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Genetic studies of human apolipoproteins

VIII. Role of the apolipoprotein H polymorphism in relation to serum lipoprotein concentrations

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Summary. Apolipoprotein H (APO H) has recently been identified as a structural component of chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Although the precise metabolic function of APO H in lipid metabolism is not certain, it has been suggested that APO H may be involved in triglyceride (TG) metabolism. In addition to the previously described quantitative polymorphism, we have recently detected a common qualitative polymorphism at the APO H structural locus. To test the role of APO H genetic variation in determining lipoprotein and lipid levels, we have estimated the allelic effects of APO H variation on TG, VLDL, LDL, HDL, HDL3, and total cholesterol on 356 Nigerian blacks (189 males, 167 females). While no significant effect of phenotype was observed on lipoprotein levels, the effect of interaction between phenotype and gender was significant. Therefore, data on males and females were analyzed separately using analysis of variance after adjusting for age and body mass index. Logarithmic transformation of pertinent variables was done to bring the distribution of the variables closer to normality. A statistically significant effect of phenotype was observed on triglyceride levels in females only ($P < 0.05$). Further analysis of this phenotypic effect revealed that it is due to the impact of the *APO H*3* allele, which raises triglycerides by 9.92 mg/dl as compared to the common allele, *APO H*2*. These findings are in accordance with the postulated role of APO H in triglyceride metabolism. On the basis of its sex-specific effect, we propose a hypothesis that may explain the combined influence of the quantitative and qualitative polymorphisms at the APO H locus on triglyceride levels in females.

Introduction

Apolipoprotein H (APO H) was first isolated by Schultze et al. (1961) who proposed the name β2-glycoprotein I (β-2-GPI). The present name, APO H, was proposed by Nakaya et al. (1980), who reported that β-2-GPI is an activator of the enzyme, lipoprotein lipase. APO H is associated with chylomicrons, low-density lipoproteins (LDL), very low-density lipoproteins (VLDL) and high-density lipoproteins (HDL: Polz and Kostner 1979a, b; Lee et al. 1983). The physiological function of APO H in lipid metabolism is not completely understood, but it may be involved in triglyceride metabolism (Burstein and Legmann 1977; Polz and Kostner 1979a). Additional evidence suggests that APO H binds to platelets and regulates adenylate cyclase activity (Schousboe 1980).

The protein is a monomeric glycoprotein with 326 amino acid residues and has five attached glycosamine-containing oligosaccharide side chains. By comparison to other apolipoproteins, APO H is rich in cysteine and proline; the cysteine residues are linked at regular intervals by disulfide bonds. Its estimated molecular weight ranges from 43,000 to 50,000 (Lee et al. 1983; Lozier et al. 1984). The normal level of APO H in plasma is about 20 mg/100 ml. However, variable levels have been observed in individuals, which are controlled genetically by two codominantly expressed alleles, designated *Bg*N* (normal), and *Bg*D* (deficient: Cleve 1968; Koope et al. 1970; Propert 1978; Walter et al. 1979). Individuals homozygous for the deficient allele have been found immunologically negative or nearly so. While heterozygous deficient individuals have a concentration of about 10 mg/100 ml.

Recently, Kamboh et al. (1988) have described a genetically determined structural polymorphism in the APO H molecule with the occurrence of three common alleles in U.S. whites and U.S. blacks. In addition, U.S. blacks were found to be unique due to the presence of a fourth allele, *APO H*4*.

The role of lipids and apolipoproteins in the atherogenic process is well established, and it has been shown that a large proportion of the variation in lipid and lipoprotein levels is attributable to genetic differences among individuals (Breslow 1988). However, the role of APO H in determining lipid profiles has not been determined. With the structural polymorphism observed in the APO H molecule, it seems plausible to investigate the role of APO H genetic variation in lipid metabolism. We report here an analysis of the effect of this polymorphism on the quantitative levels of total cholesterol, total HDL-C, HDL3-C, LDL-C, and triglycerides in a sample of 356 unrelated individuals from Nigeria using the measured genotype approach (Sing and Davignon 1985; Boerwinkle et al. 1986).

