Genotypic associations of the hepatic secretion of VLDL apolipoprotein B-100 in obesity

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Abstract **We examined the effect of genetic polymorphisms of proteins regulating intrahepatic processing of apolipoprotein B-100 (apoB) and the supply of neutral lipids to the liver on the hepatic secretion of very low density lipoprotein (VLDL) apoB in obesity. Hepatic secretion of very low density apolipoprotein B-100 (VLDL apoB) was measured using an infusion of [1-^{13}C]leucine in 29 obese men. Isotopic enrichment and turnover of VLDL apoB was determined using gas chromatography–mass spectrometry and multi-compartmental modelling, respectively. Visceral fat was measured by magnetic resonance imaging. Genotypes for the apoB signal peptide (SP27/SP24 alleles), microsomal triglyceride transfer protein promoter (MTP, −493 G/T alleles), apoE (E2, E3, E4 alleles), hepatic lipase promoter (−514 C/T alleles), and cholesteryl ester transfer protein (CETP, Taq1B B1/B2 alleles) were determined using polymerase chain reaction. Statistically significant associations were found between hepatic secretion of apoB and allelic combinations of *i*) apoB SP with apoE ($P = 0.02$), hepatic lipase ($P = 0.02$), and CETP ($P = 0.006$) genes, *ii*) MTP promoter with CETP genes ($P = 0.03$); the association with apoBSP/MTP promoter allelic combinations just failed to reach significance ($P = 0.06$), however. The CETP/apoBSP allelic combination was the most significant predictor of apoB secretion, and this was independent of visceral fat, plasma lathosterol and insulin levels, and dietary fat. SP24 carriers who were homozygous for CETP B1 had 60% lower apoB secretion than B2 heterozygotes who were non-carriers of SP24 (10.5 ± 1.74 mg/kg fat free mass/day, n = 7 vs. 26.1 ± 3.16, n = 22). The data suggest that variation in both the apoB and CETP genes may be a major genetic determinant of the hepatic secretion of apoB in men with visceral obesity.**—Watts, G. F., F. M. Riches, S. E. Humphries, P. J. Talmud, and F. M. Bockxmeer. **Genotypic associations of the hepatic secretion of VLDL apolipoprotein B-100 in obesity.** ***J. Lipid Res.*** **2000.** 41: **481–488.**

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Apolipoprotein B-100 (apoB) is a constitutively expressed glycoprotein that is synthesized exclusively in hepatocytes and secreted into plasma as VLDL (1). Assembly and secretion of VLDL-apoB is complex. It essentially takes place in two steps (2, 3): first, the nascent polypeptide is produced after cotranslational lipidation and translocation into the endoplasmic reticulum (ER); second, neutral lipids and phospholipids are added to the nascent particle in the Golgi to produce mature VLDL. Up to 65% of newly synthesized apoB is degraded intracellularly prior to hepatic secretion (4). Degradation of apoB involves ubiquination and is mediated by proteosomes and facilitated by molecular chaperones in the cytosol and ER (4). The weight of evidence suggests that the physiological regulation of apoB secretion takes place post-translationally. Increased availability of neutral lipids, for example, facilitates the translocation of nascent apoB across the ER, decreases post-translational degradation of the protein, and enhances secretion of the mature apoB-containing VLDL particle by the hepatocyte (1–4). The relative importance of triglyceride cholesterol and cholesteryl esters in the regulation of apoB secretion continues to be debated (5, 6).

Elevated plasma concentrations of apoB are an important risk factor for coronary disease (7) and may explain the increased risk of atherosclerosis in subjects with visceral obesity. We have previously shown that the hepatic secretion of apoB is elevated in men with visceral obesity (8), and that the degree of elevation may depend on a genetic interaction between apoE and apoB signal peptide genotypes (9). The regulatory action of apoE genotype on hepatic apoB secretion was consistent with a previous report (10) and with the effect of the apoE4 allele in in-

Abbreviations: apoB, apolipoprotein B-100; apoE, apolipoprotein E; BMI, body mass index; CETP, cholesteryl ester transfer protein; CV, coefficient of variation; ER, endoplasmic reticulum; FFA, free fatty acid; FFM, fat-free mass; GCMS, gas chromatography–mass spectrometry; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; L4, 4th lumbar vertebra; MRI, magnetic resonance imaging; MTP, microsomal triglyceride transfer protein; SP, signal peptide; VLDL, very low density lipoprotein; VAT, visceral adipose tissue.

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creasing the supply of lipid substrates to the liver (11). The influence of apoB signal peptide was consistent with its role in determining intracellular handling of secretory proteins (12), with demonstration of a positive association between the SP27/27 allele and elevated plasma lipid levels (13), and with in vitro data indicating that SP24 decreases the secretion of apoB17 in rat hepatoma cells and as a consequence enhanced its intracellular proteosomic degradation (14).

Microsomal triglyceride transfer protein (MTP) is a heterodimer that consists of a multifunctional protein-disulfide-isomerase and a unique 97-kDA subunit (15). The latter is responsible for the transport of neutral lipid between intracellular membranes (16). MTP appears to be obligatory for hepatic secretion of apoB (17, 18). High lipid substrate supply and insulin resistance (19), as seen in subjects with visceral obesity, may increase MTP activity and its potential interaction with apoB (20). A common functional G/T polymorphism 493bp upstream from the transcriptional start site of MTP was recently described, with subjects homozygous for the T variant having decreased plasma levels of apoB containing lipoproteins (21). This allele is associated with increased MTP promoter activity in vitro and may enhance hepatic secretion of larger VLDL species with reciprocal decrease in secretion of small VLDL apoB (21). Given the close interactive roles of MTP and apoB in the assembly of VLDL, we predicted that variations in the genetic expression of these proteins may determine the influence of visceral obesity on hepatic secretion of apoB.

