Promoter (4G/5G) Plasminogen Activator Inhibitor-1 Genotype and Plasminogen Activator Inhibitor-1 Levels in Blacks, Hispanics, and Non-Hispanic Whites
The Insulin Resistance Atherosclerosis Study

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Background—The 4G/5G polymorphism of the plasminogen activator inhibitor-1 (PAI-1) gene has been related to cardiovascular disease.

Methods and Results—Insulin resistance was measured with a frequently sampled intravenous glucose tolerance test in the Insulin Resistance Atherosclerosis Study (IRAS), and PAI-1 4G/5G promoter genotype was established by allele-specific polymerase chain reaction amplification of genomic DNA. There were 287 subjects with the 4G/4G genotype (18.4%), 691 heterozygote subjects (44.2%), and 586 carriers of the 5G/5G genotype (37.5%). The genotype distribution was different across the 3 ethnic groups (P=0.001). PAI-1 levels were lower in blacks than in non-Hispanic whites and Hispanics and lower in non-Hispanic whites than in Hispanics (all P=0.0001). Subjects homozygous for the 4G allele had the highest plasma PAI-1, heterozygote subjects were intermediate, and 5G homozygotes had the lowest levels of PAI-1. These patterns remained unaffected by adjustments for age, gender, clinical center, glucose tolerance status, body mass index, waist, triglycerides, and insulin resistance. Multiple linear regression analyses showed that the 4G/5G genotype explained very little of the variation in PAI-1 levels (0.63% in non-Hispanic whites, 0.99% in Hispanics, and 2.37% in blacks), and interaction analyses revealed no significant differences in the relation of circulating PAI-1 levels to the 4G/5G genotype by ethnicity (P=0.4).

Conclusions—We have shown ethnic differences in the PAI-1 4G/5G polymorphism along with corresponding differences in circulating PAI-1 levels. The association of the genotype with PAI-1 levels was seen consistently among all 3 ethnic groups and was unaffected by metabolic covariates, including insulin resistance. (Circulation. 2003;107:2422-2427.)

Key Words: epidemiology ■ insulin ■ genetics ■ plasminogen activator inhibitor
body weight, and insulin resistance, are major determinants of circulating PAI-1 levels, and ethnic differences exist with respect to these features. We also studied in the 3 ethnic groups the relation of the 4G/5G genotype to PAI-1 levels considering metabolic covariates, including serum lipids, body weight, and insulin resistance (S). The aim of the present study was to investigate in a large, tri-ethnic population comprising non-Hispanic whites, Hispanics, and blacks the frequency of the 4G/5G PAI-1 promoter genotype, the relation of the genotype to circulating PAI-1 levels, and the relative contribution of the genotype to PAI-1 levels, taking into account metabolic cofactors, including S, as measured directly by a frequently sampled intravenous glucose tolerance test (FSIGTT).

Methods

The IRAS is a multcenter, epidemiological study aiming to explore relationships between S, cardiovascular risk factors, and disease across different ethnic groups and varying states of glucose tolerance. A full description of the design and methods of the IRAS has been published. The IRAS protocol was approved by local institutional review committees, and all subjects gave informed consent. A total of 1625 individuals participated in the IRAS. The present report includes data on 1564 subjects in whom PAI-1 levels were measured and PAI-1 4G/5G genotyping was performed. The IRAS examination required 2 visits. Patients were asked before each visit to fast for 12 hours, to abstain from heavy exercise and alcohol for 24 hours, and to refrain from smoking the morning of the examination.

Assessment of Glucose Tolerance and Insulin Sensitivity

A standard 75-g oral glucose tolerance test was performed, and glucose tolerance status was based on the World Health Organization criteria. An FSIGTT with minimal model analysis was performed to assess insulin sensitivity (S), with 2 modifications of the original protocol as described previously.

Measures of Body Fat and Body Composition

Height was recorded to the nearest 0.5 cm; weight was measured to the nearest 0.1 kg. Body mass index (BMI) was calculated as weight/height² (kg/m²) and was used as an estimate of overall adiposity. Waist circumference was considered an estimate of visceral fat mass.

Laboratory Measurements

Glucose and insulin levels to assess S and triglyceride levels were measured by standard methods as described previously. PAI-1 was measured in citrated plasma with a 2-site immunoassay that is sensitive to free active and latent PAI-1 but not to PAI-1 complexed with tissue plasminogen activator. The citrate sample was centrifuged for a minimum of 10 minutes with 3000 g. The amplified product was electrophoresed on 2.5% DNA typing grade agarose (Gibco), stained with 100 μL/mL ethidium bromide, visualized under ultraviolet light, and recorded on Polaroid film. Each patient was classified into 1 of 3 genotype groups: 4G/4G, 4G/5G, or 5G/5G.

