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ScienceDirect
Metabolism Clinical and Experimental 57 (2008) 1719–1724
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Genetic variation in phospholipid transfer protein modulates lipoprotein profiles in hyperalphalipoproteinemia

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Received 28 December 2007; accepted 17 July 2008

Abstract

We previously demonstrated the role of a phospholipid transfer protein (PLTP) gene variation (rs2294213) in determining levels of high-density lipoprotein cholesterol (HDL-C) in hypoalphalipoproteinemia (HypoA). We have now explored the role of PLTP in hyperalphalipoproteinemia (HyperA). The human PLTP gene was screened for sequence anomalies by DNA melting in 107 subjects with HyperA. The association with plasma lipoprotein levels was evaluated. We detected 7 sequence variations: 1 previously reported variation (rs2294213) and 5 novel mutations including 1 missense mutation (L106F). The PLTP activity was unchanged in the p.L106F mutation. The frequency of the rs2294213 minor allele was markedly increased in the HyperA group (7.0%) in comparison with a control group (4.3%) and the hypoalphalipoproteinemia group (2.2%). Moreover, rs2294213 was strongly associated with HDL-C levels. Linear regression models predict that possession of the rs2294213 minor allele increases HDL-C independent of triglycerides. These findings extend the association of rs2294213 with HDL-C levels into the extremes of the HDL distribution.

1. Introduction

We define *hyperalphalipoproteinemia* (HyperA [Online Mendelian Inheritance in Man 143470]) as a level of high-density lipoprotein cholesterol (HDL-C) greater than the population-based 80th percentile. In addition, it is characterized by altered HDL particle composition, metabolism, and function, including impaired antioxidant activity [1,2]. Rare causes of familial HyperA include genetic deficiencies of plasma cholesteryl ester transfer protein or hepatic lipase [3]. With the exception of hepatic lipase [4], the contributions of common functional polymorphisms to HyperA remain largely unknown. However, the fact that the heritability of HDL-C levels is estimated at approximately 50% [5] suggests that such polymorphisms exist.

Increasing evidence indicates an important role for phospholipid transfer protein (PLTP) in determining the plasma level of HDL-C [6]. The involvement of PLTP in lipoprotein metabolism is multifold and complex. This includes the transfer of phospholipids between plasma lipoproteins, specifically, phospholipids from triglyceride (TG)-carrying lipoproteins into HDL, and the remodeling of HDL particles in both size and composition [7,8]. In reverse cholesterol transport, adenosine triphosphate–binding cassette A1 promotes the efflux of phospholipids and unesterified cholesterol from cell membranes in the peripheral tissues to pre–β-HDL [9].

Pre–β-high-density lipoprotein is described either as discoidal in particle shape with 2 to 3 molecules of apolipoprotein A-I (plus phospholipids and unesterified cholesterol) or as a monomolecular lipid-free/poor apolipo-

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0026-0495/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2008.07.031

protein A-I [10]. Limited reports from human studies suggest that PLTP activity may be positively correlated to HDL; moreover, it may also be independently and positively related to coronary artery disease [11]. Phospholipid transfer protein overexpression in PLTP transgenic mice increases the influx of phospholipids and cholesterol into HDL, causing an increase in pre–β-HDL and decreased HDL levels [11]. In our previous study [12], we established the influence of a common PLTP gene variant (rs2294213, c.69+26G>C) in a case-control study of hypoalphalipoproteinemia (HypoA). The role of common gene polymorphisms in quantitative variation in HDL-C levels, including both HypoA and HyperA [13], suggests that examination of PLTP variations in HyperA may provide novel insight into its role in HDL metabolism. Herein, we examine the role of PLTP gene variation in HyperA as a means to demonstrate the contribution of this gene locus to quantitative variation in HDL-C.

In a case-control study of HyperA, we screened for sequence anomalies in the PLTP gene. We also tested for genetic association of discovered variations with HypoA and biochemical measurements (ie, lipoprotein compartments). Genetic lesions leading to coding sequence missense changes were examined for potential differences in biochemical properties. We report a number of new sequence anomalies within the PLTP gene and provide further evidence that this gene is involved in determining HDL-C levels. Our findings showed that the rs2294213 minor allele is associated with elevated levels of HDL-C.

