Polymorphism of the Soluble Epoxide Hydrolase Is Associated With Coronary Artery Calcification in African-American Subjects

The Coronary Artery Risk Development In Young Adults (CARDIA) Study

Myriam Fornage, PhD; Eric Boerwinkle, PhD; Peter A. Doris, PhD; David Jacobs, PhD; Kiang Liu, PhD; Nathan D. Wong, PhD

Background—Modulation of endogenous epoxide levels by soluble epoxide hydrolase (sEH) in the endothelium represents an important mechanism in the regulation of cardiovascular function. We examined the relationship between a common, functional polymorphism of the human sEH gene and coronary artery calcification (CAC) in young, largely asymptomatic African-American and non-Hispanic white subjects.

Methods and Results—Multiple logistic regression and Tobit regression models were used to assess the relationship between the sEH Arg287Gln polymorphism and presence and quantity of CAC. Models adjusting for race (except in race-specific analyses), age, sex, smoking, body mass index, systolic blood pressure, LDL cholesterol, and HDL cholesterol were estimated. Allele and genotype frequency distributions were not significantly different between the 2 ethnic groups ($P=0.22$; $P=0.17$, respectively). The Arg287Gln polymorphism of the sEH gene was a significant predictor of CAC status in African-American participants, either alone or after adjusting for other risk factors. African-American subjects with at least 1 copy of the Gln287 allele had a 2-fold greater risk of having CAC compared with those not carrying this allele (95% CI, 1.1 to 2.9; $P=0.02$). There was no relationship between Arg287Gln polymorphism and the probability of having CAC in white participants (OR, 0.8; 95% CI, 0.5 to 1.3; $P=0.49$). Inferences from multivariable Tobit regression were similar to those obtained in the logistic regression models, indicating that the Arg287Gln polymorphism was a significant independent predictor of both presence and quantity of CAC in African-American but not white subjects.

Conclusions—These data suggest an intriguing and possibly novel role for sEH in the pathogenesis of atherosclerosis, which deserves additional investigation. (Circulation. 2004;109:335-339.)

Key Words: genetics ■ calcium ■ atherosclerosis

Epoxyeicosatrienoic acids (EETs) are lipid metabolites of arachidonic acid that are synthesized in vascular endothelial cells by the cytochrome P450 system. They function as potent vasodilators and have been postulated to serve as endothelial-derived hyperpolarizing factors in the regulation of vascular tone. They have antiinflammatory properties. They modulate platelet function during hemostasis, promote cell proliferation, and have been implicated in blood pressure control. The soluble epoxide hydrolase (sEH) is a ubiquitous enzyme that catalyzes the degradation of EETs into their corresponding diols and, thus, plays a critical role in the control of EET levels. Moreover, dihydroeicosatrienoic acids, the metabolic products of EETs generated by sEH, have been shown to have vasoactive properties in the coronary circulation. We have recently obtained evidence that sequence variation in the sEH gene and altered levels of expression of this gene in kidney and brain may contribute to vascular diseases in an animal model. However, the effect of sequence variation in this gene on cardiovascular phenotypes in humans has not been investigated. A common polymorphism in exon 8 of the sEH gene, which results in an amino acid substitution from arginine to glutamine at codon 287, has been identified. In vitro functional characterization of this polymorphism by transient transfection assays and expression of the recombinant proteins in mammalian cell lines showed that the Gln287 isoform exhibited decreased enzymatic ac...
Coronary artery calcification (CAC), which can be quantified noninvasively and accurately by computed tomography (CT), is a measure of the calcified component of atherosclerotic plaque. Several studies have shown a strong relationship between CAC and the extent of atherosclerosis. The presence and extent of CAC has also been shown to be an independent predictor of angiographically defined coronary heart disease (CHD). Furthermore, its ability to predict future clinical events, independent of other CHD risk factors, has been reported in several studies.

Given the mounting evidence of the role of endogenous epoxide intermediates in processes intimately connected to the pathophysiology of atherosclerosis, we have examined the association between the Arg287Gln polymorphism in the sEH gene and CAC in young individuals from the population-based CARDIA cohort.

Methods

Study Population
Participants were selected from the Coronary Artery Risk Development in Young Adults (CARDIA) study. CARDIA is a prospective multicenter investigation of the natural history and etiology of cardiovascular disease in African-American and non-Hispanic white patients 18 to 30 years of age at the time of initial examination. The initial examination included 5115 participants selectively recruited to represent proportionate racial, gender, age, and education groups from 4 communities: Birmingham, Ala; Chicago, Ill; Minneapolis, Minn; and Oakland, Calif. Details of the study design have been published previously. Five sequential examinations have been conducted from the time of initiation of the study in 1985 to 1986 through year 15 (2000 to 2001).

