12-1-2005

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Disparate Associations of a Functional Promoter Polymorphism in *PCK1* With Carotid Wall Ultrasound Traits

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Background and Purpose—Cytosolic phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32), encoded by *PCK1*, catalyzes the first committed step in gluconeogenesis. We previously showed that a –232C>G promoter polymorphism within a *cis*-acting element required for basal and cAMP-mediated *PCK1* gene transcription results in loss of negative regulation by insulin, contributing to worsened metabolic control in the context of insulin resistance. We hypothesized that this polymorphism would be associated with carotid atherosclerosis in a sample of 150 aboriginal Canadians.

Methods—Dependent variables were 2 distinct carotid traits, namely intima-media thickness (IMT) assessed using B-mode ultrasound and total carotid plaque volume (TPV) assessed using 3D ultrasound.

Results—Multivariate analysis showed significant but opposite associations of *PCK1* genotype with these traits. Specifically, subjects with the *PCK1*–232G/G genotype had more carotid IMT (0.80 ± 0.02 versus 0.73 ± 0.03 mm; $P=0.007$) but less TPV (0.10 ± 0.09 versus 0.38 ± 0.13; $P=0.03$) than subjects with other genotypes.

Conclusions—The findings connect the key enzyme in gluconeogenesis with atherosclerosis. The meaning of the opposing associations of *PCK1* genotype with IMT and TPV is unclear; more work is required to confirm whether these might be distinct quantitative traits with different biological determinants. **(*Stroke*. 2005;36:2566-2570.)**

Key Words: atherosclerosis ■ diabetes mellitus ■ genetics ■ risk factors

Diabetes mellitus and related disturbances such as hyperglycemia and insulin resistance are potent risk factors for atherosclerosis.[1] Biochemical and genetic advances have specified many candidate proteins for hyperglycemia, insulin resistance, and type 2 diabetes.[2] A key candidate protein for glycemia and diabetes is cytosolic phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32), which catalyzes the first committed step in hepatic gluconeogenesis.[3,4] In the *PCK1* gene that encodes PEPCK, we identified a common promoter single nucleotide polymorphism (SNP), namely –232C>G, within a *cis*-acting element, that governs basal and stimulated *PCK1* gene transcription.[5] In vitro, the –232G-containing promoter showed 5- to 100-fold increased basal expression with no downregulation by insulin compared with the –232C-containing promoter.[5] Furthermore, in 2 independent populations, the odds ratios for type 2 diabetes mellitus was ≈2-fold greater in subjects with –232G than in subjects with –232C.[5] Given the reported dysfunction and genetic associations, we evaluated the relationship between *PCK1*–232C>G SNP promoter genotype with measures of carotid atherosclerosis in 150 Canadian Oji-Cree individuals.

Methods

Study Sample

The Sandy Lake community is located at the 55th parallel of latitude in the subarctic boreal forest of central Canada. Baseline demographic, clinical, and biochemical attributes from an ongoing study of diabetes risk and complications have been described.[6,7] Seventy-two percent of community members >10 years of age were studied with medical history and physical examination. In 2001, 278 adult community had ultrasound (US) assessment of the carotid arteries. Of these, 150 had baseline demographic data and sufficient DNA for *PCK1* genotyping, and this subset was demographically representative of the overall sample (data not shown). All subjects provided informed consent, and the study received approval from the Sandy Lake Band Council and from the institutional review boards of the universities of Toronto and Western Ontario.

DNA Analysis *PCK1*–232C>G

We genotyped the *PCK1*–232C>G promoter SNP, as described.[5] Briefly, we amplified the *PCK1* promoter using primers F: 5′- TCT

Received July 8, 2005; final revision received August 18, 2005; accepted August 28, 2005.

