Contribution of the type 1 plasminogen activator inhibitor 4G/5G gene polymorphism to impaired fibrinolysis in vital exhaustion

Roland von Känel, Friedrich E. Maly*, Karl Frey, Joachim E. Fischer

*Institute for Behavioral Sciences, Swiss Federal Institute of Technology, *Institute of Clinical Chemistry, University Hospital, Zürich, Switzerland*

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Coronary artery disease; Fibrinolysis; Plasminogen.

Background. **Inappropriate coping with chronic stress may result in a state of "vital exhaustion" that has been associated with coronary artery disease. Impaired fibrinolysis due to an increase in type 1 plasminogen activator inhibitor (PAI-1) might mediate this link. Genetic and environmental factors both regulate the plasma PAI-1 levels. We investigated the contribution of the PAI-1 4G/5G gene polymorphism to the plasma levels of PAI-1 in exhaustion.**

Methods. **The study participants were 258 (mean age 40.9 ± 9.1 years) apparently healthy subjects of an airplane manufacturing plant in Germany who completed the Shortened 9-item Maastricht Vital Exhaustion Questionnaire. A median split was performed on exhaustion scores rendering two groups of exhausted and non-exhausted subjects. The PAI-1 4G/5G polymorphism in the promoter region of the PAI-1 gene and several variables related to the insulin resistance syndrome known to affect plasma PAI-1 levels were assessed.**

Results. **Across all subjects, exhausted individuals had higher PAI-1 antigen levels than non-exhausted subjects (46.6 ± 20.7 vs 38.3 ± 21.4 ng/ml, p = 0.002). There were no significant differences in the PAI-1 antigen levels between exhausted and non-exhausted individuals with both the 4G/4G and the 4G/5G polymorphism. With the 5G/5G polymorphism, however, exhausted subjects had higher PAI-1 antigen levels than non-exhausted subjects (44.9 ± 22.9 vs 31.2 ± 13.1 ng/ml, p = 0.017). These results did not change when controlling for the variables of insulin resistance.**

Conclusions. **The findings suggest that the PAI-1 4G/5G gene polymorphism might affect the plasma PAI-1 levels related to exhaustion severity. With the 5G/5G polymorphism, exhausted subjects might have less fibrinolytic capacity than non-exhausted subjects.**

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Address:

Roland von Känel, MD

Institute for Behavioral Sciences Swiss Federal Institute of Technology Turnerstrasse 1 8092 Zürich Switzerland E-mail: roland.vonkaenel@ifv.gess.etzh.ch

Introduction

A state of "vital exhaustion" (as of now termed "exhaustion") as characterized by undue fatigue, irritability and feelings of general malaise may emerge as a consequence of maladaptation to sustained psychological stress[1]. In prospectively designed studies, exhaustion predicted a first time myocardial infarction[2] and subsequent cardiac events in patients who had undergone percutaneous transluminal coronary angioplasty[3].

Subjects who are exhausted appear to have impaired fibrinolysis as evidenced by an increase in plasma levels of type 1 plasminogen activator inhibitor (PAI-1)[4-6]. The serine protease PAI-1 is the main inhibitor of plasminogen activation as it inhibits tissue-type plasminogen activator and urinary-type plasminogen activator. An increased PAI-1 concentration thus results in reduced plasmin formation giving rise to fibrin accumulation within the vascular bed and related atherothrombotic events[7]. In fact, increased plasma levels of PAI-1 are now viewed as an intermediate endpoint measure of coronary risk with the potential of predicting subsequent coronary events[8].

Both insulin resistance and genetic factors as well as their interaction determine the plasma PAI-1 levels[9]. For instance, PAI-1 is commonly correlated with the waist-to-hip ratio, high blood pressure, and an unfavorable lipid profile all clustering in the insulin resistance syndrome[7]. On the other hand, interventions to improve insulin resistance may decrease PAI-1 levels[10]. These findings suggest a role for PAI-1 in atherothrombotic events via an association with classic cardiovascular risk factors in the presence of underlying insulin resistance[11].