As visceral obesity increases the hepatic availability of lipid substrates, another important question to address is the influence on apoB secretion of genes that regulate the supply of neutral lipids to the liver. The potential role of apoE was described previously (9). Hepatic lipase is a lipolytic enzyme that hydrolyzes triglyceride-rich lipoproteins and enhances release and hepatic uptake of cholesteryl esters from HDL (22). A common C to T polymorphism in position −514 in the hepatic lipase promoter region has been described (23), the T allele being associated with lower enzyme activity (24) and decreased potential to deliver neutral lipids to the liver (22). Cholesteryl ester transfer protein (CETP) is also involved in reverse cholesterol transport by transferring cholesteryl ester out of HDL to triglyceride-rich lipoproteins and LDL (25). A common Taq1B polymorphism affecting the 277th nucleotide in the first intron of the CETP gene has been described (26), with the B1 and B2 alleles being associated with high and low CETP activity, respectively. High CETP has been correlated with increased concentration of cholesterol in apoB-containing lipoproteins and low HDL cholesterol (25). The potential roles of genetic variations in hepatic lipase and CETP in the regulation of hepatic secretion of apoB in obesity has not been previously explored.

Our aim in this study of obese subjects was to examine the associations between hepatic secretion of apoB and variations in genes that regulate the intracellular processing of apoB and the supply of neutral lipids to the liver, as well as to test these associations in relation to variations in rates of cholesterogenesis, insulin resistance, and degree of visceral obesity.

METHODS

These have been detailed previously (6) and are summarized below with some modifications.

Subjects and clinical methods

The clinical and biochemical characteristics of the 29 men with visceral obesity studied were: mean age ± SD 47.1 ± 9.2 yr; BMI 34.0 ± 3.1 kg/m^2; waist circumference 114.4 ± 7.8 cm; plasma cholesterol 5.9 ± 0.9 mmol/L; triglyceride 2.8 ± 1.8 mmol/L; HDL cholesterol 1.0 ± 0.2 mmol/L; insulin 16.1 ± 9.2 mU/L; glucose 5.6 ± 0.6 mmol/L; free fatty acids 0.80 ± 0.17 mmol/L; lathosterol 15.4 ± 8.3 mg/L. All subjects consumed ad libitum diets; daily intakes: mean energy ± SD 10262 ± 2233 kJ; fat 103 ± 31.1 g; carbohydrate 219 ± 62.2 g; protein 122 ± 22.9 g; alcohol 25 ± 26.3 g; cholesterol 423 ± 147.1 mg.

Visceral adipose tissue (VAT) area was estimated using magnetic resonance imaging (MRI) and an in-house software method (6). Plasma VLDL apoB turnover was examined after subjects fasted for 14 h using a primed (1 mg/kg), constant (1 mg/kg/h) intravenous infusion (10-h duration) of 1-[^{13}C]leucine, as described previously (5, 6).

Genotyping methods

MTP genotypes were determined as described by Karpe et al. (21) with the exception that the $MgCl_2$ concentration during the polymerase chain reaction (PCR) was 3.5 mmol/L and the *Hph 1* restriction (5 units @ 37°C) fragments of the PCR product were analyzed by polyacrylamide gel electrophoresis (12% T, 3.3% C). ApoB signal peptide and apoE genotypes were determined as described by Xu et al. (13) and Hixson and Vernier (27), respectively. The hepatic lipase promoter gene −514 CT polymorphism was determined using Hsp 92II restriction analysis of PCR products obtained by the method of Guerra et al. (23). The cholesteryl ester transfer protein (CETP) gene Taq I B1/B2 polymorphism was examined as described by Fumeron et al. (28).

Biochemical and modelling techniques

VLDL apoB was isolated at d 1.006 kg/L using preparative ultracentrifugation and precipitated with isopropanol (29). After delipidation and protein hydrolysis, amino acids were extracted by cation-exchange chromatography. After derivatization with MTBSTFA, isotopic enrichment of apoB was determined by gas chromatography–mass spectrometry (GCMS) analysis (Hewlett-Packard 5890) using selected ion monitoring at *m/z* 303 and 302 and electron-impact ionization. VLDL apoB concentration was determined after isopropranolol precipitation by a modified Lowry method (30). Fasting plasma insulin, free fatty acids, and lathosterol were assayed by immuno-enzymometry, enzymatic colorimetry, and GCMS (31), respectively. As indicated previously (8), plasma lathosterol was used as an estimate of the rate of in vivo cholesterol synthesis.

Enrichments were converted to tracer/tracee mass ratios according to the equation $Z(t) = [E(t) / \{E(I) - E(t)\}]$, where $E(t)$ = isotopic enrichment of apoB at time t and $E(I)$ = isotopic enrichment of infusate (32). SAAM-II (SAAM Institute, Seattle, WA) was used to fit a three-compartment model to the tracer/tracee data, as described previously (9). In this model compartment 1 = forcing function for precursor pool based on ^{13}C enrichment of plasma leucine, compartment 2 = adjusted delay for apoB synthesis, compartment 3 = plasma compartment for apoB secre-

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tion. Hepatic secretion rate of VLDL apoB was calculated as fractional turnover rate × pool size, where pool size = plasma volumes x VLDL apoB concentration, plasma volume being estimated from body weight (8, 9).