Materials and methods

Serum samples

The study population consisted of two groups. The first group were first-year medical students, nursing students, and mid-

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wifery students at the University of Benin, Benin City, Nigeria. The second group was a random sample of civil service workers from Benin City, and teachers from the Oroyo College Secondary School, Benin City. The two groups provided a total sample of 356 unrelated subjects. The mean ± SD height and weight of 189 males was 168.0 ± 7.5 cm and 62.87 ± 9.44 kg, respectively. The mean ± SD height and weight of 167 females was 159.5 ± 6.10 cm and 58.11 ± 9.74 kg, respectively. They ranged in age from 17 to 54 years with a mean ± SD age of 30.2 ± 9.5 years. Detailed descriptions of the sample populations are given in Adams-Campbell et al. (1988) and Bunker et al. (1989).

Fasting blood samples were obtained from all the subjects, and remained at room temperature for 2 h. Then, the samples were centrifuged and the serum pipetted into glass tubes and frozen at −47°C for 2 weeks. The samples were transported to Pittsburgh in a styrofoam ice chest with frozen cold packs. The lipids were analyzed within 2–4 months after collection in the Nutrition Laboratory, University of Pittsburgh, Graduate School of Public Health.

Triglycerides and total serum cholesterol were measured manually using an enzymatic method (Bucolo and David 1973; Allan et al. 1974). Total high-density lipoprotein cholesterol (THDL-C) was determined by heparin-manganese chloride precipitation methods, whereas subfraction HDL3 cholesterol concentrations were measured after the precipitation of the HDL2-cholesterol subfraction by dextran sulfate (Bucolo and David 1973; Gidez et al. 1979; Warwizk and Albers 1978). LDL cholesterol was calculated using the Friedewald equation (Friedewald et al. 1972).

Polyacrylamide gel and isoelectric focusing (IEF)

The polyacrylamide gels of 5% by weight (monomer 4.85%, bis 15%) containing 3 *M* urea were prepared as described previously (Kamboh et al. 1988). We mixed 5 ml acrylamide (29.1%); 5 ml bis (0.9%); 5.4 g urea; 0.45 ml Pharmalyte, pH 4–6.5; 0.90 ml Pharmalyte, pH 5–8; and 20 µl riboflavin (0.1%) and brought the final volume to 30 ml with deionized water. Photopolymerization was achieved by overnight exposure to fluorescent light and the gel was used the following morning.

Gels were loaded with serum samples absorbed on 5 mm × 4 mm Whatman 3 MM filter paper wicks, close to the cathode. Electrode strips were saturated with 1 *M* H_3PO_4 for the anode and 1 *M* NaOH for the cathode. Gels were placed into an LKB 2217 Ultrophor electrofocusing unit connected to a Lauda RM6 cooling unit operating at 9°C and an LKB power supply at 250 mA maximum current and 10 W constant power. The average initial voltage reading was about 540 V and the final reading about 1550 V.

Immunoblotting

Following IEF, proteins were transferred to either a 0.20 µm or 0.45 µm pore size nitrocellulose filter by passive diffusion (Kamboh and Ferrell 1986) for 20 min. After partial protein transfer, the filter was carefully removed from the gel and washed in TBS buffer (0.25 *M* NaCl, 0.03 *M* Tris-HCl, pH 8.0) for a few minutes followed by a 15-min incubation with 5% (w/v) nonfat dry milk dissolved in deionized water to saturate remaining protein-binding sites. The filter was then exposed for 30 min to goat anti-human β2-GPI (Atlantic Antibodies) at 1 µl/1 ml dilution, followed by three 5-min washes in TBS. The filter was then probed with a second antibody, rabbit anti-goat IgG, conjugated with alkaline phosphatase for 30 min in TBS at 1µl/5 ml dilution. The filter was then washed with three 5-min washes in TBS and stained histochemically using 25 mg β-naphthyl phosphate, 25 mg Fast Blue BB salt, and 60 mg magnesium sulfate in 50 ml buffer (1.8 g NaOH, 3.7 g boric acid/l).