Data Collection
Participating’s age, race, and sex were self-reported during the recruitment phase and verified during the baseline clinic visit. Body weight was measured to the nearest 0.2 lb using a calibrated scale, with the participant in light clothing without shoes. Height was measured to the nearest 0.5 cm with a verticle ruler. Body mass index was calculated as weight (kg)/height (m^2). LDL cholesterol was estimated using the Friedewald equation. Blood pressure was measured at each examination on the right arm using a random-zero sphygmomanometer with the participant seated and after a 5-minute rest. Systolic and diastolic pressures were recorded as phase I and phase V Korotkoff sounds. Three measurements were taken at 1-minute intervals. The average of the second and third measurements was taken as the blood pressure value. Participant’s tobacco use was obtained from a CARDIA-specific tobacco questionnaire. Details of the procedures for data collection have been previously described.

Coronary calcium was measured at the year-15 examination by CT from the chest. Electron beam CT (Chicago and Oakland field centers) and multidetector CT (Birmingham and Minneapolis field centers) scanners were used to obtain 40 contiguous 2.5- to 3-mm-thick transverse images from the root of the aorta to the apex of the heart in 2 sequential scans. Participants were scanned over a hydroxy-apatite phantom to allow monitoring of image brightness and noise and adjust for scanner differences. Data from both scans were transmitted electronically to the CARDIA CT Reading Center, where a trained cardiovascular radiologist examined each image and identified potential foci of coronary calcium using specially developed image-processing software. A calcium score was calculated for each calcified lesion by multiplying the area of the focus by a coefficient based on the peak CT number in the focus. This coefficient ranged from 1 to 4, where 1 = 131 to 200 HU, 2 = 201 to 300 HU, 3 = 301 to 400 HU, and 4 = 401 or greater HU. This was summed across all lesions within a given artery and across all arteries to obtain the total calcium score for the patient. Each scan set with at least 1 nonzero score and a random sample of those with 0 score was reviewed by an expert investigator without knowledge of the scan scores to confirm presence or absence of coronary calcium. CAC data were obtained on 3041 individuals, of whom 2799 had a DNA sample, including 1240 African-American and 1559 white participants.

Genotype Determination
Genotyping of the Arg287Gln polymorphism was performed using the TaqMan assay (Applied Biosystems). A 64-bp product was amplified by polymerase chain reaction from 15-ng DNA using 0.9 μmol/L each of forward primer (AGATCCCTCTCTCTGGCC) and reverse primer (TCTCCATAGCCTTCTCATGCTCA). The sequence-specific probes (FAM-TAGGACCcGGAAGACC and VIC-CTAGGACCcGGAAGACC) were used in the allele discrimination assay, and allele detection and genotype calling were performed using the ABI7900 instrument and Sequence Detection System software. Genotype data were obtained on 2707 individuals.

Statistical Methods

Genotype frequencies were estimated by gene counting. Agreement of the Arg287Gln genotype frequencies with Hardy Weinberg equilibrium expectations was tested using a χ2 goodness-of-fit test. Within each ethnic group, means and proportions of risk factor variables were compared between individuals carrying at least 1 copy of the Gln287 allele and those not carrying the Gln287 allele by t tests and χ² tests, respectively.

Multiple logistic regression models were used to assess the relationship between presence of CAC and the sEH Arg287Gln polymorphism. Maximum likelihood estimates of the odds of having coronary calcium in relation to sEH genotype category were obtained from models containing the following variables: race (except in race-specific analyses) and Arg287Gln genotype category (model 1); variables in model 1, age, and sex (model 2); and variables in model 2 and established CHD predictors, including body mass index (BMI), systolic blood pressure, LDL cholesterol, HDL cholesterol, and smoking status (model 3). In all analyses, heterozygotes Arg287/Gln287 and homozygotes Gln287/Gln287 were combined in a single category because of the low frequency of the Gln287 allele. Significance of sEH genotype effects was assessed by likelihood ratio test comparing a full model including the risk factor variables and the sEH genotype category to a reduced model not including the genotypes. P < 0.05 was considered statistically significant.

To examine whether the relationship between CAC status and the sEH polymorphism was modified by the effects of other risk factors, a variable representing the interaction between genotype category and each of the risk factors was included in the analysis models. Significance of each interaction term was assessed by likelihood ratio test.