The gene for PAI-1 on chromosome 7 has several polymorphic loci, of which the 4G/5G insertion/deletion -675 bp from the start site of the promoter[12] has been most widely investigated in relation to coronary

artery disease[13]. In a recent meta-analysis on the relationship between the PAI-1 4G/5G gene polymorphism and coronary risk including both retrospective studies and prospective case-control studies, the 4G allele was significantly associated with an increased risk of myocardial infarction[14]. PAI-1 levels in subjects homozygous for the 4G allele are approximately 25% higher than they are in subjects homozygous for the 5G allele[15].

Interestingly, and emphasizing the gene-environmental interplay in plasma PAI-1 regulation, one study found that the interaction between the 4G/5G polymorphism and the triglyceride concentration (i.e., marker of insulin resistance) was the strongest predictor of PAI-1 activity in the plasma[16]. It is assumed that the 4G/5G promoter site exhibits genotype-specific responses to triglycerides with the highest levels of PAI-1 found in 4G/4G individuals with elevated triglyceride levels[15].

Just as triglycerides, exhaustion could be hypothesized as an environmental factor contributing to PAI-1 expression via a stimulating effect on the PAI-1 gene promoter region. Therefore, we investigated the contribution of the PAI-1 promoter 4G/5G genotype on the plasma PAI-1 concentration in exhausted as compared to non-exhausted subjects taking into account the variables of insulin resistance. We speculated that plasma PAI-1 levels would be different across the three gene polymorphisms 4G/4G, 4G/5G, and 5G/5G in exhausted as compared to non-exhausted subjects.

Methods

Setting and participants. The data reported in this paper were obtained from a cohort study in an airplane manufacturing plant in Southern Germany[17]. The study protocol was approved by the institutional review board. Of a total of 1760 employees, a stratified random sample of 647 men and women was invited to participate. Of these, 537 subjects agreed to complete psychosocial questionnaires. The two main reasons why 110 subjects did not complete the psychosocial questionnaires were because they were unable to leave their workplace or because they were on leave when the testing day was scheduled. After having completed the psychosocial questionnaires, their blood pressure was assessed. The systolic and diastolic blood pressures were measured twice by sphygmomanometry and with the patient seated within 5 min, following a 15-min rest. The mean blood pressure was computed using the following formula: diastolic blood pressure + 1/3 [systolic blood pressure – diastolic blood pressure].

The subjects then were asked to volunteer in a medical examination and assessment of biological variables. A maximum of 2 weeks after having delivered the psychosocial data, 332 subjects presented at the laboratory within 2 hours after awakening and prior to work (i.e., between 7:00 and 8:45 a.m.) to have their fasting blood samples collected.

For the present study, we excluded all subjects with a history of hypertension, coronary artery disease and any other atherosclerotic disease, cardiac surgery, type 2 diabetes and/or elevated plasma glucose, liver disease and nephropathy. We did not exclude, however, the 53 subjects who took any prescribed and/or non-prescribed medication preventing us from further compromising the sample size.

These criteria left a sample of 258 participants (224 males, 34 females, mean age 40.9 ± 9.1 years) with a complete data set in terms of vital exhaustion scores, PAI-1 antigen, and the PAI-1 4G/5G gene polymorphism. With regard to cardiovascular risk factors, the waist-to-hip ratio and the serum levels of glycosylated hemoglobin A1c, data were missing for 2 subjects each, and the smoking status was not available for 8 subjects.

Biochemical measures. Venous blood for PAI-1 was collected in ice-cooled citrate tubes to minimize post-collection liberation of PAI-1 from platelets. The plasma was immediately centrifuged at 4°C and snap-frozen at -70°C until further processing. The plasma levels of PAI-1 antigen were measured using a commercially available enzyme-linked immunosorbent assay (Asserachrom Stago, Asnières, France). Blood samples for high-density lipoprotein cholesterol and low-density lipoprotein cholesterol, and for glycosylated hemoglobin A1c were processed using standard laboratory techniques and analyzed by a laboratory company (Synlab, Augsburg, Germany) within 4 hours of blood collection.