Statistical methods

Allele frequencies were estimated by gene counting. A contingency chi-square test was used to test for homogeneity between the two population samples. For each lipid and lipoprotein variable, adjustment were done to remove the effects of concomitant variables which were: age, age^2, height, height^2, weight, and body mass index (BMI). The adjustment was performed in two steps. First, significant concomitant variables were identified by stepwise regression analysis. Second, the multiple regression equation using the significant concomitant variables was estimated for each lipid and lipoprotein variable, and was used to compute the residuals (that is, the adjusted values of the variable).

The adjusted lipid and lipoprotein variables were then examined for closeness to the normal distribution by computing skewness and kurtosis coefficients. Suitable transformations of the adjusted variables were performed to induce normality to the extent possible.

To test whether the mean lipid/lipoprotein profiles are equal between genders and among APO H phenotypes, a multivariate analysis of variance was performed by considering jointly the vectors of mean values of the lipid/lipoprotein variables.

Analyses of variance were used to test differences in mean values of adjusted variables between genders and among APO H phenotypes. The standard statistical analyses were performed using SAS, BMDP, and MINITAB program packages. Computational formulae used for the average excess of the APO H allele have been given elsewhere (Templeton 1987).

Results

Table 1 shows APO H phenotypes and allele frequencies in a sample of 189 males and 167 females from Benin City, Nigeria. Observed phenotype frequencies showed no significant deviation from those expected under Hardy-Weinberg equilibrium. Contingency χ^2 analysis revealed no significant differences in genotype frequencies between the two sampled groups and they were treated as a single homogeneous sample for all analyses.

Before estimating the effects of the APO H polymorphism on lipid and lipoprotein levels, stepwise regression analyses were performed for each lipid variable: total cholesterol (TC), total HDL-cholesterol (THDL-C), HDL subfraction cholesterol (HDL3-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides. The stepwise regression analyses helped to identify the significant concomitant variables. The significant (at 5% level) concomitant variables for total cholesterol and LDL-C were age and BMI; and for THDL-C, HDL3-C, and triglycerides were weight, age, and age^2, respectively. The concomitant variables not entered into the regression equation were those that were found to have nonsignificant effects

Table 1. Distribution of apolipoprotein H (APO H) phenotypes and allele frequencies in male and female Nigerian blacks

Sex	Number tested	Phenotypes				Allele frequencies			
		2–1	2–2	3–2	4–2	*APO H*1*	*APO H*2*	*APO H*3*	*APO H*4*
Male	189	4	153	24	8	0.011	0.905	0.063	0.021
Female	167	4	130	26	7	0.012	0.889	0.078	0.021
Pooled	356	8	283	50	15	0.011	0.897	0.071	0.021

Table 2. Adjusted mean values[a] (± SE) of serum lipid variables in the sample of 356 Nigerian blacks

Variable (in mg/dl)	Apolipoprotein H phenotype				
	2–1	2–2	3–2	4–2	Pooled mean
Total cholesterol	167.30 ± 11.68	162.67 ± 1.85	162.21 ± 4.88	160.06 ± 8.23	162.60 ± 1.82
Total high-density lipoprotein–cholesterol	42.28 ± 4.00	46.53 ± 0.68	49.59 ± 1.68	44.81 ± 2.88	46.79 ± 0.61
High-density lipoprotein 3–cholesterol	27.21 ± 3.18	30.20 ± 0.41	29.77 ± 1.08	30.36 ± 2.11	30.08 ± 0.38
Low-density lipoprotein–cholesterol	112.27 ± 10.15	102.53 ± 1.56	98.24 ± 4.32	101.75 ± 6.95	102.06 ± 1.67
Triglycerides	63.06 ± 9.50	68.41 ± 1.53	71.76 ± 4.42	67.65 ± 6.16	68.73 ± 1.68

[a] Adjusted mean value is unadjusted grand mean plus mean of the adjusted residual

Table 3. Skewness and kurtosis coefficients of the distribution of untransformed and transformed values of adjusted lipid variables

