Gas Chromatograph–Mass Spectrometer (VG Biotech TRIO-2; VG Biotech, Aetriucham, UK) using positive-electron–impact ionization. Plasma *a*-KIC was derivatized with pyridine and *NO*-bis(trimethylsilyl)trifluoroacetamide, and isotopic enrichment was determined by selected ion monitoring at *m/z* 233 and 232 (GCMS analysis: Hewlett Packard 5890A, Bracknell, UK). Leucine and *a*-KIC enrichments were calculated using the following formula[22]:

$$\text{enrichment (E)} = \left\{\left[\frac{R_t}{R_t + 1}\right] - \left[\frac{R_0}{R_0 + 1}\right]\right\} \times 100,$$

where R_t is the $^{13}C/^{12}C$ ratio at time t and R_0 is the $^{13}C/^{12}C$ ratio at baseline before infusion of 1-^{13}C-leucine. The coefficient of variation of the complete method, assessed by taking replicate samples (five) at two time points in four of the studies, was less than 8% for isotopic enrichment of both leucine and *a*-KIC.

Quantification of VLDL apo B and MVA. VLDL samples were pooled from six separate hourly time points during the infusion to measure VLDL apo B concentration (milligrams per liter) by the Lowry method[21,23] after precipitation of apo B in these fractions by the tetramethyl-urea (TMU) method (interassay coefficient of variation, 4.5%). Plasma MVA was analyzed using a modification[24] of the method reported by Scoppola et al.[14] Briefly, after addition of [$^2H_3$]-MVA to each sample as internal standard, MVA was converted into the lactone form using Dowex 50 (H^+) and then extracted into dichloromethane-propan-2-ol. After purification and conversion back to the acid form, MVA was esterified to the 3,5-bis(trifluormethyl)benzyl ester and the trimethylsilyl derivative prepared using bis(trimethylsilyl)-trifluoroacetamide. Derivatized samples were analyzed by a Finnigan 4500 quadropole mass spectrometer (Finnigan-MAT SSQ 7000, San Jose, CA). This used electron capture and selected ion-mode monitoring of ions at *m/z* 291 and 294 for detecting the derivatized MVA and [$^2H_3$]-MVA, respectively; the interassay coefficient of variation was 6.7%.

Calculation of VLDL apo B secretion rate. The fractional secretion rate (FSR) of VLDL apo B (pools per day) was determined by a modified monoexponential function,[25] $E(t) = F(1 - e^{k(t-d)})$, where E(t) is enrichment at time t, F is precursor enrichment, d is intrahepatic delay time, and k is the FSR of VLDL apo B. Initial estimates of intrahepatic delay time and FSR were derived from the plot of E(t) of VLDL apo B versus time data. F was estimated from E(t) of *a*-KIC versus time data. Curve-fitting of the data relied on finding the minimum value for the standard error of the estimated parameter (P) expressed as a percentage of P: values for the accepted curve fits were less than 10% for F and d and less than 15% for k. Absolute secretion rates ([ASRs] milligrams per kilogram per day) of VLDL apo B were calculated as the product of FSR and pool size (milligrams). Pool size was derived by multiplying VLDL apo B concentration by plasma volume. Plasma volume (liters) was calculated by multiplying body weight (kilograms) by 0.045.[26]

Statistical Analysis

The correlation between hepatic secretion of VLDL apo B and plasma MVA concentration was examined by simple linear regression analysis, with logarithmic transformation of VLDL apo B to correct for skewness. Adjustments of apo E phenotype and nutrient intake were made by a multiple regression method.

RESULTS

Table 1 lists clinical and biochemical characteristics of the subjects. There were five men and three women; most were middle-aged. None were obese or had a plasma LDL cholesterol concentration greater than 4.0 mmol/L. Five subjects were E3/E3 homozygotes. Daily nutrient intake (mean ± SD) was as follows: 32.2% ± 10.7% energy as total fat, 46% ± 12.7% energy as carbohydrate, and 296 ± 91.1 mg as cholesterol.

Figure 1 shows the isotopic enrichment of VLDL apo B during infusion of ^{13}C-leucine in the patients as a group. Steady-state isotopic enrichment of VLDL apo B was reached after 5 hours. Plateau enrichment of *a*-KIC was reached within 2 hours and remained constant throughout the infusion. There was no statistically significant difference between steady-state isotopic enrichments of VLDL apo B and *a*-KIC.