2. Methods

2.1. Study design

The design was a retrospective case-control study using a non-Hispanic white sample of subjects from the University of California, San Francisco (UCSF) Genomic Resource in Arteriosclerosis (GRA) [14]. The GRA is a repository of DNA samples from more than 12,000 patients, along with their blood samples and clinical data. Subjects with HyperA (n = 107) were identified from the GRA as individuals with HDL-C levels greater than 80 mg/dL. Healthy controls (n = 365) had normal lipoprotein profiles [15]. All subjects gave informed consent in a protocol approved by the UCSF Committee on Human Research. Clinical and demographic data were available for all subjects, and baseline lipoprotein measurements were obtained when patients had not taken lipid-lowering medications for at least 1 month.

2.2. Measurements

2.2.1. Genotypic and phenotypic studies

Genomic DNA was prepared from whole blood obtained from patients in the GRA population of UCSF [14]. Blood was drawn after a 10-hour fast; lipoprotein quantification was carried out using standard protocols [16-19]. Standards were provided by the Centers for Disease Control (Atlanta, GA). Baseline lipoprotein measurements were obtained when patients had received no lipid-lowering medication for at least 1 month, a standard "washout" period for such medications [14].

2.2.2. Molecular gene scan of PLTP gene

Denaturing high-performance liquid chromatography (dHPLC) [20] and denaturing gradient gel electrophoresis (DGGE) [21] mutation detection were both used to scan the 10 amplicons that spanned the 16 exons encoding the full-length PLTP transcript (BC01984) using standard protocols. Exon 5, which is absent in 1 of the 2 known PLTP splice variants [22], was included in the analysis. Direct DNA sequencing was used further to identify the underlying DNA variations seen with wave (dHPLC) or electrophoresis (DGGE). Nomenclature guidelines for position and sequence changes described herein are available at http://archive.uwcm.ac.uk/uwcm/mg/docs/mut_nom.html.

In our previous report [12], conditions for molecular genetic analysis of the PLTP gene (ie, primer sequences) are provided. Sequence anomalies (ie, mutations, polymorphisms) were screened against public sequence databases (ie, www.genome.ucsc.edu, www.ensembl.org) to determine if they were novel or previously deposited as identified by Reference Sequence ID.

2.2.3. Site-directed mutagenesis

For expression of "wild-type" and mutant PLTP (wild-type PLTP in pSVL plasmid was a gift from Christian Ehnholm, National Public Health Institute, Helsinki, Finland), a pSVL construct was used. Site-directed mutagenesis was carried out using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

2.2.4. Cell culture

COS-7 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, L-glutamine, streptomycin, and penicillin. On the day before transfection, the cells (1.2×10^6) were seeded on 6-cm culture dishes. They were then transiently transfected using PolyFect Reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol and grown in serum-free medium overnight. Supernatants were removed after 72 hours, centrifuged to remove detached cells, and used for PLTP transfer activity and mass analysis.

2.2.5. Specific PLTP activity

Using a radiometric assay [23,24], PLTP activity was measured. In this assay, the ability of PLTP to transfer phospholipids from [C^{14}] dipalmitoyl phosphatidylcholine (DPPC) donor vesicles to ultracentrifugally isolated HDL_3 acceptor particles was measured. Phospholipid transfer protein mass was obtained from Western blots. Culture supernatants were concentrated using acetone precipitation, separated on sodium dodecyl sulfate gels, transferred onto nitrocellulose membranes, and blotted using a monoclonal anti-PLTP antibody JH59 [25] (a gift from Drs Matti Jauhiainen and Christian Ehnholm, National Public Health

Institute, Helsinki, Finland). The relative PLTP mass compared with the wild type was obtained by scanning and quantitation of the PLTP bands. Specific PLTP activity was obtained by dividing the activity by mass and is expressed relative to the wild-type PLTP (wild-type PLTP activity was 727 ± 148 nmol/mL-h and was set to a value of 1.0). All mutations were assayed in 4 replicates and included both positive and negative controls.