Statistical analyses

Skewed variables were log.transformed where appropriate to normalize distributions. Binary variables were used to describe MTP, apoB signal peptide, apoE, hepatic lipase, and CETP genotypes. Subjects were grouped according to their carrier status of the SP24 allele (i.e., 0 = SP27/27; 1 = SP24/27, SP24/24), MTP T allele (i.e., 0 = G/G; 1 = G/T, T/T;) apoE2 allele (i.e., 0 = E3/E3, E4/E3, E4/E4; 1 = E3/E2, E4/E2), hepatic lipase allele (i.e., 0 = C/C; 1 = C/T, T/T), and CETP allele (i.e., 0 = B1/B1; 1 = B1/B2, B2/B2). Subjects were then classified into the following allelic combinations: (a) apoB SP and MTP: 0 = SP27/24, SP24/24 + G/T, T/T; 1 = other combinations (b) apoB SP and apoE: 0 = SP27/27 + E3/E3, E4/3, E4/E4; 1 = other combinations (c) apoB SP and hepatic lipase: 0 = SP27/24, SP24/24 + C/T, T/T; 1 = other combinations (d) apoB SP + CETP: 0 = SP27/24, SP24/24 + B1/B1; 1 = other combinations (e) MTP and apoE: 0 = G/T, T/T + E3/E2, E4/E2; 1 = other combinations (f) MTP and hepatic lipase: 0 = G/T, T/T + C/T, T/T; 1 = other combinations (g) MTP and CETP: 0 = G/G + B1/B2, B2/B2; 1 = other combinations. All classifications by genotypes and allelic combinations were hypothesis based, according to previously reported associations with apoB-containing lipoproteins (9, 13–15, 21) or according to the putative influence of functional activity due to altered expression of the protein on hepatic secretion of apoB (22–26, 33). A Student's *t*-test was used to compare groups. Associations between the hepatic secretion of VLDL apoB and other variables were examined using simple and multiple linear regression methods. In multiple linear regression analyses, a dummy variable was used to code allelic combinations as described above. Statistical significance was defined at the 5% level based on two-tailed tests of the null hypothesis.

RESULTS

The plasma leucine and VLDL apoB enrichment curves in the obese subjects were shown previously (8, 9). **Table 1** shows the kinetic parameters for apoB, visceral adipose tissue area measured by MRI at the 4th lumbar vertebra (L4), and genotypes for MTP, apoB SP, apoE, hepatic lipase, and CETP in the obese subjects. Hepatic secretion of VLDL apoB was significantly correlated with visceral fat at L4 ($r = 0.41$, $P = 0.03$) and with plasma triglyceride ($r = 0.47$, $P = 0.01$), but not with plasma insulin, lathosterol, free fatty acids or nutrient intake.

Twelve subjects were MTP G/G homozygotes, 16 were G/T heterozygotes, and 1 was T/T homozygote (rare allele frequency 0.3). Ten subjects were homozygous for the SP27 allele, 15 were heterozygous for the SP24 allele,

TABLE 1. Kinetic parameters for VLDL apoB metabolism, visceral adipose tissue area, and genotypes for apoB signal peptide, MTP promoter, apoE, hepatic lipase promoter and CETP

Subject	VLDL ApoB	VLDL ApoB Pool size	Fractional Catabolic Rate	Hepatic Secretion Rate	VAT at L4	ApoB Signal Peptide	MTP Promoter	ApoE Genotype	Hepatic Lipase Promoter	CETP Genotype
	mg/L	*mg*	*pools/day*	*mg/kg fat free mass/day*	*cm²*					
1	155.6	529.0	4.9	47.7	304	SP27/27	G/T	E3/E3	C/T	B1/B2
2	111.1	400.0	2.5	14.6	316	SP27/24	G/G	E3/E2	C/C	B1/B1
3	53.9	209.7	2.8	7.3	288	SP24/24	G/T	E3/E3	C/T	B1/B1
4	181.8	709.0	4.3	43.0	479	SP27/24	G/T	E3/E3	C/C	B1/B2
5	108.9	409.5	3.8	21.4	353	SP27/27	T/T	E3/E2	C/T	B1/B1
6	34.0	120.7	12.9	25.4	285	SP27/24	G/G	E4/E3	C/C	B2/B2
7	133.0	460.2	7.1	55.0	385	SP27/24	G/G	E4/E2	C/C	B1/B2
8	384.5	1330.4	2.1	43.3	307	SP24/24	G/G	E3/E2	C/T	B2/B2
9	98.9	303.6	4.1	20.8	191	SP24/24	G/T	E4/E3	C/C	B1/B2
10	40.5	131.2	3.4	7.7	203	SP27/24	G/G	E4/E3	C/C	B2/B2
11	45.3	170.3	13.2	31.3	179	SP27/27	G/G	E3/E3	C/C	B1/B1
12	43.3	161.5	4.2	10.8	224	SP27/24	G/T	E3/E3	T/T	B1/B1
13	37.4	129.8	5.5	10.8	161	SP27/24	G/G	E3/E3	C/C	B1/B1
14	33.4	111.2	5.3	9.4	242	SP27/24	G/T	E3/E3	C/C	B1/B1
15	53.1	182.1	6.4	17.3	238	SP27/24	G/G	E3/E3	C/C	B1/B1
16	74.5	260.8	6.1	29.0	300	SP27/24	G/T	E3/E3	C/T	B1/B1
17	54.5	211.5	3.2	11.3	239	SP27/24	G/T	E4/E3	C/C	B1/B2
18	151.1	501.7	5.1	39.6	207	SP24/24	G/G	E4/E4	C/C	B1/B2
19	60.1	207.9	9.9	30.3	175	SP27/27	G/G	E3/E3	C/C	B1/B2
20	65.0	256.1	2.1	7.4	209	SP27/24	G/T	E3/E3	C/T	B2/B2
21	98.9	351.1	3.5	19.7	238	SP27/24	G/T	E3/E2	C/C	B2/B2
22	310.6	1052.9	2.9	42.6	232	SP27/27	G/T	E4/E3	T/T	B1/B1
22	73.4	264.2	2.3	10.5	277	SP27/27	G/T	E3/E3	C/C	B1/B1
24	58.9	221.5	1.0	3.2	223	SP27/24	G/G	E3/E2	C/T	B1/B1
25	65.0	236.6	5.6	21.6	322	SP27/27	G/T	E3/E3	C/C	B1/B1
26	100.0	356.0	1.5	8.6	335	SP27/24	G/T	E3/E3	C/C	B1/B2
27	98.3	377.5	5.8	37.7	443	SP27/27	G/G	E4/E3	C/C	B1/B2
28	48.3	171.9	6.5	10.0	194	SP27/27	G/T	E4/E2	C/T	B2/B2
29	53.9	191.9	7.2	10.3	206	SP27/24	G/T	E3/E2	C/T	B1/B2
Mean	97.5	345.5	4.8	22.3	267.4					
± SD	80.1	275.9	3.0	14.7	78.7					

VAT, visceral adipose tissue; L4, 4th lumbar vertebra.