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Stroke **is available at http://www.strokeaha.org** **DOI: 10.1161/01.STR.0000190833.43791.be**

AAG TGA GTT TGG TCG GAG G –3′ and R: 5′- CTG CAG AGT GCT GCT AAG GG –3′. Samples were amplified for 30 cycles, each of which consisted of denaturing at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds. Digestion of the 1640-bp promoter product with *Mae*III yielded fragments of 494, 483, 393, 180, 38, 34, and 18 bp for the –232C allele and fragments of 674, 483, 393, 38, 34, and 18 bp for the –232G allele. Sequence-proven controls were run with each reaction and fragments were resolved in 8% polyacrylamide gels.

General US Logistics

General procedures to measure intima-media thickness (IMT) and total carotid plaque volume (TPV) were described previously.[8–10] Briefly, US images were obtained using an HDI-5000 US machine and an L12–5 transducer (both from Advanced Technology Laboratories) that had been flown to the community and housed there. A single certified operator used the same instrument over a 4-week period to obtain carotid US images for determinations of IMT and TPV.

IMT Measurement

A single blinded observer measured combined IMT of the far wall of both common carotid arteries, with technical details as described.[8–10] Still images were analyzed using computerized edge-detection software (Prowin)[11] using a stepwise algorithm, edge detection, and linear interpolation as described.[8–10] Mean IMT was computed from 80 to 120 measurements over a 10-mm span ending 5 mm proximal to the transition between the common carotid and bulb regions. Intraoperator and interoperator coefficients of variation of 3.0% and 3.1%, respectively, and intraoperator and interoperator intraclass correlations were both 0.97.

TPV Measurement

Two-dimensional US images of the carotid arteries were obtained and immediately reconstructed into a 3D volume to verify scan quality, as described.[12,13] Three-dimensional US images were acquired with a freehand scanning system scanning system and analyzed with L3Di visualization software (Life Imaging Systems). Each 3D image was displayed using multiplanar texture mapping, and plaque volumes were measured using manual planimetry as described.[8–10] Plaques were identified based on visible morphological changes, in which local intimal thickening exceeded 1.0 mm. Plaque boundaries were traced using a mouse-driven cross-haired cursor, as described.[8–10] Slice areas were summed and multiplied by interslice distance to give plaque volume; TPV was the sum of plaque volumes between clavicle and angle of the jaw for both carotids. Intraobserver and interobserver reliabilities were 0.94 (n=40) and 0.93 (n=40), respectively.

Statistical Analysis

SAS version 8 (SAS Institute) was used to evaluate the association of carotid US traits and *PCK1*–232C>G genotypes. IMT and TPV measurements were significantly nonnormally distributed in this data set, so both variables were transformed: 1/IMT and the natural logarithm (log) of TPV were normally distributed. ANOVA (general linear models procedure) was used to determine sources of variation. F-tests were computed from the type III sums of squares, which applies to unbalanced study designs and reports effects of independent variables after adjusting for all other variables in the model.[9,10,14–17] Dependent variables were transformed IMT and TPV. Independent variables were *PCK1*–232C>G genotype (assuming a recessive model), age, sex, body mass index, diabetes status, current smoking, hypertension, and the ratio of plasma apolipoprotein B (apoB):A1. The general linear model procedure for least-squares means (also called population marginal means) was used to determine the level of significance in pairwise comparisons. Least-squares means are the values for class means after adjustment for all covariates. χ^2 analysis was used to evaluate deviation of genotype frequencies from Hardy–Weinberg law.

Results

Baseline Demographic Features

Clinical attributes of the 150 subjects overall and according to sex are shown in Table 1. None of the discrete or quantitative traits were significantly different between males and females. The simple Pearson correlation coefficient between untransformed carotid artery quantitative traits was 0.505 ($P<0.0001$). This increased somewhat to 0.633 ($P<0.0001$) when transformed values (ie, 1/IMT and log TPV) were used.