To determine the PAI-1 4G/5G gene polymorphism, we extracted genomic DNA from the leukocyte-containing pellets remaining after centrifugation of coagulated blood using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The PAI-1 4G/5G gene polymorphism was assessed by means of the fluorescent real-time polymerase chain reaction with melting curve analysis on a LightCycler (Roche Diagnostics, Rotkreuz, Switzerland) using the PAI-1 4G/5G ToolSet for LightCycler (Genes-4U, Neftenbach, Switzerland) containing specific primers and fluorescent mutation detection oligonucleotide probes, in conjunction with the Roche LightCycler HybProbe Master Mix (Roche Diagnostics) according to the manufacturer's protocols.

Assessment of exhaustion. To assess exhaustion, we used the Shortened 9-item Maastricht Vital Exhaustion Questionnaire[18], which was derived from the original 21-item Maastricht Questionnaire. There is an excellent correlation between scores of the abbreviated and of the original instrument ($r = 0.94$, $p < 0.001$, $n = 452$)[18]. For the purpose of this study, the 9 items were translated into German in collaboration with the authors of the original scale. The possible responses to each item were

"no" (score = 0), "don't know" (score = 1) or "yes" (score = 2), resulting in a maximum score of 18. The items of the 9-item questionnaire are provided (Table I).

Statistical analysis. Calculations were performed using the SPSS Inc. (version 9.0) statistical software package (Chicago, IL, USA). Descriptive data are presented as mean ± SD, unless in the figure, which depicts mean ± SEM. Results were considered statistically significant at the $p \leq 0.05$ level with all tests being two-tailed. Normality testing of continuous variables by means of QQ-plots showed that the assumption of a normal distribution was not violated for any variable.

The Student's t-test and χ^2 testing were applied to compute differences between continuous and categorical variables, respectively. Unadjusted and adjusted (for health factors in Table II) bivariate correlation analyses were performed to test for a continuous relationship between exhaustion scores and PAI-1 antigen levels both in the entire study population and in the three groups of the 4G/5G genotype. Analyses of covariance were computed for the entire study population as well as for each group of individuals with a specific PAI-1 gene polymorphism (i.e., 4G/4G, 4G/5G, 5G/5G). For the latter analyses, we applied the Bonferroni correction for multiple comparisons setting the significance level at $p = 0.05/3 = 0.017$. In these analyses, PAI-1 antigen was considered as the dependent variable and exhaustion severity as per a median split (i.e., exhausted vs non-exhausted) as the independent variable. The covariates were those demographic variables and cardiovascular risk factors which showed a significant bivariate correlation (Pearson) with the plasma PAI-1 antigen levels.

Table I. The 9 items of the Shortened Maastricht Vital Exhaustion Questionnaire.

Often tired
Difficulty falling asleep
Wake up repeatedly during the night
Feel weak all over
Feel more listless recently than before
Little things irritate more than they used to do
Body is like a battery that is losing power
Discouraged
Wake up tired/exhausted

The questionnaire renders a total exhaustion score ranging between 0 and 18 points. "No" (0 points); "don't know" (1 point), "yes" (2 points).

Results

Allele frequencies and Hardy-Weinberg equilibrium. As reported in epidemiological studies, the frequency of both the 4G and 5G alleles of the PAI-1 4G/5G gene polymorphism in the general population is 0.5[19]. In our study population the allele frequencies of the 4G and 5G alleles were 0.57 and 0.43 respectively. Hardy-Weinberg proportions are the expected genotype frequencies at a locus in a population under given assumptions. Many methods in population genetic analyses rest on the assumption that genotypes are in Hardy-Weinberg proportions within the population under investigation. χ^2 testing suggested that the observed and the expected frequencies under Hardy-Weinberg equilibrium were the same (χ^2 $df_1 = 0.08$; $p > 0.05$) meaning that in our study population the genetic locus was not out of Hardy-Weinberg equilibrium.

Health variables, type 1 plasminogen activator inhibitor antigen and 4G/5G gene polymorphism. Table II shows the plasma PAI-1 antigen levels, demographic variables, and cardiovascular risk factors across the three PAI-1 4G/5G gene polymorphisms. Consistent with the literature[14], though not reaching statistical significance, the absolute plasma values of PAI-1 antigen were highest with the 4G/4G polymorphism and lowest with the 5G/5G polymorphism. There also were no significant differences in any other health variable across the three genotypes.