Variable	Untransformed residuals		Transformed residuals	
	Skewness	Kurtosis	Skewness	Kurtosis
Total cholesterol	0.31	0.18	−0.63	0.62
Total high-density lipoprotein–cholesterol	0.44	0.48	0.08	0.42
High-density lipoprotein 3–cholesterol	0.26	0.25	0.05	0.18
Low-density lipoprotein–cholesterol	0.19	0.41	−3.20	28.99
Triglycerides	1.85	5.75	0.65	1.71

at the 5% level after adjustments were made for the effects of the variables already in the equation. The multiple regression equations obtained from the stepwise regression analyses were then used to adjust the values of the lipid variables.

The average adjusted lipid and lipoprotein levels among individuals with different phenotypes, (i.e., APO H 2–2, APO H 2–1, APO H 3–2, APO H 4–2) are presented in Table 2. Analyses of variance were performed to test equality of adjusted mean values of lipid variables between genders and among APO H phenotypes. However, before the analyses of variance were performed, the skewness and kurtosis coefficients of the adjusted lipid variables were computed. The adjusted values were also transformed logarithmically (to base 10), and the skewness and kurtosis coefficients of the transformed value were computed to find out whether a logarithmic transformation brought the distribution closer to the normal distribution. Some other transformations were also tried, but the log (to base 10) transformation was the best. The results are given in Table 3, which shows that the logarithmic transformation was useful for THDL-C, HDL3-C, and triglycerides but not for TC and LDL-C. The distributions of transformed THDL-C and HDL3-C and untransformed TC and LDL-C were fairly close to the normal distribution. The analyses of variance were, therefore, performed on the untransformed, adjusted values of TC and LDL-C and on the transformed values of THDL-C, HDL3-C, and triglycerides. The analyses of variance models included gender and APO H phenotypes as the main effects and also the interaction between the two. Due to small sample sizes in the APO H 2–1 and 4–2 phenotype classes, the analyses were performed separately either including or excluding both of these classes. This was done to avoid vagaries of small sample sizes.

Before testing equality of mean values for individual lipid variables, we tested whether the mean lipid profiles were equal between genders and among APO H phenotypes. This analysis was performed primarily to determine whether consideration of individual lipid variables was warranted. The Wilks' Λ values for the multivariate analyses of variance and the corresponding approximate F-ratios (Rao 1973) are given in the first row of Table 4. While the difference between sexes is significant, the differences among APO H phenotypes are not significant. Since gender differences in lipid profiles were noted, we have also performed univariate analysis of variance for each of the lipid variables separately (Table 4). The sex effect was significant on TC and LDL-C, but the effect of APO H phenotypes was not significant on any of the lipid parameters. The interaction effect between sex and phenotype was only significant for HDL3-C. Because of this significant interaction, separate adjusted mean lipid values were estimated for males and females (Table 5). Since the interaction term was significant only for HDL3-C, we performed one-way analyses of variance separately for each sex, including and excluding APO H 2–1 and APO H 4–2 phenotypes. The effect of phenotype in males was not significant. When in females APO H 2–1 and 4–2 phenotypes were included, the effect of phenotype was significant ($F = 3.02$, $P < 0.05$) on HDL3-C. However, if these two phenotypes were excluded, the effect of phenotype was significant ($F = 4.43$, $P < 0.05$) for triglycerides in females. We estimated the average excesses of the APO H alleles on HDL3-C (including APO H 2–1 and 4–2 phenotypes). The average excesses of the *APO H*1* and *APO H*2* alleles were −6.25 mg/dl and −0.08 mg/dl, respectively, while the average excesses of the *APO H*3* and *APO H*4* alleles were 0.20 mg/dl and 6.16 mg/dl, respectively. We estimated also the average excesses of APO H alleles on triglycerides (excluding APO H 2–1 and 4–2 phenotypes).