Table 2 lists variables describing the kinetics of VLDL

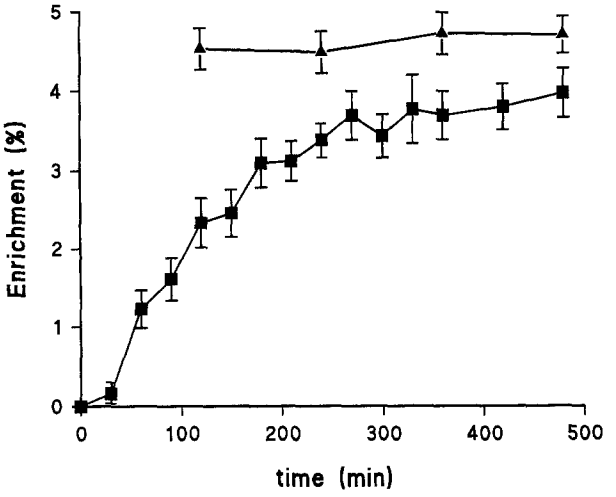

Fig 1. Rates of ^{13}C enrichment of VLDL apo B (■) and plasma α-KIC (▲) in 8 normolipidemic subjects during infusion of L-[1-^{13}C]leucine. Mean ± SEM.

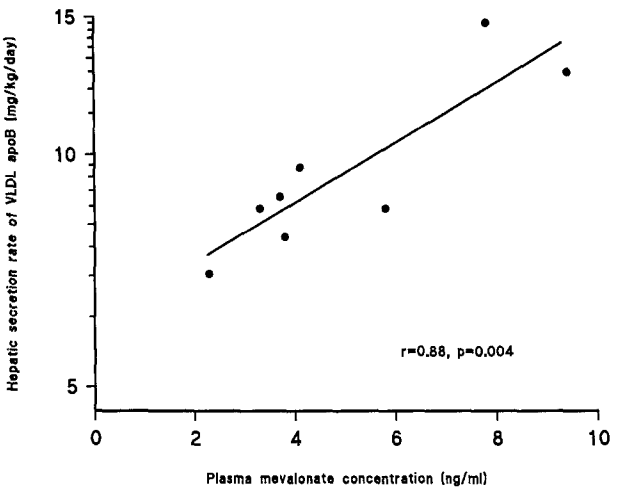

Fig 2. Direct correlation between hepatic secretion rate of VLDL apo B and plasma concentration of MVA in 8 normolipidemic subjects.

apo B with corresponding fasting plasma concentrations of MVA. Values (mean ± SD) for the ASR of VLDL apo B and plasma MVA concentration, 9.7 ± 2.6 mg/kg/d and 5.0 ± 2.5 ng/ml, respectively, were comparable to other reference data.[14,24,25,27,28] There was a highly significant positive correlation between ASR of VLDL apo B and plasma MVA (log[ASR apo B] = 0.04MVA + 0.78, standard error for regression coefficient, 0.009, P = .004, r = .88; Fig 2). MVA was significantly associated with apo E phenotype (P = .04), with subjects with the E2/E2 or E2/E3 phenotypes having a higher plasma concentration than those with the E3/E3 or E4/E4 phenotypes. The association between ASR of apo B and MVA remained significant (regression coefficient 0.04 [standard error, 0.02], P = .039, r = .83) after adjusting for apoE phenotype. Nutrient intake, plasma lipid and lipoprotein concentrations, age, and body mass index were not significantly correlated with the ASR of VLDL apo B or with plasma MVA concentration.

DISCUSSION

We provide the first demonstration of a direct correlation between the rate of cholesterol synthesis and rate of hepatic secretion of VLDL apo B in normolipidemic individuals. Hepatic secretion of VLDL apo B was measured directly using a stable-isotope/mass spectrometry method. Use of plasma MVA to reflect *in vivo* cholesterol synthesis is well supported by other studies.[15] Our finding is consistent with observations in perfused rat liver[7,8,10] and in patients with cholesterol ester storage disease,[11] in which pharmacologic inhibition of HMG CoA reductase, a rate-limiting enzyme in cholesterol synthesis, results in decreased secretion of apo B.