Table II. Health factors across the three type 1 plasminogen activator inhibitor (PAI-1) gene polymorphisms.

	4G/4G (n=84)	4G/5G (n=128)	5G/5G (n=46)
Plasma PAI-1 antigen (ng/ml)	44.1 ± 22.8	42.6 ± 21.2	38.1 ± 19.7
Exhaustion score (range 0-18)	6.9 ± 4.6	6.7 ± 5.1	7.7 ± 5.5
Age (years)	39.0 ± 8.6	41.8 ± 9.2	41.8 ± 9.3
Sex (M/F)	76/8	106/22	42/4
Waist-to-hip ratio	0.92 ± 0.06	0.91 ± 0.08	0.92 ± 0.07
Low/high-density lipoprotein cholesterol ratio	2.86 ± 0.98	2.83 ± 0.98	2.93 ± 1.10
Mean blood pressure (mmHg)	98.2 ± 10.8	98.1 ± 9.8	96.0 ± 9.8
Glycosylated hemoglobin A1c (%)	5.11 ± 0.47	5.19 ± 0.51	5.14 ± 0.45
Current/former or never smokers*	24/58	32/92	7/37

Values are expressed as mean ± SD. * smoking status was not available for 8 subjects. There were no significant differences in any risk factor between groups (analyses of variance, χ^2).

Analyses in the entire study population. Dichotomization of exhaustion values rendered scores of 2.9 ± 2.1 and of 11.3 ± 3.3 for the non-exhausted and exhausted individuals respectively. The 124 exhausted individuals had significantly higher plasma PAI-1 antigen levels than the 134 non-exhausted subjects (46.6 ± 20.7 vs 38.3 ± 21.4 ng/ml, p = 0.002). Also, crude correlation analyses showed a significant association between exhaustion scores and PAI-1 antigen levels (r = 0.15, p = 0.014, n = 258).

PAI-1 antigen levels showed a significant correlation with the waist-to-hip ratio (r = 0.31, p < 0.001), low-density lipoprotein cholesterol/high-density lipoprotein cholesterol ratio (r = 0.25, p < 0.001), mean blood pressure (r = 0.15, p = 0.016), and glycosylated hemoglobin A1c (r = 0.12, p = 0.048). In addition, men had higher PAI-1 antigen levels than women (43.8 ± 21.6 vs 32.2 ± 17.6, p = 0.003). After having covaried for these correlates of PAI-1, exhausted individuals still had higher PAI-1 antigen levels than non-exhausted subjects (F = 7.8, p = 0.006). As opposed to the crude correlation analysis above, the continuous relationship between exhaustion scores and PAI-1 antigen levels became a trend when adjusted for the significant correlates of the PAI-1 antigen (r = 0.11, p = 0.088, n = 247).

Analyses in the type 1 plasminogen activator inhibitor 4G/5G gene polymorphism subgroups. As a first step, we tested whether the relationship between exhaustion severity and the plasma PAI-1 levels found in the entire study population would differ in relation to a particular PAI-1 4G/5G gene polymorphism. Table II shows that the exhaustion scores were not significantly different between the three polymorphic genotypes. Similarly, figure 1 shows that PAI-1 antigen levels did not significantly differ between exhausted and non-exhausted subjects with both the 4G/4G and the 4G/5G polymorphisms. However, when having the 5G/5G polymorphism, exhausted subjects had significantly higher PAI-1 antigen levels than non-exhausted subjects (44.9 ± 22.9 vs 31.2 ± 13.1 ng/ml, p = 0.017). Crude correlation analyses between exhaustion scores and PAI-1 antigen levels showed a trend for a significant association in the 5G/5G genotype (r = 0.26, p < 0.081, n = 46), while associations in the 4G/4G genotype (r = 0.16, p = 0.151, n = 84) and in the 4G/5G genotype (r = 0.13, p = 0.152, n = 128) did not reach statistical significance.