2.3. Statistical methods

Statistical analyses were conducted using the SPSS for Windows (version 11.0.1, 2001; SPSS, Chicago, IL) system [26]. The gene-counting method was used to determine allele and genotype frequencies directly. Tests for Hardy-Weinberg equilibrium in controls, and allelic or genotypic association in cases vs controls, were evaluated by χ^2 test. Two-group comparisons of means of transformed or normally distributed variables used the independent-samples Student *t* test (2-tailed). Natural log transformation was found to be appropriate for the normalization of variables. Two-group comparisons of means of untransformed, nonnormally distributed variables used the Wilcoxon 2-sample test. The general linear model was used to fit regression models. Power transformations of potential predictor variables were examined to maximize the explanatory power of the overall model (by maximizing the F statistic). Selected interaction effects and covariate-adjusted means of the transformed responses for levels of categorical factors were tested using procedure general linear model. Interaction effects with *P* less than .10 were retained. Data in tables are presented as the mean ± 1 standard deviation.

3. Results

3.1. Baseline characteristics of the study populations

The clinical characteristics of subjects with HyperA, subjects with HypoA, and healthy controls are described in Table 1. Compared with healthy controls, HyperA subjects were older, had lower body mass index (BMI), and were predominantly women. Hyperalphalipoproteinemia subjects also had higher total cholesterol, low-density lipoprotein cholesterol (LDL-C), and HDL-TG, and lower very low-density lipoprotein (VLDL)–TG and VLDL-cholesterol (VLDL-C) (Table 1).

Table 2
Phospholipid transfer protein rs2294213 allelic and genotype frequencies

	HypoA	Control	HyperA
C/C	264	335	92
C/G + G/G	12	29	15
Total	276	365[a]	107[b]
G allele	2.2%	4.3%	7.0%

Clinical characteristics for the control and HypoA comparison groups reproduced from our previous report [12]. Allelic and genotypic frequencies determined directly by gene counting.

[a] $\chi^2 = 4.436$, 1 *df*, $P = .035$. Odds ratio = 2.035; 95% CI, 1.039-3.985.

[b] $\chi^2 = 11.005$, 1 *df*, $P = .001$. Odds ratio = 3.224; 95% CI, 1.561-6.661.

3.2. Results of PLTP molecular gene scan

Mutational analysis of the 16 exons and flanking regions of the PLTP gene in the HyperA group detected several sequence anomalies, including the single nucleotide polymorphism (SNP) in intron 1 that we reported previously (rs2294213) [12]. One novel missense mutation was identified in exon 4 (p.L106F, c.316C>T). Four novel (c.329+86T>C; c.410-21insT; c.613+121_123delTGT; c.1218+87G>T) and 1 previously identified (c.613+84_89delAGTCCT) [12] mutations were identified in 8 subjects with HyperA.

Relative frequencies of rs2294213 in the HypoA, control, and HyperA groups are 2.2%, 4.3%, and 7.0%, respectively (Table 2). There is a significantly greater frequency of the minor G allele in the HyperA group compared with the control group ($\chi^2 = 4.436$, 1 *df*, $P = .035$; odds ratio [OR] = 2.035; 95% confidence interval [CI], 1.039-3.985) and the