Table 4. Two-way analysis of variance for testing significance and the effect of sex, apolipoprotein H phenotype, and interaction between sex and phenotype. * Significant at 5% level

Variable	*F*-ratio for the effect of					
	Sex		Phenotype		Sex × phenotype	
	Including 2–1 & 4–2 phenotypes	Excluding 2–1 & 4–2 phenotypes	Including 2–1 & 4–2 phenotypes	Excluding 2–1 & 4–2 phenotypes	Including 2–1 & 4–2 phenotypes	Excluding 2–1 & 4–2 phenotypes
All (multivariate)[a]	3.71* (5,344)[b]	3.63* (5,325)	1.10 (15,950)	1.92 (5,325)	1.24 (15,950)	1.73 (5,325)
Total cholesterol	8.82* (1,348)	8.11* (1,329)	0.10 (3,348)	0.01 (1,329)	0.09 (3,348)	0.24 (1,329)
Total high-density lipoprotein–cholesterol	2.12 (1,348)	1.11 (1,329)	1.61 (3,348)	2.98 (1,329)	1.47 (3,348)	0.31 (1,329)
High-density lipoprotein 3–cholesterol	0.02 (1,348)	0.24 (1,329)	0.55 (3,348)	0.18 (1,329)	2.74* (3,348)	0.53 (1,329)
Low-density lipoprotein–cholesterol	8.80* (1,348)	8.59* (1,329)	0.65 (3,348)	0.93 (1,329)	0.07 (3,348)	0.01 (1,329)
Triglycerides	3.07 (1,348)	2.62 (1,329)	0.39 (3,348)	0.58 (1,329)	1.89 (3,348)	2.76* (1,329)

[a] *F*-ratio for the multivariate test are approximate (Rao 1973)
[b] Figures in parentheses indicate degrees of freedom

Table 5. Adjusted mean values[a] (± SE) of serum lipid variables in the sample of Nigerian black males (n = 189) and females (n = 167)

Variable (in mg/dl)	Sex	Apolipoprotein H phenotype				
		2–1	2–2	3–2	4–2	Pooled mean
Total cholesterol	M	162.87 ± 22.24	160.80 ± 2.48	157.16 ± 6.27	159.77 ± 12.81	160.34 ± 2.60
	F	168.43 ± 12.94	164.80 ± 2.65	167.19 ± 7.25	162.43 ± 9.72	165.16 ± 2.53
Total high-density lipoprotein–cholesterol	M	43.23 ± 5.87	45.58 ± 0.97	47.35 ± 2.15	40.17 ± 3.92	45.77 ± 0.87
	F	41.53 ± 6.56	47.22 ± 0.93	51.46 ± 2.37	52.15 ± 2.37	47.95 ± 0.85
High-density lipoprotein 3–cholesterol	M	30.31 ± 6.47	30.87 ± 0.60	29.68 ± 1.71	26.54 ± 2.34	30.53 ± 0.56
	F	23.72 ± 0.67	29.40 ± 0.55	29.76 ± 1.36	35.34 ± 2.70	29.57 ± 0.50
Low-density lipoprotein–cholesterol	M	107.66 ± 16.17	99.26 ± 2.38	94.86 ± 5.92	103.02 ± 10.92	99.04 ± 2.37
	F	114.80 ± 13.82	106.20 ± 2.39	102.22 ± 6.18	99.15 ± 8.90	105.49 ± 2.31
Triglycerides	M	60.30 ± 17.06	78.33 ± 2.66	74.67 ± 4.88	82.76 ± 10.28	77.67 ± 2.53
	F	60.60 ± 8.23	56.91 ± 1.68	68.75 ± 7.22	51.58 ± 2.95	58.62 ± 1.87

[a] Adjusted mean value is unadjusted grand mean plus mean of the adjusted residual

The average excess of the *APO H*2* allele was −0.90 mg/dl, while that of the *APO H*3* allele was 9.92 mg/dl in females.