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TABLE 2. Hepatic secretion rate of VLDL apoB in obese subjects according to pre-specified allelic combinations for the apoB signal peptide, MTP promoter, apoE, hepatic lipase promoter, and CETP genes

Gene	Allelic Combinations	N	Hepatic Secretion of VLDL ApoB	*P* Value for Difference
			mg/kg fat free mass/day	
ApoB signal peptide	SP27/27	10	28.2 ± 3.99	0.056
	SP27/24, SP24/24	19	19.2 ± 3.40	
MTP promoter	G/G	12	26.4 ± 4.61	0.356
	G/T, T/T	17	19.5 ± 3.26	
ApoE	E3/E3, E4/3, E4/E4	21	22.4 ± 3.0	0.698
	E3/E2, E/E2	8	22.2 ± 6.8	
Hepatic lipase promoter	C/C	18	23.0 ± 3.25	0.395
	C/T, T/T	11	21.2 ± 5.02	
CETP	B1/B1	13	17.7 ± 3.11	0.180
	B1/B2, B2/B2	16	26.1 ± 4.09	

Mean ± SEM shown.

and 4 were homozygous for the SP24 allele (rare allele frequency SP24 0.4). Fourteen subjects were apoE3/E3 homozygotes, 6 were E4/E3 heterozygotes, 6 were E3/E2 heterozygotes, 2 were E4/E2 heterozygotes, and 1 was E4/E4 homozygote (allele frequency apoE4 0.17, apoE2 0.12). Eighteen subjects were homozygous for the hepatic lipase CC allele, 9 were heterozygous for C allele, and 2 were homozygous for the T allele (allele frequency 0.34). Thirteen subjects were homozygous for the CETP B1 allele, 10 were heterozygous for the B1 allele, and 6 were homozygous for the B2 allele (allele frequency 0.4).

Table 2 shows the hepatic secretion of VLDL apoB in the subjects according to pre-specified allelic combinations for individual genes. Carriers of the SP24 allele of the apoB signal peptide had 32% lower apoB secretion than non-carriers of the allele ($P = 0.06$). Carriers of the T allele of the MTP promoter also tended to have a 20% lower apoB secretion than non-carriers of this allele ($P = 0.22$). Hepatic secretion of apoB was similar in relation to carrier status of the apoE2 and hepatic lipase T alleles. Carriers of the B2 allele of the CETP gene had 47% higher secretion than non-carriers of the allele ($P = 0.11$). In a regression analysis including all allelic combinations, increased hepatic secretion of apoB was independently associated with homozygosity for the apoB SP27 allele ($P = 0.03$) and heterozygosity for the CETP B2 allele ($P = 0.049$). When the fractional catabolic rate of VLDL apoB was compared between allelic combinations for each gene, it was only found to be significantly different with hepatic lipase, being 40% lower with carriers of the T allele: CT, TT 3.34 ± 0.425 versus CC 5.64 ± 0.786 pools/day, $P = 0.039$. There were no signifi-

TABLE 3. Hepatic secretion rate of VLDL apoB in obese subjects according to allelic combinations of both the apoB signal peptide and MTP promoter genes and allelic combinations of each of these genes with polymorphisms of the apoE, hepatic lipase promoter, and CETP genes

Genes	Allelic Combinations	N	Hepatic Secretion of VLDL ApoB	*P* Value for Difference
			mg/kg fat free mass/day	
ApoB SP + MTP	SP27/24, SP24/24 + G/T, T/T	10	14.8 ± 3.5	0.061
	Other combinations	19	26.3 ± 3.5	
ApoB SP + apoE	SP27/27 + E3/E3, E4/E3, E4/E4	8	31.3 ± 4.17	0.022
	Other combinations	21	18.9 ± 3.15	
ApoB SP + hepatic lipase	SP27/24, SP24/24 + C/T, T/T	6	13.7 ± 6.03	0.019
	Other combinations	23	24.6 ± 2.94	
ApoB SP + CETP	SP27/24, SP24/24 + B1/B1	7	10.5 ± 1.74	0.006
	Other combinations	22	26.1 ± 3.16	
MTP + apoE	G/T, T/T + E3/E2, E4/E2	4	15.3 ± 3.03	0.547
	Other combinations	25	23.4 ± 3.08	
MTP + hepatic lipase	G/T, T/T + C/T, T/T	9	20.7 ± 5.22	0.652
	Other combinations	20	23.1 ± 3.26	
MTP + CETP	G/G + B1/B2, B2/B2	7	34.1 ± 5.68	0.026
	Other combinations	22	18.6 ± 2.71	

Mean ± SEM shown.

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TABLE 4. Multiple linear regression model showing association between the hepatic secretion of VLDL apoB (mg/kg FFM/day) and allelic combinations of apoB signal peptide (SP) and CETP genes, and degree of visceral fat at L4 vertebra (A), and after adjusting plasma lathosterol and insulin concentrations and dietary fat intake (B)

Predictor Variable	Regression Coefficient (SE)	β-coefficient	*P* Value
A			
ApoB SP/CETP allelic combination[a]	0.731 (0.266)	0.44	0.011
Visceral adipose tissue at L4 (cm^2)	0.003 (0.001)	0.33	0.049
R^2 = 35.7%			
B			
ApoB SP/CETP allelic combination[a]	0.692 (0.264)	0.42	0.015
Visceral adipose tissue at L4 (cm^2)	0.003 (0.002)	0.36	0.043
Lathosterol (mg/L)	0.025 (0.014)	0.29	0.085
Insulin (mU/L)	0.014 (0.012)	0.17	0.281
Dietary fat (% energy)	0.016 (0.017)	0.15	0.350
R^2 = 46.3%			

[a] 0 = SP27/24, SP24/24 + B1/B1; 1 = other allelic combinations.

cant differences in the cholesterol and triglyceride content of VLDL among the genotypes tested.