Table 2. Kinetics of VLDL ApoB Metabolism and Plasma Concentrations of MVA in Eight Subjects

Subject No.	VLDL Apo B Pool Size (mg)	FSR of VLDL Apo B (pools per day)	ASR of VLDL Apo B (mg/kg/d)	MVA (ng/mL)
1	35.3	14.6	7.0	2.3
2	112.2	8.6	12.7	9.4
3	78.4	13.5	14.7	7.8
4	97.5	8.5	9.6	4.1
5	115.8	5.5	8.8	3.7
6	23.2	24.1	8.5	3.3
7	29.7	16.8	8.5	5.8
8	40.8	10.9	7.8	3.8
Mean ± SD	66.6 ± 38.6	12.8 ± 5.9	9.7 ± 2.6	5.0 ± 2.5

The turnover of apo B has previously been studied under physiologic conditions using radioisotopic methods[27] and more recently using stable isotopes.[12,28] However, the correlation between apo B turnover and *in vivo* cholesterol synthesis has not been previously examined. We elected to study the kinetics of VLDL apo B, because in normolipidemia most of the apo B secreted by the liver appears in VLDL.[29] Our reference values for the fractional turnover rate are compatible with findings of other stable-isotope studies,[25,28] but are higher than those obtained with some radioisotopic techniques.[27] Exogenous labeling does not achieve uniform incorporation of radioisotope into VLDL apo B subspecies,[13] and conventional radiokinetic methods infer production rates indirectly from catabolic rates.[27] Although labeling with endogenous tracer produces higher transport rates for VLDL apo B than exogenous labeling techniques,[30] it has been proposed that the former may also underestimate the true hepatic secretion of VLDL apo B.[31] However, this has not been directly confirmed.

Our stable-isotope method may have some shortcomings, especially with regard to analysis of the data.[32] Estimation of the precursor pool enrichment during the primed, constant infusion of isotope may be inaccurate. In deriving this from the enrichment of plasma *a*-KIC, we have assumed that the latter is in equilibrium with intrahepatic *a*-KIC and that it also reflects enrichment of hepatic leucyl-t-RNA, the direct precursor of apo B. However, these two assumptions are supported by both *in vitro* and *in*

vivo studies.[33,34] Although *a*-KIC enrichment was not measured during the first 2 hours of ^{13}C-leucine infusion, our data show that at 2 hours it was similar to the steady-state isotopic enrichment of VLDL apo B. We have also used a modified monoexponential function to model the isotopic enrichment data instead of multicompartmental analysis. However, monoexponential analysis examines the integrated metabolism of VLDL apo B subspecies and gives fractional secretion rates that are comparable to results of multicompartment models.[25] Use of a delay function to model secretion of VLDL apo B is supported by experimental observations.[35] Linear regression analysis has been shown to underestimate the flux rate of apo B.[25] Our hypothesis for this study was primarily based on experimental evidence for the role of intrahepatic cholesterol synthesis in regulating VLDL apo B secretion.[7] Khan et al[7,8] showed that lovastatin, an HMG CoA reductase inhibitor, decreased hepatic secretion of VLDL apo B in rats and that this effect was related to a decreased concentration of hepatic cholesteryl esters that resulted from diminished cholesterol synthesis. Cianflone et al[6] demonstrated that in HepG2 cells the rates of cholesterol ester synthesis and apo B secretion were closely coupled after a fatty acid challenge. Increased influx of cholesterol into these cells may also increase secretion of apo B.[36] Relative contributions of the different sources and intrahepatic pools of cholesterol to the regulation of apo B metabolism remain to be established.[37] Given that de novo cholesterol synthesis stimulates acyl-CoA:cholesterol transferase, plasma MVA concentration does not sufficiently distinguish between the effects of rates of synthesis of free cholesterol and esterified cholesterol. *In vivo* studies in man also suggest that the availability of cholesterol may be more important than triglyceride substrate in determining hepatic apo B secretion.[38] In the HepG2 cell, the reverse suggestion has recently been made,[9] with discrepancies probably being due to differences in experimental model and study design. It is likely that both types of lipid alter intrahepatic processing of apo B under different experimental conditions.[4] *In vitro* studies suggest that hepatic apo B secretion is also dependent on the availability of free fatty acids that determine the synthetic rates of both cholesteryl esters and triglycerides.[6,39] Given the limitations of correlational analysis, our study suggests that in fasting normolipidemic subjects the hepatic synthesis of cholesterol probably plays a major role in regulating secretion of apo B. Whether this is in turn dependent on the turnover of free fatty acids remains to be established.