Comparison of PAI-1 levels by genotype were performed with an upstream control primer (5'-TGCAGCCAGCCACGTGATTGTCTAG-3'). Two PCR reactions were run per sample (1 for each allele). A 257-bp control band resulted from the upstream and downstream primers. Each allele-specific primer and downstream primer amplified 136-bp bands. 4G/4G homozygotes yielded 257- and 136-bp bands for the 4G allele-specific reaction and a 257-bp band for the 5G allele-specific reaction. 4G/5G heterozygotes demonstrated 257-bp bands for both reactions, whereas 5G/5G homozygotes demonstrated a 257-bp band for the 4G allele-specific reaction and 257- and 136-bp bands for the 5G allele-specific reaction.

The 4G allele-specific PCR reaction mixture (25 μL volume) contained 1 ng/mL DNA, 0.4 U of Taq DNA polymerase, 1× PCR buffer (PE Biosystems), 0.8 mmol/L MgCl₂, 50 μmol/L dNTP (Gibco), 200 nmol/L upstream control primer, and 400 nmol/L each allele-specific and downstream control primers (IDTDNA). The 5G allele-specific PCR reaction was identical except 1.1 mmol/L MgCl₂ was used.

The 4G allele-specific thermal cycling conditions (32 cycles) were denaturation (94°C for 35 seconds), annealing (65°C for 35 seconds), and extension (72°C for 70 seconds). The 5G allele-specific thermal cycling conditions were identical except the final 22 cycles were annealed at 58°C. The amplified product was electrophoresed on 2.5% DNA typing grade agarose (Gibco), stained with 100 μL of ethidium bromide, visualized under ultraviolet light, and recorded on Polaroid film. Each patient was classified into 1 of 3 genotype groups: 4G/4G, 4G/5G, or 5G/5G.

Statistical Analysis

Statistical analyses were performed with the SAS statistical software system. Table 1 shows descriptive data stratified by ethnicity. Comparison of PAI-1 levels by genotype were performed with ANOVA (Table 2). Because age, gender, diabetic status, and metabolic covariates (body weight, S, and triglycerides) were related to PAI-1, the coefficient of variation from external blind duplicates was 14%. Samples for PAI-1 were frozen and stored at -70°C at the centers not later than 90 minutes after the blood had been drawn. Frozen samples were shipped on a monthly basis to the Laboratory for Clinical Biochemistry Research, University of Vermont (R.P.T.), where measurements were performed.

Determination of PAI-1 4G/5G Genotype

PAI-1 4G/5G promoter genotype was established for each subject by allele-specific polymerase chain reaction (PCR) amplification of genomic DNA by a method similar to that of Falk et al. The PCR reaction used an upstream control primer (5'-

<table>
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<tr>
<th>TABLE 1. Descriptive Data Stratified by Ethnicity: The IRAS</th>
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<tr>
<td><strong>Glucose tolerance, %</strong></td>
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<tr>
<td><strong>NHW</strong>/<strong>Blacks</strong>/<strong>HIS</strong></td>
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<tr>
<td><strong>Female/male gender, %</strong></td>
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<tr>
<td><strong>Age, y</strong></td>
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<tr>
<td><strong>BMI, kg/m²</strong></td>
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<td><strong>Waist circumference, cm</strong></td>
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<td><strong>Fasting glucose, mg/dL</strong></td>
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<tr>
<td><strong>Triglyceride, mg/dL</strong></td>
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<td><strong>S(0) × 10⁻⁴</strong></td>
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NHW indicates non-Hispanic whites; HIS, Hispanic whites; NGT, normal glucose tolerance; IGT, impaired glucose tolerance; and DM, type 2 diabetes mellitus. Values are mean (SEM).

<table>
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<th>TABLE 2. PAI-1 Levels (ng/mL) in the Overall Population</th>
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<td><strong>Unadjusted</strong>/<strong>Model 1</strong>/<strong>Model 2</strong></td>
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<tr>
<td><strong>4G/4G</strong>/<strong>4G/5G</strong>/<strong>5G/5G</strong></td>
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<tr>
<td><strong>28.09 (1.5)</strong>/<strong>21.43 (1.25)</strong>/<strong>19.69 (1.08)</strong></td>
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<tr>
<td><strong>26.68 (1.0)</strong>/<strong>20.52 (0.78)</strong>/<strong>19.72 (0.72)</strong></td>
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Model 1 was adjusted for age, gender, clinical center, and diabetic status; model 2 was additionally adjusted for BMI, waist circumference, S, and triglycerides. Data are mean (SEM).
to PAI-1 levels in previous reports and/or the IRAS population, we performed multivariate analyses (ANCOVA) that included demographic covariates (model 1) and additionally BMI, waist, $S_i$, and triglycerides (model 2). For these and further analyses, logarithmically transformed values of PAI-1 were used because the distribution of the residuals from the fitted models became normally distributed after log transformation. Adjusted means in Table 2; Figures 2 through 4 are presented after back-transformation of the data.