Table 3 shows the hepatic secretion of VLDL apoB according to allelic combinations of pre-specified pairs of genes. Carriers of both the apoB SP24 allele and the T allele of MTP promoter had 44% lower apoB secretion (P = 0.06) than non-carriers of both of these alleles. Subjects who were both homozygous for the SP27 allele and not carriers of the apoE2 allele had significantly higher apoB secretion (P = 0.022) than carriers of the SP24 and E2 alleles, as reported previously (9). Carriers of both the SP24 allele and the T allele of hepatic lipase promoter showed 56% lower apoB secretion (P = 0.019) than other allelic combinations of this gene pair. Carriers of the SP24 allele who were also B1B1 homozygous for the CETP promoter had 59% lower apoB secretion ($P < 0.001$) than subjects who were both non-carriers of SP24 and heterozygous for the B2 allele. Carriers of both the T allele of MTP and the E2 allele tended to have a lower apoB secretion than other allelic combinations of these genes, but the difference was not significant (P = 0.086). Subjects who were both homozygous for the MTP G allele and carriers of the CETP B2 had significantly higher apoB secretion rate (P = 0.012) than carriers of the T allele and non-carriers of the B2 allele. There was no significant association between hepatic secretion of apoB and allelic combinations of hepatic lipase and MTP promoter genes. Plasma cholesterol, triglyceride, HDL cholesterol, and apoB concentrations and dietary fat intake did not differ significantly between allelic combinations.

In stepwise regression, the SP24/CETP B1B1 allelic combination was the most significant predictor of apoB secretion (R^2 25.2%, regression coefficient (SE) 0.834 (0.277), P = 0.006). In multivariate analysis, the hepatic secretion of apoB was independently associated with the SP24/CETP B1B1 allelic combination (P = 0.01) and the degree of visceral fat at L4 (P = 0.049) (**Table 4A**). These associations remained significant after adjusting for plasma lathosterol and insulin concentrations and dietary fat intake, Table 4B summarizing the best regression model for the whole data set.

DISCUSSION

Our results indicate that genes that are involved in the regulation of the intrahepatic processing of apoB and the rate of lipid supply to the liver may determine the hepatic output of apoB in obesity. The most significant correlate of hepatic output of apoB was the combination of genetic polymorphisms in apoB signal peptide and CETP. This novel association was independent of the degree of visceral adiposity, rate of cholesterogenesis, dietary fat intake, and fasting insulin. Because plasma concentrations and hepatic output of apoB are elevated in relation to coronary disease (7, 34), the mutations identified in the present study may also be predictive of risk of cardiovascular disease in obese subjects.

We have extended our previous study (9) with evidence that apoB SP24 interacts with the MTP T, hepatic lipase T, and CETP B1 alleles to decrease hepatic output of apoB in obesity. The apoB SP24/CETP B1B1 combination was more strongly associated with apoB secretion than the apoB SP/apoE genotypic combination reported previously. As with other signal peptides (35), the apoB SP is involved in regulating protein translocation in the endoplasmic reticulum. The signal sequence probably interacts with a signal recognition particle to bind the ribosome and nascent apoB chain to the endoplasmic reticulum. Our results indicate that the apoB SP24 variant may regulate this process, and this is consistent with data from our group showing decreased secretion of apoB-17 with this mutation in rat hepatoma cells (14).

The precise molecular mechanism whereby CETP may regulate apoB is unclear. The CETP B1 allele is associated with increased CETP activity, but this may be conditional on environmental factors such as alcohol intake (28). In hypertriglyceridemic subjects, high CETP activity increases transfer of triglycerides to LDL and HDL and of cholesterol from these particles to $VLDL_1$ (25, 36). This implies that reverse cholesterol transport to the liver via HDL and possibly LDL pathways would be decreased (37), and this in turn would reduce the hepatic output of apoB (33). In randomly selected healthy subjects, increased CETP activity

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has been associated with an increased serum concentration of apoB (28). The potentially favorable effect of the CETP B1 allele on apoB metabolism also has to be interpreted in the light that this mutation is associated with progression of coronary disease (38), but in this study of Kuivenhoven et al. obese patients were not examined.

Hepatic lipase enhances hepatic uptake of cholesteryl esters from apoB-containing lipoproteins, as well as from HDL particles (22, 39); the lower apoB secretion associated with the hepatic lipase T allele in our study is consistent with decreased enzyme activity (24) and a rate-limiting effect of cholesterol substrate availability on apoB secretion in obesity. Lipoprotein-derived cholesteryl ester pools may regulate VLDL apoB secretion by hepatocytes (40), but how this compares quantitatively with the effect of de novo synthesized lipids remains unclear.

MTP is obligatory for the hepatic assembly of apoB-containing lipoproteins (16–18), and particularly for the first step of assembly of nascent apoB particles (41). The second step involves addition of core lipids to nascent apoB (2, 3), and it is possible that in the setting of visceral obesity the supply of lipid substrates to the liver, as determined by CETP, apoE and hepatic lipase, may have a rate-limiting effect on apoB secretion. Our results support this hypothesis. The interaction between MTP and apoB in the process of assembly and secretion of the VLDL is well described (16, 20), but our findings suggest that proteins that regulate cholesterol supply to the liver may also play an important role in vivo. Whilst our findings in respect of the MTP promoter polymorphisms are consistent with those of Karpe et al. (21) in non-obese individuals, they are also paradoxical because the T allele increases expression of MTP (21). The effect observed here may be due to the consequences of insulin resistance and increased availability of lipid substrates on the expression of MTP itself (4, 19, 42), or to linkage disequilibrium of the T allele with other regulatory regions of the MTP promoter region, although a search for other significant promoter polymorphisms has proved uninformative (43).