Allele and Genotype Frequencies

Frequencies for *PCK1*–232C/C, –232C/G, and –232G/G genotypes were 0.24, 0.48, and 0.28, respectively, with no difference between genders. –232G allele frequency was 0.52, and the observed genotype frequencies did not deviate from Hardy–Weinberg expectations. In this sample, the odds of diabetes related to –232G/G genotype was 1.26 (95% CI, 0.61 to 2.59; NS). Furthermore, there were no significant between-genotype differences in age, body mass index, proportion of females, ratio of apoB:A1, plasma glucose concentration, smoking, or hypertension (data not shown).

Determinants of Carotid IMT and TPV

Representative IMT and TPV images are shown in the Figure. ANOVA in Table 2 showed that transformed IMT was significantly associated only with age and with *PCK1*–232C>G genotype in this sample, each with a nominal

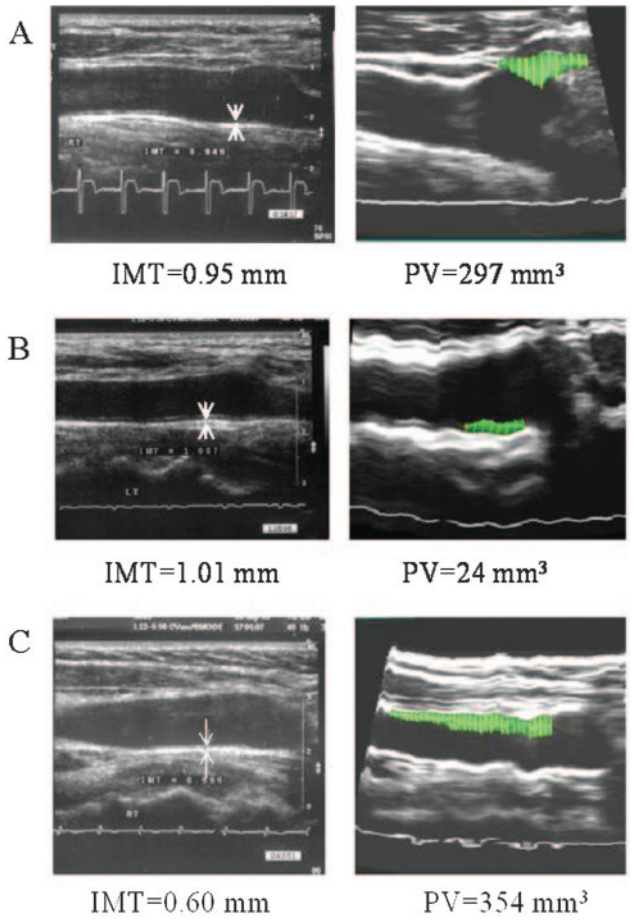
TABLE 1. Baseline Demographics of 150 Oji-Cree Study Subjects

	All Subjects	Males	Females
Discrete traits			
No.	150	59	91
Female	60.7%		
Diabetes or IGT	53.0%	39.7%	62.6%
Hypertension	30.0%	37.2%	25.2%
Current smoking	13.7%	11.9%	14.9%
Quantitative traits, mean±SD			
Age, y	38.0±14.9	36.2±14.6	39.2±15.0
Body mass index, kg/m^2	29.2±5.0	27.8±4.5	30.1±5.2
Ratio of apo B:A1	0.82±0.26	0.90±0.31	0.77±0.20
Carotid IMT, mm	0.78±0.18	0.78±0.18	0.77±0.16
Carotid TPV, mm^3	0.25±0.07	0.21±0.12	0.26±0.10

IGT indicates impaired glucose tolerance; IMT, carotid intima-media thickness; TPV, total volume of carotid plaques seen on 3D US, logarithmically transformed.

Diabetes refers to physician-diagnosed diabetes and current treatment with insulin or an oral hypoglycemic agent (verified by nursing station chart review), or to physician-diagnosed diabetes or a fasting blood sugar of >11.1 mmol/L or diabetes diagnosed using a 75-g oral glucose tolerance test. Impaired glucose tolerance (IGT) was diagnosed according to 2–h post-load plasma glucose between 7.8 and 11.1 mmol/L. Hypertension refers to documented hypertension on treatment or systolic blood pressure >140 (average of 2 measurements) or diastolic blood pressure >90 mm Hg. Each measurement was performed twice, and the average of the 2 was used in the analysis. Ratio of apo B:A1 refers to the ratio of plasma concentrations of apoB to apoA1.