To estimate the relative contribution of the 4G/5G genotype to circulating levels of PAI-1 versus metabolic covariates, stepwise multiple linear regression analyses were performed. Waist, BMI, triglycerides, and $S_i$ were included as independent variables in addition to the genotype and demographic covariates in a model that was used to analyze PAI-1 levels as the dependent variable (Table 3). These analyses were also performed with stratification by ethnicity group (Table 3). We also tested for possible interactions of ethnicity on the association of the 4G/5G genotype with PAI-1 levels by fitting a multivariate model for PAI-1 levels as the dependent variable. To further explore this interaction, stratified analyses were performed (Figures 2 through 4). Probability values less than 0.05 (2-sided) were considered statistically significant.

**Results**

**Genotype Distribution**

Overall, there were 287 subjects with the 4G/4G genotype (18.35%), 691 heterozygote subjects (44.18%), and 586 carriers of the 5G/5G genotype (37.47%). Overall, the genotype frequencies were statistically different from those predicted by Hardy-Weinberg equilibrium, whereas within the 3 ethnic groups, the equilibrium was met. The allele frequencies were 0.40 and 0.60 for 4G and 5G, respectively.

The genotype distribution was significantly different across the 3 ethnic groups (Figure 1; $\chi^2 P=0.001$). The allele frequencies for 4G and 5G, respectively, were 0.52 and 0.48 in non-Hispanic whites, 0.38 and 0.62 in Hispanics, and 0.28 and 0.72 in blacks.

**PAI-1 Levels and Relation of Genotype to PAI-1 Levels**

Overall, PAI-1 levels were lower in blacks than in non-Hispanic whites and Hispanics (mean [SE] 13.38 [0.71], 18.19 [0.80], and 25.00 [1.04] ng/mL) and lower in non-Hispanic whites than in Hispanics (all $P=0.0001$ for pairwise comparisons; Figure 2, unadjusted model). After adjustment for demographic covariates, the differences remained highly significant between blacks and non-Hispanic whites or Hispanics, respectively ($P<0.0001$), whereas the difference between non-Hispanic whites and Hispanics reached borderline significance only ($P=0.12$; Figure 2, model 1). Further adjustment for BMI, $S_i$, or both did not significantly affect any of these differences (Figure 2, model 2).

In the overall population, subjects who were homozygous for the 4G allele had the highest plasma PAI-1 levels,
heterozygote subjects were intermediate, and 5G homozygotes had the lowest levels of PAI-1 (Table 2). Similar patterns were also seen within the 3 ethnic groups (Figures 3 and 4 for the fully adjusted model). These patterns remained unaffected by adjustments for age, gender, clinical center, glucose tolerance status and additional adjustment for BMI, waist, triglycerides, and SHR (Table 2; Figures 3 and 4).

Multiple linear regression analyses (Table 3) showed that the 4G/5G genotype explained 0.63% of the variability of circulating PAI-1 levels in non-Hispanic whites, 0.99% in Hispanics, and 2.37% in blacks. Formal interaction analyses, however, did not reveal statistical differences in the relation of circulating PAI-1 levels to the 4G/5G genotype by ethnicity (P=0.4 for the demographic model). The addition of fasting insulin to these models did not change the results appreciably (data not shown).

Discussion

The present study yielded several novel findings. We found in the IRAS a different distribution of the 4G/5G polymorphism among non-Hispanic whites, blacks, and Hispanics and a weak but significant relation of the 4G/5G genotype to circulating PAI-1 levels, which was seen in all 3 ethnic groups. Circulating PAI-1 levels differed among these 3 ethnic groups, and metabolic covariates, including SHR, as measured directly, did not significantly affect these relations.

Previous studies investigating the relation of the 4G/5G genotype to PAI-1 antigen or activity have yielded conflicting results. An association of the 4G allele with higher PAI-1 levels/antigen has been shown in patients with coronary artery disease,2,3 patients with diabetes mellitus,7 healthy subjects,4 and postmenopausal women,3 whereas only a weak relation was found in healthy women but not men,42 and no association was found in patients46 or first-degree relatives of patients with type 2 diabetes,43 or in families with premature and severe coronary heart disease.45 Differences in the study populations with regard to factors affecting PAI-1 levels or the relation of the 4G/5G genotype and PAI-1, such as gender,42 various underlying disease conditions (coronary artery disease, diabetes mellitus, and insulin resistance),46 and concomitant medications, such as estrogen replacement therapy4 or drugs that affect the renin-angiotensin-aldosterone system,5,46 may account for these differences to some extent. Furthermore, ethnicity may be a contributing factor, as described previously in Afro-Caribbeans, Asians, Pima Indians, Japanese, and, more recently, blacks.7,3-9,47 In the present study, the strength of the relation differed only slightly among ethnic groups, being somewhat stronger in blacks than in non-Hispanic whites and Hispanics.