A novelty of our approach is that we quantified visceral fat using magnetic resonance imaging (44) and showed a positive correlation with hepatic oversecretion of apoB. This association may be a consequence of hepatic insulin resistance, increased portal supply of free fatty acids, and increased neutral lipid accumulation in the liver (45–47). Obesity and insulin resistance may be associated with increased CETP activity (25, 48) and it is possible that this is enhanced by the CETP B1 allele, with the consequence of decreased delivery of cholesteryl esters to the liver via the HDL pathway. Visceral adiposity has also been correlated with increased hepatic lipase activity (49) which in turn may increase the uptake of cholesteryl esters and triacylglycerol by the liver. We speculate that the hepatic lipase T allele may diminish this process. Insulin has also been shown to negatively regulate MTP gene expression in HepG2 cells (19), but in our study did not appear to contribute to the association of apoB secretion with the MTP or apoB SP genotype. The positive correlation between apoB secretion and plasma lathosterol and triglyceride concentrations has been reported previously (8, 50). Our findings suggest that in obesity the hepatic availability of neutral sterols may determine the processing and secretion of apoB independent of the genotypes examined in the study. Neutral lipid availability may be particularly important in the second step of the assembly of apoB (2, 3) and may in part be dependent on the delivery pathways that involve hepatic lipase, CETP, and apoE genotypes. Dietary fats may increase transcription of MTP experimentally (42), but in our study we did not confirm that it influenced the genetic associations reported. Our results might have been different, particularly in respect of apoE genotypes, had we studied hepatic apoB secretion in the postprandial state (33).

In addition to the study limitations discussed previously (9), we did not examine the conversion of VLDL apoB to IDL or LDL apoB, or the direct hepatic secretion of LDL apoB. We would anticipate an increase in the synthesis of these lipoproteins from VLDL in subjects who are not carriers of both the MTP T or apoB S24 alleles (21). The conversion rates of VLDL apoB to other apoB-containing lipoproteins are likely to have been influenced by genetic polymorphisms of hepatic lipase (22), as evidenced by the dependence of the catabolic rate of VLDL on the hepatic lipase T allele. Lipoprotein lipase gene polymorphisms may also influence VLDL apoB catabolism (51), but their frequencies in this sample population were too low to include them in statistical analyses. We were unable to make inferences regarding VLDL species, but would expect the impact of the MTP T variant to be greatest on the small size subfraction ($VLDL_2$), as suggested elsewhere (21). Because an accumulation of visceral adipose tissue results in clustering of metabolic abnormalities (45), our study design was also insufficient to dissociate the independent effects of different lipid substrates and their genetic interactions on hepatic secretion of apoB. Finally, we consider it unlikely that the statistically significant observations reported here are due to a type I error as our search for statistical associations was based on a priori hypotheses. Nevertheless, the small sample size of the study may have diminished the full impact of certain genotypes, particularly the MTP T allelic variant.

In conclusion, genetic variation in apoB SP appears to interact with apoE, hepatic lipase, and CETP variants to regulate apoB secretion in obesity. MTP and CETP polymorphisms may also interact, but the impact on apoB secretion may be less significant than the genetic influences of the apoB SP. The results underscore the potential roles of genes that regulate intrahepatic processing of apoB and lipid substrates supply to the liver in determining apoB output in insulin-resistant and possibly diabetic states. The impact of visceral adiposity and rate of de novo cholesterol synthesis may also be independent predictors of apoB secretion in obesity. Our findings need to be confirmed in a larger sample of unselected subjects and in diverse populations. Whether these genotypic associations determine the response of apoB metabolism to weight reduction and risk of cardiovascular disease in obesity also

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merits examination, as do also the precise molecular mechanisms responsible for the findings that we have reported.