None of the between-gender comparisons was statistically significant.



Representative US images for determinations of right carotid artery anatomy using IMT and TPV from 3 study subjects (A, B, and C). The left panels for each study subject show typical images used to determine IMT, with arrows at the far carotid wall indicating IMT. The right panels show images used to determine TPV, which are defined as colored regions. Values of each trait for each subject are shown. Among these 3 subjects, the relationship between IMT and TPV was modest. For instance, subjects A and B each had IMT of ≈1 mm, whereas subject C had considerably less IMT. However, the quantity of TPV was markedly different between subjects A and B, whereas subject C, who had a small IMT measurement, had a markedly high TPV throughout the carotid arterial system. PV indicates plaque volume.

$P<0.05$. The ratio of apoB:A1 and hypertension each tended to be associated with IMT. ANOVA in Table 2 also showed that transformed TPV was significantly associated only with age and *PCK1*–232C>G genotype, each with a nominal $P<0.05$. The ratio of apoB:A1 and diabetes each tended to be associated with TPV. Significant associations detected by ANOVA were evaluated by comparing least-squares means for genotype classes (Table 3). Subjects homozygous for –232G had significantly more carotid IMT than others (0.80±0.02 versus 0.73±0.03 mm; $P=0.007$) but significantly less carotid TPV than others (0.10±0.09 versus 0.38±0.13; log transformed; $P=0.030$).

Discussion

In Canadian Oji-Cree, we found disparate associations between *PCK1* promoter genotype and quantitative carotid US phenotypes. Specifically, homozygotes for the –232G allele, which is associated with higher in vitro expression of PEPCK at baseline and a failure to normally downregulate in response to insulin, was associated with significantly greater carotid IMT but significantly less carotid plaque volume measured in the same individuals. As with any genetic association finding in a small study sample, the results may have represented a chance finding and thus require replication. However, if replicated, the findings indicate that a functional polymorphism in the focal enzyme of gluconeogenesis is associated with carotid intermediate traits of atherosclerosis. The distinctive nature of these carotid US traits is highlighted by their moderate degree of correlation with each other (r≈0.6) and by the fact that the same functional genetic polymorphism has an opposite association with each. Indeed, we have previously shown that IMT and TPV have different relationships with specific risk factors.[8,9] Also, we previously found an analogous disparate relationship of the association

TABLE 2. Determinants of Carotid US Traits in Oji-Cree (ANOVA)

Source of variation	*df*	F	*P*>F
Dependent variable: inverse of mean carotid IMT			
Age	1	74.0	<0.0001
Sex	1	0.72	NS (0.40)
Body mass index	1	0.01	NS (0.99)
Diabetes	1	0.82	NS (0.37)
Ratio of apoB:A1	1	3.63	NS (0.059)
Current smoking	1	0.60	NS (0.44)
Hypertension	1	2.89	NS (0.09)
PCK1 –232C>G genotype	1	7.77	0.006
Dependent variable: natural logarithm of total carotid plaque volume			
Age	1	67.8	<0.0001
Sex	1	0.01	NS (0.98)
Body mass index	1	1.86	NS (0.17)
Diabetes	1	3.70	NS (0.057)
Ratio of apo B:A1	1	2.94	NS (0.09)
Current smoking	1	0.52	NS (0.47)
Hypertension	1	0.31	NS (0.58)
PCK1 –232C>G genotype	1	4.81	0.030

P>F indicates probability of a greater between-group F-value from ANOVA using type III sums of squares.