Table 1
Clinical characteristics of the study cohort

Trait		HypoA (n)[c]	Control (n)[c]	HyperA (n)	Test statistic
Age		52.7 ± 20.71 (276)	51.6 ± 20.03 (364)	58.0 ± 16.90 (107)	$t = -3.386$, $P = .001$
Sex (% female)		0.420 (276)	0.313 (364)	0.766 (107)	$\chi^2 = 9.579$, $P = .002$
TG[b]	Total	172.4 ± 76.80 (276)	124.7 ± 61.56 (364)	113.5 ± 53.21 (107)	NS
	VLDL[a]	113.8 ± 72.70 (210)	74.5 ± 57.56 (261)	52.5 ± 37.67 (71)	$t = 3.795$, $P < .001$
	LDL[a]	44.3 ± 18.71 (208)	34.8 ± 13.28 (259)	36.0 ± 15.99 (71)	NS
	HDL	16.3 ± 5.80 (276)	16.6 ± 6.35 (364)	21.6 ± 9.72 (107)	$t = -5.032$, $P < .001$
Cholesterol[b]	Total	210.9 ± 72.93 (276)	193.3 ± 37.30 (364)	263.6 ± 61.25 (107)	$t = -11.197$, $P < .001$
	VLDL[a]	27.0 ± 15.60 (221)	17.9 ± 14.52 (271)	12.0 ± 9.21 (71)	$t = 4.111$, $P < .001$
	LDL	152.3 ± 67.64 (221)	128.4 ± 32.90 (271)	162.1 ± 62.27 (71)	NA
	HDL	31.3 ± 6.30 (276)	48.1 ± 13.97 (364)	94.9 ± 13.67 (107)	NA
BMI		27.9 ± 3.212 (193)	26.0 ± 4.77 (216)	24.5 ± 5.43 (86)	$z = -3.219$, $P = .001$

Values are mean ± SD. NS indicates not significant; NA, not applicable.

[a] Natural log transformation.

[b] Lipid measurements expressed in milligrams per deciliter.

[c] Clinical characteristics for the control and HypoA comparison groups reproduced from our previous report [12].

HypoA group (χ^2 = 11.005, 1 *df*, *P* = .001; OR = 3.224; 95% CI, 1.561-6.661). No significant differences were seen in the minor allele frequency between sexes in the HyperA group (χ^2 = 1.724, 1 *df*, *P* = .19). The genotype distribution did not deviate significantly from Hardy-Weinberg expectations (χ^2 = 0.122, 1 *df*, *P* = .73).

Differences in demographic and clinical characteristics and lipoprotein components were examined by rs2294213 genotype (GG vs GC + CC) and are provided in Table 3. Carriers of the minor allele displayed elevated VLDL-C, as well as modestly elevated levels of TG and VLDL-TG. Minor allele carriers also displayed modestly decreased HDL-C.

3.3. Functional studies of PLTP missense mutations

To study defects in lipid transfer activities of mutant proteins, site-directed mutagenesis was carried out, followed by transient transfection of COS7 cells and subsequent PLTP functional assay (radioisotopic method). Protein content was determined by Western blotting. This variant showed specific transfer activity comparable with wild-type protein (p.L106F, c.316C>T: 111.4% ± 23.2%, *P* > .05, n = 4). The results are mean values ± SE of 4 independent experiments and are expressed as percentages of the activity of wild-type PLTP and assessed by Student unpaired *t* test, 2-tailed. The mutation was synthesized and secreted from the cells as efficiently as the wild-type PLTP.

Table 3
Hyperalphalipoproteinemia group demographic and clinical characteristics according to PLTP rs2294213 genotype

		GG (n = 92)	CG + CC (n = 15)	Statistical test
Age (y)		57.9 ± (16.92)	58.9 ± (17.34)	*t* = −0.219 *P* = .827
TG[b]	Total[a]	109.9 ± (52.54)	135.9 ± (53.6)	t = −1.736, *P* = .085
	VLDL[c]	50.4 ± (37.53)	74.3 ± (34.82)	*z* = −1.861, *P* = .063.
	LDL[c]	36.1 ± (16.28)	35.2 ± (13.62)	*z* = −0.062, *P* = .951
	HDL	21.0 ± (9.02)	25.6 ± (12.88)	*z* = −1.020, *P* = .308
Cholesterol[b]	Total[a]	263.9 ± (63.19)	262.0 ± (49.48)	*t* = −0.027, *P* = .979
	VLDL[c]	11.5 ± (9.34)	17.3 ± (5.72)	*z* = −2.071, *P* = .038
	LDL[c]	161.5 ± (64.00)	168.3 ± (42.61)	*z* = −0.641, *P* = .522
	HDL	95.8 ± (13.78)	89.7 ± (12.12)	*z* = −1.944, *P* = .052
BMI[d]		24.1 ± (4.95)	26.9 ± (7.59)	*t* = −1.223, *P* = .244

[a] Natural log transformation.
[b] Lipid measurements expressed in milligrams per deciliter.
[c] Measurements for this lipoprotein compartment were only available in 65 participants with the GG genotype and 6 participants with the GC or CC genotypes.
[d] Measurements for BMI were only available in 74 participants with the GG genotype and 12 participants with the GC or CC genotypes.