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REFERENCES

1. Yao, Z., and R. S. McLeod. 1994. Synthesis and secretion of hepatic apolipoprotein B-containing lipoproteins. *Biochim. Biophys. Acta.* **1212:** 152–166.
2. Pease, R. J., and J. M. Leiper. 1996. Regulation of hepatic apolipoprotein-B-containing lipoprotein secretion. *Curr. Opin. Lipidol.* **7**: 132–138.
3. Boren, J., S. Rustaeus, and S-O. Olofsson. 1994. Studies on the assembly of apolipoprotein B-100 and B-48-containing very low density lipoprotein in McArdle RH-7777 cells. *J. Biol. Chem.* **269:** 25879–25888.
4. Yao, Z., K. Tran, and R. S. McLeod. 1997. Intracellular degradation of newly synthesized apolipoprotein B. *J. Lipid Res.* **38:** 1937–1953.
5. Wu, R., N. Sakata, E. Lui, and H. N. Ginsberg. 1994. Evidence for a lack of regulation of the assembly and secretion of apolipoprotein B-containing lipoprotein from HepG2 cells by cholesteryl ester. *J. Biol. Chem.* **269:** 12375–12382.
6. Zhang, Z., K. Cianflone, and A. D. Sniderman. 1999. Role of cholesterol ester mass in regulation of secretion of apoB100 lipoprotein particles by hamster hepatocytes and effects of statins on that relationship. *Arterioscler. Thromb. Vasc. Biol.* **19:** 743–752.
7. Lamarche, B., S. Moorjani, P. J. Lupien, B. Cantin, P-M. Bernard, G. R. Dagenais, and J-P. Després. 1996. Apolipoprotein A-I and B levels and the risk of ischemic heart disease during a five-year follow-up of men in the Québec Cardiovascular Study. *Circulation*. **94:** 273–278.
8. Riches, F. M., G. F. Watts, R. P. Naoumova, J. M. Kelly, K. D. Croft, and G. R. Thompson. 1998. Hepatic secretion of very-low-density lipoprotein apolipoprotein B-100 studied with a stable isotope technique in men with visceral obesity. *Int. J. Obesity.* **22**: 414–423.
9. Riches, F. M., G. F. Watts, F. M. van Bockxmeer, J. Hua, S. Song, S. E. Humphries, and P. J. Talmud. 1998. Apolipoprotein B signal peptide and apolipoprotein E gentotypes as determinants of the hepatic secretion of VLDL apoB in obese men. *J. Lipid Res.* **39:** 1752–1758.
10. Demant, T., D. Bedford, C. J. Packard, and J. Shepherd. 1991. Influence of apolipoprotein E polymorphism on apolipoprotein B-100 metabolism in normolipemic subjects. *J. Clin. Invest.* **88:** 1490–1501.
11. Davignon, J., R. E. Gregg, and C. F. Sing. 1988. Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis.* **8:** 1–21.
12. Sturley, S. L., P. J. Talmud, R. Brasseur, M. R. Culbertson, S. E. Humphries, and A. D. Attie. 1994. Human apolipoprotein B signal peptide sequence variants confer a secretion-defective phenotype when expressed in yeast. *J. Biol. Chem.* **269:** 21670–21675.
13. Xu, C., M. J. Tikkanen, J. K. Huttunen, P. Pietinen, R. Butler, S. Humphries, and P. Talmud. 1990. Apolipoprotein B signal peptide insertion/deletion polymorphism is associated with Ag epitopes and involved in the determination of serum triglyceride levels. *J. Lipid Res.* **31:** 1255–1261.
14. Benhizia, F., M. Reina, S. Sturley, S. Vilaro, H. Ginsberg, S. Humphries, and P. Talmud. 1997. Variations in the human apolipoprotein B signal peptide alter apoB secretion and degradation in McArdle RH-7777 rat hepatoma cells. *Atherosclerosis.* **134:** 65 (abstract).
15. Wetterau, J. R., K. A. Combs, S. N. Spinner, and B. J. Joiner. 1990. Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex. *J. Biol. Chem.* **265**: 9801–9807.
16. Gordon, D. A. 1997. Recent advances in elucidating the role of the microsomal triglyceride transfer protein in apolipoprotein B lipoprotein assembly. *Curr. Opin. Lipidol.* **8:** 131–137.
17. Sharp, D., L. Blinderman, K. A. Combs, B. Kienzle, B. Ricci, K. Wager-Smith, C. M. Gil, C. C. Turck, M. E. Bouma, D. J. Rader, L. P. Aggerbeck, R. E. Gregg, D. A. Gordon, and J. R. Wetterau. 1993. Cloning and gene defects in microsomal triglyceride transfer protein associated with abetalipoproteinaemia. *Nature.* **365:** 65–69.
18. Jamil, H., C-H. Chu, J. K. Dickson, Y. Chen, M. Yan, S. A. Biller, R. E. Gregg, H. R. Wetterau, and D. A. Gordon. 1998. Evidence that microsomal triglyceride transfer protein is limiting in the production of apolipoprotein B-containing lipoproteins in hepatic cells. *J. Lipid Res.* **39:** 1448–1454.
19. Hagan, D. L., B. Kienzle, H. Jamil, and N. Hariharan. 1994. Transcriptional regulation of human and hamster microsomal triglyceride transfer protein genes. *J. Biol. Chem.* **269:** 28737–28744.
20. Wu, X., M. Zhou, L. Huang, J. Wetterau, and H. N. Ginsberg. 1996. Demonstration of a physical interaction between microsomal triglyceride transfer protein and apolipoprotein B during the assembly of apoB-containing lipoproteins. *J. Biol. Chem.* **271:** 10277–10281.
21. Karpe, F., B. Lundahl, E. Ehrenborg, P. Eriksson, and A. Hamsten. 1998. A common functional polymorphism in the promoter region of the microsomal triglyceride transfer protein gene influences plasma LDL levels. *Arterioscler. Thromb. Vasc. Biol.* **18:** 756–761.
22. Santamaria-Fojo, S., C. Handenschild, and M. Amar. 1998. The role of hepatic lipase in lipoprotein metabolism and atherosclerosis. *Curr. Opin. Lipidol.* **9:** 211–219.
23. Guerra, R., J. Wang, S. M. Grundy, and J. C. Cohen. 1997. A hepatic lipase (LIPC) allele associated with high plasma concentrations of high density lipoprotein cholesterol. *Proc. Natl. Acad. Sci. USA.* **94:** 4532–4537.
24. Tahvanainen, E., M. Syvanne, M. Heikki Frick, S. Murtomäki-Repo, M. Antikanien, Y. A. Kesaniemi, H. Kauma, A. Pasternak, M. R. Taskinen, and C. Ehnholm. 1998. Association of variation in hepatic lipase activity with promoter variation in the hepatic lipase gene. *J. Clin. Invest.* **101:** 956–960.
25. Tall, A. R. 1993. Plasma cholesteryl ester transfer protein. *J. Lipid Res.* **34:** 1255–1274.
26. Kondo, I., K. Berg, D. Drayna, and R. Laura. 1989. DNA polymorphism at the locus for human cholesteryl ester transfer protein (CETP) is associated with high density lipoprotein cholesterol and apolipoprotein levels. *Clin. Genet.* **35:** 49–56.
27. Hixson, J. E., and D. T. Vernier. 1990. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. *J. Lipid Res.* **31:** 545–548.
28. Fumeron, F., D. Betoulle, G. Luc, I. Behague, S. Ricard, O. Poirier, R. Jemaa, A. Evans, D. Arveiler, P. Marques-Vidal, J-M. Bard, J-C. Fruchart, P. Ducimetiere, M. Appelbaum, and F. Cambieu. 1995. Alcohol intake modulates the effect of a polymorphism of the cholesteryl ester transfer protein gene on plasma high density lipoprotein and the risk of myocardial infarction. *J. Clin. Invest.* **96:** 1664–1671.
29. Egusa, G., D. W. Brady, S. M. Grundy, and B. V. Howard. 1983. Isopropanol precipitation method for the determination of apolipoprotein B specific activity and plasma concentrations during metabolic studies of very low density lipoprotein and low density lipoprotein apolipoprotein B. *J. Lipid Res.* **24:** 1261–1267.
30. Cummings, M. H., G. F. Watts, P. J. Lumb, and B. M. Slavin. 1994. Comparison of immunoturbidimetric and Lowry methods for measuring concentration of very low density lipoprotein apolipoprotein B-100 in plasma. *J. Clin. Pathol.* **47:** 176–178.
31. Mori, T. A., K. D. Croft, I. B. Puddey, and L. J. Beilin. 1996. Analysis of native and oxidized low-density lipoprotein oxysterols using gas chromatography-mass spectrometry with selective ion monitoring. *Redox Report*. **2:** 25–34.
32. Cobelli, C., G. Toffolo, and D. M. Foster. 1992. Tracer-to-tracee ratio