Hepatic cholesterol synthesis and LDL receptor activity are reciprocally related under physiologic conditions.[40] Since we found no significant correlation between plasma LDL cholesterol concentration and apo B secretion in the present study, we propose that the absolute rate of cholesterol uptake by the liver is a less important determinant of apo B secretion in normolipidemic subjects than the rate of intracellular cholesterol synthesis. At the molecular level, the latter probably operates by posttranslational modification of apo B.[36] Our findings may well have been different had we studied patients with familial hypercholesterolemia, in whom increased hepatic uptake of cholesterol by the receptor-independent pathway may be a more important determinant of secretion of apo B than the rate of de novo synthesis of cholesterol.[18] Consistent with this, in a recent stable-isotope study Arends et al[41] did not find that an acute reduction in the circulating mass of apo B and cholesterol significantly influenced hepatic VLDL apo B secretion, but measurements were not made in the steady state and cholesterol biosynthetic rate was not examined. It is possible that our principal finding also applies to the direct hepatic secretion of apo B as LDL, although evidence for the existence of this pathway in man has been challenged.[29] That we found no significant association between VLDL apo B secretion and plasma triglyceride concentration may reflect uncoupling of hepatic secretion of apo B and triglyceride in normolipidemic subjects or the small sample size of our study. Dissociation of hepatic secretion rates of apo B and triglyceride in VLDL has been demonstrated with a carbohydrate challenge both in HepG2 cells and in human subjects.[42,43] Whether this also applies to the fasting state will require confirmation with simultaneous study of the kinetics of VLDL apo B and VLDL triglyceride in a large group of subjects.

MVA is formed principally in the liver by the action of HMG CoA reductase, and approximately 1% of MVA synthesized is transported into plasma.[15] Plasma MVA concentration therefore reflects activity of HMG CoA reductase and is a more convenient measure of the rate of *in vivo* cholesterol synthesis than sterol-balance or isotopic techniques.[14,44] Measurement of MVA level in a single plasma sample taken at 9 AM or in a 24-hour urine collection is highly correlated with a direct estimate of whole-body cholesterol synthesis rate.[15] Cholesterol synthesis exhibits diurnal variation,[15,44] but the prolonged fast used in the present study would have reduced this[15] and potentially increased the accuracy of the 9 AM plasma MVA concentration as an integrated measure of whole-body cholesterol synthesis. Factors that regulate the activity of HMG CoA reductase may influence hepatic secretion of apo B. Increased dietary cholesterol depresses HMG CoA reductase activity *in vitro,*[45] but there was no relationship between cholesterol intake and plasma MVA in our study. This might have been due to the small sample size or cross-sectional design of the study, inaccurate dietary data, or differences in apo E phenotypes.[16] That apo E phenotype was associated with MVA concentration is consistent with regulation of cholesterol uptake by the liver,[16] but did not significantly influence the correlation between MVA concentration and apo B secretion. It is possible that apo E polymorphisms influence hepatic secretion of apo B by altering cholesterol synthesis, but this requires further examination in a larger study. Future studies should use apo E genotype rather than the phenotype method used in the present report, since the latter is less accurate and might have confounded our findings.

This report is consistent with the hypothesis that apo B is constitutively expressed and that its rate of hepatic secre-

tion varies directly with the availability of lipid substrate.[4,20] Future studies should test the effects of increased fatty acid substrate delivery to the liver, dietary interventions, and pharmacologic inhibition of HMG CoA reductase activity. An examination of the role of increased hepatic cholesterol synthesis in the pathogenesis of lipid disorders with elevated hepatic secretion of apo B, such as familial combined hyperlipidemia[20,46] and diabetic dyslipidemia, would also be of interest. Finally, given that elevated hepatic secretion of apo B may result in atherosclerosis,[2,3] it remains to be established at the clinical level whether standardized measurements of plasma MVA levels in normolipidemic subjects may be indirectly used to predict risk of coronary heart disease.

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