As a second step, we tested whether these findings were maintained when accounting for health factors, showing a significant correlation with the PAI-1 antigen levels in bivariate correlation analyses. Significant correlations emerged for the low-density lipoprotein cholesterol/high-density lipoprotein cholesterol ratio in terms of each polymorphism, for glycosylated hemoglobin A1c in terms of the 4G/4G and 5G/5G polymorphisms, for waist-to-hip ratio in terms of both the 4G/4G and the 4G/5G polymorphisms, and for gender in terms of the 4G/5G polymorphism (statistics not shown). In brief, the results did not substantially change in terms of the significances shown in figure 1. Specifically, in the 5G/5G gene polymorphism subgroup, exhausted subjects still had higher PAI-1 antigen levels than non-exhausted subjects (F = 4.13, p = 0.049). Likewise, the significance was maintained when the calculations considered only men or only subjects who did not report taking any drug (data not shown). In adjusted bivariate correlation analyses, there was a trend for a significant correlation between the exhaustion scores and PAI-1 antigen levels in subjects with the 4G/4G genotype (r = 0.19, p = 0.094, n = 78). No such correlation emerged, however, in subjects with the 5G/5G genotype (r = 0.13, p = 0.415, n = 41) and in subjects with the 4G/5G genotype (r = 0.10, p = 0.280, n = 123).

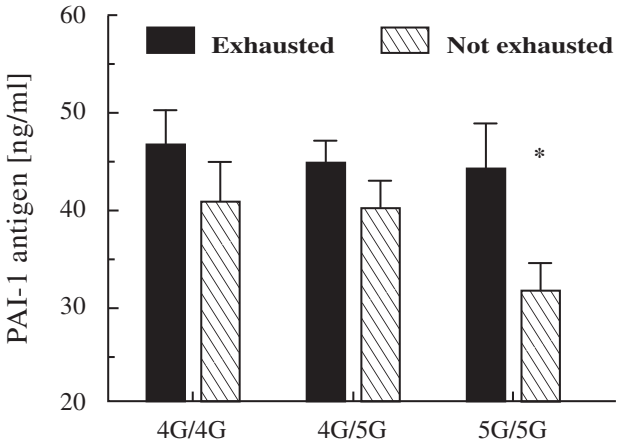

Figure 1. *Values are expressed as mean ± SEM. With the type 1 plasminogen activator inhibitor (PAI-1) 5G/5G gene polymorphism (n = 46: 23 subjects not exhausted, 23 subjects exhausted), exhausted subjects had significantly higher plasma PAI-1 antigen levels than non-exhausted subjects (* p= 0.017; Bonferroni correction at 0.5/3). For both the 4G/4G (p = 0.236; n = 84: 41 subjects not exhausted, 43 subjects exhausted) and the 4G/5G (p = 0.207; n = 128: 59 subjects not exhausted, 69 subjects exhausted) polymorphisms, the plasma PAI-1 antigen levels were not significantly different between exhausted and non-exhausted individuals.*

As opposed to a median split, one might argue that a tertile split of exhaustion scores comparing the top tertile with the bottom tertile of subjects would be a more accurate procedure to differentiate subjects in terms of exhaustion severity and the related changes in the PAI-1 levels. Due to the relatively small sample size in the group of subjects with the 5G/5G genotype (n = 46) we felt that dividing subjects in tertiles based on exhaustion severity would markedly compromise statistical power. Moreover, in our population, it appeared that performing a median split rendered results which were similar to those obtained by means of a tertile split. For instance, and similar to analyses using the median split, crude analyses in the 5G/5G genotype subgroup showed that subjects in the top tertile of exhaustion scores had higher PAI-1 antigen levels than subjects scoring in the bottom tertile of exhaustion (45.4 ± 21.6 vs 31.1 ± 13.5 ng/ml, p = 0.030, n = 32).

Discussion

PAI-1 is a now well-established risk factor for coronary artery disease[7,8,11]. Previous studies have shown increased plasma PAI-1 levels in exhaustion[4-6], and PAI-1 levels are also regulated by factors related to the insulin resistance syndrome[7,11]. The latter observation led some authors to suggest the addition of increased plasma PAI-1 levels to the cluster of atherogenic abnormalities of the insulin resistance syndrome[7].