Tobit regression models are frequently used when the dependent variable is censored below a threshold value (left censored, in this case) and is a mixture of discrete and continuous distributions. The Tobit regression model is a single equation that models the relationship of 1 or more independent variables with the probability of being uncensored and, if uncensored, the level of the continuous variable. In the present analyses, participants without detectable CAC were considered as censored observations. Tobit regression models were used to assess whether sEH genotype category predicts the probability of having detectable CAC and, if uncensored, the natural logarithm of the continuous CAC score plus 1. As for the logistic regression analyses, models adjusting for race (except in race-specific analyses), age, sex, BMI, systolic blood pressure, LDL cholesterol, HDL cholesterol, and smoking status measured at the year 15 examination were estimated.

This decreased protein stability was additionally supported by analysis of the crystal structure of the sEH.
Results

Genotype frequencies by ethnic group are presented in Table 1. Genotype frequency distributions were in accordance with Hardy-Weinberg equilibrium expectations (P = 0.09) and were not significantly different between the 2 ethnic groups (Fisher’s exact test; P = 0.17).

Within each ethnic group, there were no significant differences in means or proportions of CHD risk factors between sEH genotype categories (Table 2). In African-American subjects, there was a statistically significant difference in both CAC prevalence and mean log (CAC score + 1) between sEH genotypes (P = 0.02 and P = 0.02, respectively). Presence of CAC and quantity of CAC were significantly greater in African-American participants carrying at least 1 Gln287 allele compared with those carrying none. CAC prevalence and quantity did not significantly differ between white participants with or without the Gln287 allele (Table 2).

In the logistic regression analyses, there was a significant interaction between race and genotype categories (model 1, P = 0.03; model 2, P = 0.02; model 3, P = 0.03; not shown), indicating evidence for a race-specific effect of variation in the sEH gene on CAC risk. All analyses are therefore presented stratified by ethnic group.

The Arg287Gln polymorphism of the sEH gene was a significant predictor of CAC status in African-American participants, either alone or after adjusting for other CHD risk factors (Table 3). African-American subjects with at least 1 Gln287 allele had an approximately 2-fold greater risk of having CAC compared with those not carrying this allele (95% CI, 1.1 to 2.9; P = 0.02). There was no relationship between the Arg287Gln polymorphism and the probability of having CAC in white participants. In either ethnic group, there was no evidence of interaction effects on CAC risk between this polymorphism and any of the other CHD risk factors.

Inferences from multivariable Tobit regression were similar to those obtained in the logistic regression models, indicating that the Arg287Gln polymorphism was a significant independent predictor of both presence and quantity of CAC in African-American but not in white subjects (Table 4).

Discussion

There is accumulating evidence that modulation of endogenous epoxide levels by sEH in the endothelium represents an important mechanism in the regulation of cardiovascular and renal function23 and inflammation.3 As suggested by our recent work in an animal model, allelic variation in the sEH gene influencing enzyme structure or activity may have important physiological and pathophysiological implications.9 This prompted us to examine the relationship between a functional polymorphism of the human sEH gene and CAC, a marker of coronary atherosclerosis, in young, largely asymptomatic adults from the well-characterized CARDIA cohort.
TABLE 4. Tobit Regression Results Evaluating the Relationship of sEH Arg287Gln Genotype Category to CAC Status or Quantity by Ethnic Group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>African-American subjects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sEH genotype (model 1)</td>
<td>2.13</td>
<td>0.87</td>
<td>0.01</td>
</tr>
<tr>
<td>sEH genotype (model 2)</td>
<td>1.99</td>
<td>0.84</td>
<td>0.02</td>
</tr>
<tr>
<td>sEH genotype (model 3)</td>
<td>2.00</td>
<td>0.82</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>White subjects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sEH genotype (model 1)</td>
<td>−0.26</td>
<td>0.60</td>
<td>0.66</td>
</tr>
<tr>
<td>sEH genotype (model 2)</td>
<td>−0.41</td>
<td>0.57</td>
<td>0.47</td>
</tr>
<tr>
<td>sEH genotype (model 3)</td>
<td>−0.33</td>
<td>0.57</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Models defined in Table 3.

*Change in CAC quantity log(CAC + 1) per change of genotype status holding everything else constant.
†Wald χ² test.
‡Gln/Gln vs Arg/Arg (reference).