Insulin resistance per se and other core features of the insulin resistance syndrome, particularly obesity, have been related to increased PAI-1 expression.6-8,21-26,42-44,46 In the present study, the relation of the PAI-1 4G/5G genotype to PAI-1 levels was unaffected by metabolic covariates, including insulin resistance, as measured directly, and body weight. In a previous biopsy study, the 4G/5G polymorphism did not influence the adipose tissue secretion rate of PAI-1 in healthy individuals.48 It is therefore tempting to speculate that adipose tissue–derived PAI-1 is largely unaffected by the genotype (no change after adjustment for adiposity and SHR in multivariate analyses), whereas consequently, other sources of PAI-1 synthesis (such as hepatocytes or endothelial cells) or PAI-1 metabolism might be more closely genetically controlled. Accordingly, in experimental studies,49 human umbilical vein endothelial cells from non-Hispanic whites secreted more PAI-1 than endothelial cells derived from blacks (lower circulating PAI-1 levels and lower 4G allelic frequency than the former), which suggests that PAI-1 secretion from endothelial cells might be affected by the 4G/5G polymorphism. Other in vitro studies suggest that interleukin–1 and insulin-stimulated rather than basal PAI-1 secretion may be genotype dependent in hepatocytes1 and human umbilical vein endothelial cells.50,51

This leads to the question whether the differences in genotype frequencies among the 3 ethnic groups as shown explain the differences in circulating PAI-1 levels. Differences in circulating PAI-1 levels persisted after adjustment for features of the insulin resistance syndrome (including insulin resistance) that have been shown to differ among ethnic groups.27-34 These results do not favor the notion that in subjects with more pronounced features of the insulin resistance syndrome, the proportion of variance of PAI-1
levels caused by genetic factors is minimized.\(^{46,52}\) Also, ethnic differences seen in circulating PAI-1 levels are unlikely to be explained by these metabolic factors, and further environmental factors (not accounted for in the multivariate models or as yet unidentified) and genetic factors have to be considered. Specifically, additional polymorphisms within the PAI-1 gene could contribute to PAI-1 levels. In a previous report, 5 polymorphisms failed to show such an association,\(^{44}\) but to determine the role of each polymorphism in the variation in PAI-1 levels, a complete sequencing of the PAI-1 gene (introns and exons) would be required. However, because the overall variability explained by the 4G/5G genotype was only \(\approx 1\%\), it is unlikely that the differences in genotype distribution alone explain the ethnic differences in circulating PAI-1 levels. In a previous report, the contribution of the 4G/5G genotype to PAI-1 levels and activity ranged from 0.3% to 1.6% in men and from 2.0% to 3.3% in women.\(^{42}\) Therefore, the contribution of the 4G/5G genotype to circulating PAI-1 levels is modest at best, which emphasizes the significance of potentially modifiable environmental factors to circulating PAI-1 levels, including body weight and insulin resistance.

Differences in cardiovascular disease rates among the 3 ethnic groups of the IRAS have been reported.\(^{14–20}\) However, low PAI-1 levels in blacks and high PAI-1 levels in Hispanics contrast with the previously reported higher cardiovascular disease rates in blacks and lower or similar cardiovascular disease rates in Hispanics versus non-Hispanic whites, respectively. Therefore, ethnic differences in PAI-1 levels and the PAI-1 4G/5G genotype do not appear to explain previously observed ethnic differences in cardiovascular disease risk. This is not in contrast with the observed relation of the genotype to circulating PAI-1 levels.

The results of the present study are potentially of clinical significance. A number of drugs that are widely used for disorders commonly seen in a middle-aged population such as that of the IRAS, including hypertension, hyperlipidemia, and postmenopausal symptoms, are considered to exert pleiotropic effects via modulation of PAI-1 expression. These drugs, including ACE inhibitors, statins, and estrogen, might act differently among the 3 ethnic groups. In addition, we have shown recently an association of PAI-1 levels with incident diabetes,\(^{53}\) and blacks have higher rates of type 2 diabetes than non-Hispanic whites.\(^{54}\) Further analyses are under way to explore the association of the PAI-1 4G/5G polymorphism with incident diabetes.

In summary, we have shown ethnic differences in the PAI-1 4G/5G polymorphism along with corresponding differences in circulating PAI-1 levels. Additional studies are needed to explore the clinical significance of these findings.

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