TABLE 3. Carotid US Traits According to *PCK1* Genotype in Oji-Cree

	PCK1 Genotype		
	–232C/C plus –232C/G	–232G/G	*P* Value
No.	108	42	
IMT (mm)	0.73±0.03	0.80±0.02	0.007
TPV (mm^3)	0.38±0.13	0.10±0.09	0.030

Least-squares group means±standard errors are shown. Probability of nonrandom difference between least-square group means in pairwise comparisons using the general linear models procedure. *P* values were calculated for normalized dependent variables.

between *PPARG* genotype and IMT versus TPV.[10] The current *PCK1* findings add to the growing evidence that carotid US traits have different determinants likely reflecting different aspects of "atherosclerosis."[8–10]

Atherosclerosis is a complex, multistage, multifactorial disease process[18,19] that connotes varied phenotypes ranging from clinical events to measurements taken from images acquired noninvasively. IMT and plaque measurements such as TPV likely reflect different attributes of atherosclerosis and are not necessarily well correlated as shown in the Figure. In this sample, there was no association between *PCK1*–232C>G genotype and risk factor traits that might have explained the disparate association with the carotid arterial changes. Specifically, we found no association of *PCK1* genotype and obesity, hypertension, dyslipidemia, the metabolic syndrome defined using current criteria,[20] or diabetes (data not shown). Risk factors themselves, such as lipids and hypertension, tended to be associated with atherosclerosis traits, with none showing significance. These observations may be attributable to the relatively small sample size, but the signals for the genetic associations might also reflect the possible pleiotropic effects of this functional polymorphism through unmeasured intermediate mechanisms and pathways.

IMT is a linear variable that is determined from standardized portion of the carotid wall by measuring combined thickness of intima and media at specified intervals and then determining their mean.[21–31] As such, IMT probably more closely reflects a hypertrophic response of intimal and medial cells to hypertension or growth factors.[18] We have previously shown that among risk factors, hypertension is most strongly associated with IMT.[8] In contrast, formed plaques that contribute to TPV measurement represent a later stage of atherogenesis related to inflammation, oxidation, or myocyte proliferation.[1,2] In this context, –232G may exert differential effects on IMT compared with TPV; these are not always well correlated (Figure) and could represent different phenotypes. As always, genetic association studies performed in small samples have limitations and will require replication.[32]

The results of this small study indicate that although functional genetic variation in *PCK1* encoding the focal enzyme of gluconeogenesis PEPCK is associated with atherosclerosis, specific relationships with IMT and TPV differ somewhat. In addition to small sample size, the limitations include the relatively young age of the sample, which tended to limit the generalizability of the study. PEPCK is emerging as a key metabolic determinant of carbohydrate metabolism and glycemia, which is, in turn, a determinant of atherosclerosis risk. Also, the findings support the concept that IMT and TPV are different stages along a continuum that reflect different attributes of atherosclerosis. Therefore, their use as surrogates for atherosclerosis might lead to different conclusions in a particular sample. Carotid US phenotypes represent new and interesting quantitative markers for study of genetic and other determinants.[33,34] However, it is becoming clear that different US phenotypes are only modestly related to each other and have different determinants.[35–37] Future work in individual study samples, with careful and extensive collection of intermediate phenotypes and genetic markers, may help to clarify whether these traits actually reflect different aspects of atherosclerosis.

Acknowledgments

This work was supported by grants from the Heart and Stroke Foundation of Ontario (T4772), the Canadian Institutes for Health Research (FRN 44087 and MOP 44076), Genome Canada and the Blackburn Group. R.A.H. and A.F. hold Canada research chairs (tier I). R.A.H. is a career investigator of the Heart and Stroke Foundation of Ontario (CI4380). S.B.H. is a career scientist of the Ontario Ministry of Health. A.J.G.H. was supported through a Canadian Diabetes Scholarship and a University of Toronto Banting and Best Diabetes Centre New Investigator Award. B.Z. holds the Sam and Judy Pencer Family Chair in Diabetes Research.

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