We report an association between the sEH Arg287Gln polymorphism and CAC status and quantity in African-American subjects. Individuals with at least 1 copy of the Gln287 allele had 2-fold increased odds of having CAC compared with those carrying the Arg287 allele. The molecular mechanisms that may underlie such an effect on CAC are unclear. EETs are endogenous substrates of sEH and have been implicated in the regulation of a variety of biological processes, including vascular reactivity, vascular inflammation, vascular smooth muscle cell proliferation, as well as leukotxin-mediated alteration in vascular permeability and calcium homeostasis. More recently, inactivation of EETs by sEH was proposed to contribute to ischemic brain injury, because both sEH gene deletion and pharmacological inhibition of the enzyme reduced infarct size after focal cerebral ischemia in rodent models. EETs and their corresponding diol regioisomers represent a wide variety of compounds with different physiological potencies. The specific biological processes they each may mediate are incompletely understood. Moreover, multiple interactive and redundant pathways are known to be involved in the metabolism of these epoxide intermediates. For example, a recent study by Fang et al showed that under sEH inhibition, EET metabolism is channeled through an alternative β-oxidation pathway, whose products are themselves biologically active. Whether and how any of these pathways or any particular compound they may generate or metabolize might affect calcium deposition in the vasculature has yet to be investigated. Nonetheless, it has been shown that EETs can induce intracellular calcium influx in several cell types, including smooth muscle cells, endothelial cells, and myocytes. Additionally, much remains to be understood about the recently characterized lipid phosphatase activity of sEH, suggesting that this enzyme acts on a broader variety of substrates than previously recognized and, thus, may have additional, yet-unknown physiological functions. Although no causal relationship can be inferred from the present study, these data suggest an intriguing and possibly novel role for sEH in the pathogenesis of atherosclerosis, which deserves additional investigation.

The association of the sEH Arg287Gln variant with CAC in African-American subjects could not be explained by an association with other major CAC risk factors. In particular, we did not find any evidence of an association between this polymorphism and blood pressure levels or hypertension status in this ethnic group (not shown). Deletion of the sEH gene and pharmacological inhibition of the enzyme have been shown to decrease blood pressure in rodent models, suggesting a role of sEH in blood pressure regulation.

Although CAC is a component of and is strongly correlated with overall coronary atherosclerotic burden, not all atherosclerotic plaque is calcified and not all calcified plaque is detectable by CT. Hence, coronary atherosclerosis may have been undetected in some participants in the study. Because the clinical significance of CAC remains to be fully elucidated, so does the relationship between variation in the sEH gene and clinical disease. For example, it has yet to be determined whether the sEH genotype affects the propensity of the atherosclerotic plaque to become calcified or the risk of developing atherosclerotic plaque itself or both. Additionally, whether sEH gene variation influences the risk for the clinical manifestations of atherosclerosis or the relationship between them and CAC could not be investigated in the young, largely asymptomatic CARDIA cohort.

Racial differences in the prevalence and severity of CAC have been reported by several investigators. In particular, both epidemiological and pathological data consistently suggest that CAC is less prevalent and less severe in an African-American than in a white population, whereas the converse is true for clinically overt CHD. These findings have led to the hypothesis that the pathophysiological determinants of calcification may differ between the 2 ethnic groups. Although it is tempting to suggest that the race-specific association of the sEH Arg287Gln polymorphism with CAC reported here supports such a hypothesis, some limitations of the study warrant a more cautious interpretation. Clearly, the antecedents and promoters of CAC are complex and multifactorial, and the impact of genetic variation on CAC likely differs in the presence of other risk factors. The concomitants of race, whether social, medical, or physiological, are incompletely understood. Hence, we cannot exclude the possibility that other factors, whose prevalence differs between races but is not accounted for in our analyses, may impact the association and, thus, contribute to the observed ethnic differences. Finally, it is possible that the Arg287Gln polymorphism may simply be a marker in linkage disequilibrium with the true causative locus. Possible ethnic differences in the strength of linkage disequilibrium between the Arg287Gln locus and the causative locus would, thus, explain ethnic differences in the association of this polymorphism with CAC.

Identification of genes contributing to CAC and characterization of the risk factors with which they may interact to influence the relationship between coronary calcium and development of clinically overt disease are essential to fully elucidate the clinical and pathophysiological implications of CAC. Additional studies are needed to clarify the role of sEH gene variation in the pathogenesis of atherosclerosis and to investigate its contribution to the clinical manifestations of atherosclerosis.
Acknowledgments

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