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for analysis of stable isotope tracer data: link with radioactive kinetic formalism. *Am. J. Physiol.* **262:** E968–E975.
33. Sniderman, A. D., and K. Cianflone. 1993. Substrate delivery as a determinant of hepatic apoB secretion. *Arterioscler. Thromb.* **13:** 629–636.
34. Kesaniemi, Y. A., W. F. Beltz, and S. M. Grundy. 1985. Comparisons of metabolism of apolipoprotein B in normal subjects, obese patients and patients with coronary heart disease. *J. Clin. Invest.* **76:** 586–595.
35. Rapoport, T. A., M. M. Rolls and B. Jungnickel. 1996. Approaching the mechanism of protein transport across the ER membrane. *Curr. Opin. Cell. Biol.* **8:** 499–504.
36. Millar, J. S., and C. J. Packard. 1998. Heterogeneity of apolipoprotein B-100-containing lipoproteins: what we have learnt from kinetic studies. *Curr. Opin. Lipidol.* **9:** 197–202.
37. Fielding, C. J., and P. E. Fielding. 1995. Molecular physiology of reverse cholesterol transport. *J. Lipid Res.* **36:** 211–228.
38. Kuivenhoven, J. A., J. W. Jukema, A. H. Zwinderman, P. De Knijff, R. McPherson, A. V. G. Bruschke, K. I. Lie, and J. J. P. Kastelein. 1998. The role of a common variant of the cholesteryl ester transfer protein gene in the progression of coronary atherosclerosis. *N. Engl. J. Med.* **338:** 86–93.
39. Choi, S. Y., I. J. Goldberg, and A. D. Cooper. 1998. Interaction between apoB and hepatic lipase mediates the uptake of apoB-containing lipoproteins. *J. Biol. Chem.* **273:** 2056–2062.
40. Liscum, L., and J. R. Faust. 1994. Compartmentation of cholesterol within the cell. *Curr. Opin. Lipidol.* **5:** 221–226.
41. Gordon, D. A., H. Jamil, R. E. Gregg, S-O. Olofsson, and J. Boren. 1996. Inhibition of the microsomal triglyceride transfer protein blocks the first step of apolipoprotein B lipoprotein assembly but not the addition of bulk core lipids in the second step. *J. Biol. Chem.* **271:** 33047–33053.
42. Lin, M. C., C. Arbeeny, K. Bergquist, B. Kienzle, D. A. Gordon, and J. R. Wetterau. 1994. Cloning and regulation of hamster microsomal triglyceride transfer protein. The regulation is independent from that of other hepatic and intestinal proteins which participate in the transport of fatty acids and triglycerides. *J. Biol. Chem.* **269:** 29138–29145.
43. Herrmann, S. M., O. Poirier, V. Nicaud, A. Evans, J. B. Ruidavets, G. Luc, A. Dominique, C. Bao-Sheng, and F. Cambrien. 1998. Identification of two polymorphisms in the promoter of the microsomal triglyceride transfer protein (MTP) gene: lack of association with lipoprotein profiles. *J. Lipid Res.* **39:** 2432–2435.
44. Ross, R., L. Leger, D. Morris, J. DeGuise, and R. Guardo. 1992. Quantification of adipose tissue by MRI: relationship with anthropometric variables. *J. Appl. Physiol.* **72:** 787–795.
45. Bjorntorp, P. 1990. "Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. *Arteriosclerosis.* **10:** 493–496.
46. Lewis, G. F., K. D. Uffelman, L. W. Szeto, B. Weller, and G. Steiner. 1995. Interaction between free fatty acids and insulin in the acute control of very low density lipoprotein production in humans. *J. Clin. Invest.* **95:** 158–166.
47. Sparks, J. D., and C. E. Sparks. 1990. Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes. *J. Biol. Chem.* **265:** 8854–8862.
48. Hayashibe, H., K. Asayama, T. Nakane, N. Uchida, Y. Kawada, and S. Nakazawa. 1997. Increased plasma cholesteryl ester transfer activity in obese children. *Atherosclerosis.* **129:** 53–58.
49. Sattar, N., C. E. Tan, T. S. Han, L. Forster, M. E. Lean, J. Shepherd, and C. J. Packard. 1998. Associations of indices of adiposity with atherogenic lipoprotein subfractions. *Int. J. Obesity.* **22:** 432–439.
50. Riches, F. M., G. F. Watts, R. P. Naoumova, J. M. Kelly, K. D. Croft, and G. R. Thompson. 1997. Direct association between the hepatic secretion of very-low-density lipoprotein apolipoprotein B-100 and plasma mevalonic acid and lathosterol concentrations in man. *Atherosclerosis.* **135:** 83–91.
51. Wittrup, H. H., A. Tybjaerg-Hansen, and B. Nordestgaard. 1999. Lipoprotein lipase mutations, plasma lipids and lipoproteins and risk of ischaemic heart disease. A meta-analysis. *Circulation.* **99:** 2901–2913.

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