Aside from environmental factors – exhaustion and insulin resistance – genetic determinants of plasma PAI-1 levels have been recognized[13]. For instance, homozygous carriers of the 4G allele of the 4G/5G polymorphism in the promoter region of the PAI-1 gene express about 25% higher plasma PAI-1 levels than carriers who are homozygous for the 5G allele[5]. Although statistically non-significant, we found a similar distribution of PAI-1 levels across its polymorphisms. Such genetic regulation of plasma PAI-1 might contribute to the increased coronary risk of subjects with the 4G/4G polymorphism[14]. Even though environmental factors appear to play a much greater role than genetic determinants in regulating plasma PAI-1 levels[9], it must be emphasized that there is a yet not fully understood interaction between the environment and the genotype. For instance, plasma PAI-1 levels appear to be highest in subjects homozygous for the 4G allele and with elevated triglycerides[15].

We corroborated previous findings in this same cohort on increased plasma PAI-1 levels in exhausted versus non-exhausted subjects which we have already reported in a previous study[6]. Thus, the main purpose of the present study was to investigate whether the PAI-1 4G/5G gene polymorphism might differentially affect plasma PAI-1 levels in relation to exhaustion severity. Our main finding was that exhausted subjects homozygous for the 5G allele had significantly higher PAI-1 antigen levels than non-exhausted individuals homozygous for the 5G allele. This may suggest an interaction between the 5G/5G polymorphism and exhaustion in terms of elevated plasma PAI-1 levels similarly to what observed for the interaction between the 4G/4G polymorphism and triglycerides[15]. This observation refines the hemostatic mechanisms currently believed to link exhaustion with an increased coronary risk[4,5]. Notably, our study may also suggest that psychosocial factors might contribute to cardiovascular disease by modifying gene expression.

Transcription of PAI-1 may be upregulated by a genotype-specific response to very-low-density lipoprotein of a region adjacent to the 4G/5G promoter site[20]. We may only speculate by which mechanisms exhaustion might upregulate PAI-1 expression though cytokines could be involved. It is known that cytokines may stimulate PAI-1 synthesis *in vitro*[21] and exhausted subjects have higher plasma levels of tumor necrosis factor-α and interleukin-1β than non-exhausted subjects[22].

From a salutogenetic perspective, the figure suggests that results might also favor an alternative interpretation. One might speculate that, due to an increased profibrinolytic or, alternatively, a reduced antifibrinolytic capacity, the 5G/5G polymorphism might exert more relative protection from atherothrombotic risk in non-exhausted as compared to exhausted individuals. The observation that the absolute PAI-1 levels in exhausted subjects turned out to be similar across the three genotypes may support this notion. However, only prospectively designed studies may show whether the interaction between exhaustion and a particular genetic polymorphism of the PAI-1 promoter region will indeed translate into more harm or protection in terms of cardiovascular events.

We found no differences in PAI-1 levels among exhausted and non-exhausted subjects with the two other polymorphisms investigated. Since absolute plasma PAI-1 levels were also higher in exhausted than in non-exhausted subjects with both of these polymorphisms, this may be a consequence of the sample size. Nonetheless, the results essentially held when a range of cardiovascular risk factors related and not related to insulin resistance as well as demographic variables were controlled for. Also, medication did not appear to have a significant impact on the plasma PAI-1 regulation by exhaustion with respect to a particular genotype.

In conclusion, our study suggests that the PAI-1 4G/5G gene polymorphism may modify the exhaustion severity-dependent plasma PAI-1 levels even when variables of insulin resistance are accounted for. The finding may add to the emerging gene-environmental interactions in determining the plasma levels of the hemostasis variables related to cardiovascular events[23]. Future studies on the interactions between psychosocial variables and genotypes which regulate plasma proteins and hemostasis factors in particular[24] might advance our understanding of the deleterious impact of psychosocial characteristics on cardiovascular disease[25].

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