Table 4
Regression analysis of lipoprotein measures

Outcome	Predictor	Coefficient	Standard error	95% CI	*P* value
HDL-C (n = 494)	Genotype	3.55	1.90	−0.19 to 7.93	.063
	Sex	7.67	1.05	5.61 to 9.74	<.001
	HypoA	−20.03	2.35	−24.64 to −15.41	<.001
	HyperA	36.29	2.21	31.95 to 40.63	<.001
	Constant	59.43	0.95	57.55 to 61.30	<.001
			Overall model fit: F = 239.66, *P* < .0001, adjusted R^2 = 0.817		

Multiple linear regression analysis of HDL-C levels. Predictors evaluated in the model included age (in years), sex (female vs male), plasma TG (in milligrams per deciliter), BMI (in kilograms per square meter), clinical category (control vs HyperA, HypoA), PLTP rs2294213 genotype (GG vs GC + CC), and genotype by clinical category interaction. An interaction between clinical category and genotype was observed (*P* = .013), with carriers of the minor allele having higher levels of HDL-C in HypoA (GG: 32.69 [95% CI, 31.06-34.33]; GC + CC: 35.27 [95% CI, 28.21-42.33]) and controls (GG: 48.81 [95% CI, 47.24-50.38]; GC+CC: 59.21 [95% CI, 53.88-64.53]) vs lower HDL-C levels in HyperA (GG: 91.46 [95% CI, 88.84-94.07]; GC + CC: 89.13 [95% CI, 83.03-95.24]). Covariates appearing in the model are evaluated at the following values: age = 52.93 years, TG = 147.1 mg/dL, BMI = 26.5.

3.4. Linear regression analysis of lipoprotein measures in carriers of rs2294213

In the model fitted for HDL-C, PLTP rs2294213 genotype (GG homozygotes vs GC + CC minor allele carriers), participant's age (in years), plasma TG level (in milligrams per deciliter), sex, clinical category (HypoA, control, HyperA), and BMI were significant predictors retained in the model (F = 239.66, *P* < .0001) (Table 4). Controlling for clinical category, age, sex, TG, and BMI, minor allele carriers (GC + CC) had a mean HDL-C level that was 3.55 ± 1.90 mg/dL higher than that of common allele homozygotes (95% CI, −0.19 to 7.93; *P* = .063). An interaction between clinical category and genotype was observed (*P* = .013), with carriers of the minor allele having higher levels of HDL-C in HypoA (GG: 32.69 [95% CI, 31.06-34.33]; GC + CC: 35.27 [95% CI, 28.21-42.33]) and controls (GG: 48.81 [95% CI, 47.24-50.38]; GC + CC: 59.21 [95% CI, 53.88-64.53]) vs lower HDL-C levels in HyperA (GG: 91.46 [95% CI, 88.84-94.07]; GC + CC: 89.13 [95% CI, 83.03-95.24]). The model explained 81.7% of the variance in HDL-C levels in this sample, with genotype accounting for 0.7% of the variability in HDL-C and the clinical category by genotype interaction accounting for an additional 1.8% of the variability in HDL-C.

4. Discussion

Elevated HDL-C is a significant negative risk factor for coronary artery disease [27-29]. In the current study, the PLTP gene was chosen as a potential determinant of HDL-C

levels. The effect of PLTP on HDL-C levels is attributed to its facilitated transfer of phospholipids from TG-rich lipoproteins to HDL and the generation of pre–β-HDL in reverse cholesterol transport.