Discussion

Genetically determined structural variation in various apolipoproteins, which are structural components of lipoprotein particles, plays an important role in determining interindividual lipid variation and susceptibility to cardiovascular diseases (Breslow 1988; Humphries 1988). The precise function of APO H in lipid metabolism is unknown. However, as a constituent of various lipoprotein particles, and possessing genetic variation, the APO H gene products have a potential role in determining quantitative levels of lipoprotein lipids (Burstein and Legmann 1977; Polz and Kostner 1979a). To evaluate its role in lipid metabolism, we have screened a large number of Nigerian blacks for the APO H genetic polymorphism. Furthermore, the effect of genetic variation has been investigated on triglycerides, total cholesterol, total HDL-C, LDL-C, and HDL3-C. The present study did not demonstrate any significant effect of APO H phenotype on quantitative levels of lipid and lipoproteins in 356 Nigerians tested. However, the interaction effect between phenotype and sex was found to be significant for HDL3-C (Table 4). Therefore, separate analyses were performed for males and females. A statistically significant effect of phenotype was revealed on HDL3-C in females only. This significant effect was contributed largely by the small numbers present in the APO H 2–1 and 4–2 phenotype classes. When these two minor phenotype classes were excluded from the analysis, the phenotypic effect was non-significant for HDL3-C. It is unclear from this analysis whether the effect of APO H polymorphism on HDL3-C in females was real or due to vagaries of small sample sizes. Further studies, with larger sample sizes are needed to confirm these findings. In any case, the effects of *APO H*2* and *APO H*3* alleles on HDL3-C were negligible, while those of *APO H*1* and *APO H*4* were large and in opposite directions. While *APO H*1* lowered the level of HDL3-C, *APO H*4* had an elevating effect. The exclusion of the 2–1 and 4–2 phenotypes showed a dramatic change and yielded a significant effect (at the 5% level) of phenotype on triglycerides in females. This observation prompted us to estimate the allelic effects. The average adjusted values of triglycerides in the remaining APO H 2–2 and 3–2 phenotypes were 56.91 mg/dl and 68.75 mg/dl, respectively (Table 5), giving rise to a significant difference of 11.84 mg/dl at the 5% level. In comparison with the effect of the *APO H*2* allele on triglycerides (which was negligible), the *APO H*3* allele had a large elevating effect on triglyceride levels in females.

Our present observation on the allelic effect of APO H on triglycerides is in accordance with its postulated function in triglyceride metabolism. Burstein and Legmann (1977) first reported a correlation between APO H deficiency and ele-

vated levels of VLDL. Subsequently Nakaya et al. (1980) demonstrated that in vitro APO H increases the enzymatic activity of the lipoprotein lipase (LPL) enzyme in the presence of APO C-II. These studies are confirmed by in vivo studies where APO H was found to be involved in the clearance of triglycerides from plasma (Wurm et al. 1982). Our finding that the APO H locus influences triglyceride levels only in females needs to be confirmed in other population samples. However, from the existing information available on APO H, we may suggest the following hypothesis of its sex-specific effect on triglycerides in the Nigerians. In addition to the recently described qualitative polymorphism (Kamboh et al. 1988), APO H is known to demonstrate quantitative polymorphism (Cleve 1968; Propert 1978; Walter et al. 1979). Two alleles, *Bg*N* (normal) and *Bg*D* (deficiency), have been documented. The frequency of the deficiency allele in Caucasians and blacks differ significantly with respective values of about 5% and 20%. The average concentration of APO H is significantly higher in Caucasians than in blacks (Walter et al. 1979). While there are no data on sex differences among blacks, the Caucasian females have relatively low levels of APO H compared to males (Cleve 1968; Propert 1978). To be consistent with these observations one may speculate that black females have the lowest values of APO H. Assuming further that the *APO H*3* allele is associated with the deficiency trait (quantitative polymorphism), this would cause further decline in APO H values among females carrying this alle product. Such a drastic reduction in APO H may make this protein a poor activator of the LPL enzyme and lead to the accumulation of triglycerides or VLDL in the plasma. The same mechanism may occur in males, but since they have already comparatively higher values of APO H than females, the effect of the *APO H*3* allele may be moderate. It is likely that different hormonal profiles of females may be another contributing determinant of this sex-specific effect. The advantage of the proposed hypothesis is that it is easily testable. Studies are underway in our laboratory to evaluate the relationship between the qualitative and quantitative genetic polymorphisms and their role in lipid metabolism.

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