It is clear from the current findings that the rs2294213 variant of the PLTP gene is associated with the lipoprotein profile, with an increased level of HDL-C in healthy subjects. Interrogation of the International HapMap database (http://www.hapmap.org) yields only 1 SNP (rs2294212) in strong linkage disequilibrium (r^2 = 1.0) with rs2294213, located 128 base pairs downstream (c.69+154G>C) in intron 1. Previous examinations of the human PLTP promoter have failed to include these 2 SNPs [30-34]. Analysis of the 2 alleles represented by rs2294213 using AliBaba 2.1 (http://www.gene-regulation.com/pub/programs/alibaba2) showed the creation of the overlapping putative simian virus 40 protein–1 and yin yang protein–1 binding sites in minor allele carriers (c.69+26C). Promoter analysis of both the LDL receptor [35] and the HDL receptor [36] demonstrated that costimulation of the simian virus 40 protein–1/yin yang protein–1 sites modulates gene expression. A recent survey of SNPs and haplotypes in the PLTP gene provided evidence of an association between another intron 1 polymorphism (rs553359) and obesity-related traits [37]. Unfortunately, rs553359 (and rs2294212) was not included in the amplicons designed to analyze exon 1 and exons 2 and 3. Moreover, rs553359 was not in linkage disequilibrium (r^2 < 0.8) with rs2294213. Functional studies are required to formally demonstrate the functional impact of rs2294213.

Phospholipid transfer protein's role in phospholipid transfer from TG-rich lipoproteins, phospholipid and cholesterol acquisition from peripheral cells, and participation in hepatic synthesis of VLDL indicates that PLTP is a central effector of HDL metabolism. In the present study, we explored further the impact of rs2294213, mapping to intron 1 of PLTP, which we previously demonstrated as being associated with antiatherogenic changes in lipoprotein profiles.

Previously, we reported decreased PLTP activity in 1 (p.R235W) of 4 (p.E72G, p.S119A, p.S124Y, and p.R235W) missense mutations in the HypoA group using an in vitro activity assay [12]. Whereas the L106F missense mutation was only identified in 1 HyperA subject, it has the potential to further our understanding of PLTP structure and function. Predictions regarding the potential impact of this missense mutation on PLTP structure can be made within the context of the structural model proposed by Desrumaux and colleagues [38]. This mutant is predicted to be "benign" when analyzed online by PolyPhen (at http://genetics.bwh.harvard.edu/cgi-bin/pph/polyphen.cgi). The L106F mutation does not induce any gross structural abnormalities affecting secretion of the mutant protein. Although it appears to be benign, it is unclear if the L106F mutation results in more subtle functional differences that the current in vitro assay is not sufficiently sensitive to discern. Although the PLTP activity assay is used to assay the impact of these missense mutations, additional analysis of these sequence variations with respect to PLTP-mediated remodeling of HDL [7], adenosine triphosphate–binding cassette A1–mediated efflux of cholesterol from peripheral tissues to HDL [9], and its participation in hepatic VLDL synthesis [39-41] is warranted.

In our previous report, we detailed potential study limitations that are also relevant to the current report [12]. These include the fact that the participants recruited into the GRA were enrolled primarily from tertiary lipid clinics, which are most commonly referred for elevated LDL-C and could explain their elevated LDL-C levels in this study. Lastly, although measurement of sequence anomalies by DGGE and dHPLC has detection sensitivity approaching 100% [42], some sequence anomalies may have gone undetected.

This is the first study to screen the entire PLTP gene for sequence anomalies in individuals with HyperA. Our findings show that the rs2294213 minor allele was enriched in individuals with HyperA. The inverse relationship between HDL-C and risk of coronary heart disease is firmly established. This and our previous report [12] are the first in-depth sequence analyses of the PLTP gene in humans and identify a novel polymorphism associated with altered lipoprotein profiles. The strikingly progressive increase in representation of this minor allele with ascending HDL-C levels presents strong inference that PLTP is an important determinant of HDL metabolism and makes PLTP a potential target for pharmacologic intervention.

Acknowledgment

The National Institutes of Health (NIH) Training Grant in Genetics T32 HL07731, American Heart Association grant 0465005Y, and the Hellman Family Award supported BEA. The NIH K01 NR07888 supported MBE and YN. This work was also supported by grants from Pfizer, the Joseph Drown Foundation, the Valentine Foundation, and the Foundation Leducq; a gift from Donald Yellon; and NIH grants AA11205, HL50779, and HL50782.

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