Plasma Levels of Activated Protein C in Healthy Subjects and Patients With Previous Venous Thromboembolism

Relationships With Plasma Homocysteine Levels

Marco Cattaneo, Franca Franchi, Maddalena L. Zighetti, Ida Martinelli, Daniela Asti, P. Mannuccio Mannucci

Abstract—The proteolytic enzyme activated protein C (APC) is a normal plasma component, indicating that protein C (PC) is continuously activated in vivo. High concentrations of homocysteine (Hcy) inhibit the activation of PC in vitro; this effect may account for the high risk for thrombosis in patients with hyperhomocysteinemia (HyperHcy). We measured the plasma levels of APC in 128 patients with previous venous thromboembolism (VTE) and in 98 age- and sex-matched healthy controls and correlated them with the plasma levels of total Hcy (tHcy) measured before and after an oral methionine loading (PML). Forty-eight patients had HyperHcy and 80 had normal levels of tHcy. No subject was known to have any of the congenital or acquired thrombophilic states at the time of the study. Because the plasma levels of APC and PC were correlated in healthy controls, the APC/PC ratios were also analyzed. Plasma APC levels and APC/PC ratios were significantly higher in VTE patients than in controls (P=0.03 and 0.0004, respectively). Most of the increase in APC levels and APC/PC ratios were attributable to patients with HyperHcy. Patients with normal tHcy had intermediate values, which did not differ significantly from those of healthy controls. There was no correlation between the plasma levels of tHcy or its PML increments and APC or APC/PC ratios in controls. The fasting plasma levels of APC and APC/PC ratios of 10 controls did not increase 4 hours PML, despite a 2-fold increase in tHcy. This study indicates that APC plasma levels are sensitive markers of activation of the hemostatic system in vivo and that Hcy does not interfere with the activation of PC in vivo. (Arterioscler Thromb Vasc Biol. 1998;18:1371-1375.)

Key Words: homocysteine ■ protein C ■ thromboembolism ■ activated protein C ■ hypercoagulability

The protein C anticoagulant system is of major physiological importance in the regulation of the hemostatic mechanism.¹ The zymogen protein C is converted to the active protease, activated protein C (APC), through proteolytic cleavage by thrombin bound to its endothelial membrane receptor thrombomodulin.² The demonstration that APC is a normal plasma component,³ whose enzymatic activity can be detected with specific and sensitive methods,⁴,⁵ indicates that the protein C anticoagulant pathway is continuously activated in vivo. Measurement of APC plasma levels might therefore be helpful in determining the in vivo integrity of the protein C anticoagulant pathway. More generally, APC levels might mirror the in vivo activation of the coagulation system and serve as a marker of thrombin activity in the circulation.⁶

The mechanism(s) by which a moderate elevation of plasma levels of homocysteine (Hcy) increases the risk for arterial and venous thrombotic disease is still unclear.⁶,⁷ In vitro studies showed that Hcy inhibits the thrombomodulin-dependent protein C activation to APC and interferes with the expression of thrombomodulin on human umbilical vein endothelial cells.⁸-¹⁰ These findings may be relevant to unravel the thrombogenic mechanism of Hcy, because congenital or acquired disorders characterized by impaired production or function of APC are associated with a high risk for venous thromboembolism (VTE).¹¹ It must be noted, however, that these in vitro findings have been obtained by using very high concentrations of Hcy, at least 1 order of magnitude higher than the plasma concentrations found in patients with homozygous homocystinuria.¹²,¹³ Their clinical relevance is therefore uncertain and awaits confirmation from ex vivo and/or in vivo studies in humans. In this study, we compared the plasma levels of APC with those of the prothrombin fragment F₁+₂, a marker of thrombin generation,¹⁴ in healthy subjects and patients with previous episodes of VTE and tested whether the levels are affected by plasma Hcy concentrations.

Methods

Materials
1-Methionine, tri-n-butylphosphine, 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABDF), L-cystine, L-homocystine, ovalbumin, Tween 20, Tween 80, benzamidine, and HEPES were from Sigma. (4-Amidinophenyl)-methanesulfonfluoride (APMSF) was from Boehringer, BSA from Calbiochem, and the chromogenic substrate

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From the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Institute of Internal Medicine, IRCCS Ospedale Maggiore, University of Milano, Italy.
Correspondence to Marco Cattaneo, MD, Hemophilia and Thrombosis Center, Via Pace 9, 20122 Milano, Italy. E-mail marco.cattaneo@unimi.it
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S-2366 from Chromogenics. The monoclonal antibody directed against the light chain of protein C (C3-Mab) was a kind gift of Dr H.P. Schwarz (Immuno, Vienna, Austria). All other chemicals were of reagent grade.

**Subjects**

We studied 128 patients with previous VTE and 98 healthy controls. All diagnoses of thrombotic episodes, excluding those of superficial veins, had been confirmed by objective methods: compression ultrasonography or venography for deep vein thrombosis; and ventilation/perfusion scintigraphy for pulmonary embolism. The contemporary presence of deep vein thrombosis in patients with superficial vein thrombosis had not been excluded by objective methods. Table 1 shows the characteristics of the patients studied. They belonged to a cohort of 315 patients who had been screened for thrombophilic states at our Center between December 1993 and July 1995 and were selected on the basis of the following characteristics: (1) absence of congenital or acquired thrombophilic states except hyperhomocysteinemia (HyperHcy) (see below); (2) oral anticoagulant therapy discontinued at least 1 month before screening; (3) at least 4 months elapsed since the last thrombotic episode; and (4) willingness to participate in the study. The screening for thrombophilia included the following tests: prothrombin time; activated partial thromboplastin time; thrombin time; plasma levels of fibrinogen, protein C, protein S, and antithrombin; APC resistance; and screening for antiphospholipid syndrome and plasma levels of total homocysteine (tHcy) before and 4 hours after an oral methionine load. Patients with abnormal APC resistance were also screened for factor V Leiden. The study was designed and completed before the demonstration that the mutation G20210A of the prothrombin gene is a risk factor for deep vein thrombosis. This mutation therefore was looked for retrospectively only in those subjects whose DNA was still available for analysis (all controls and 50 patients); 5 patients (10%) and 2 controls (2.1%) were heterozygous for the mutation.

Of the 128 patients enrolled in the study, 48 had hyperhomocysteinemia (VTE-HyperHcy) according to the diagnostic criteria outlined below, and 80 had normal Hcy levels (VTE-NormoHcy). The healthy controls, who were age and sex matched with the patients (male/female, 50/45; median age, 40 years [range, 20 to 73 years]), had been chosen from the same geographical area and with the same socioeconomic background as the patients. Previous episodes of thrombosis had been ruled out by a validated structured questionnaire. No subject had abnormal liver or renal function, or overt autoimmune or neoplastic disease. Informed consent to participate in the study was obtained from all subjects. The study was approved by the ethics committee of the University of Milano.

<table>
<thead>
<tr>
<th>Table 1. Demographic Characteristics of Patients With Previous VTE-HyperHcy and Patients With Previous VTE-NormoHcy</th>
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<tbody>
<tr>
<td>VTE-HyperHcy</td>
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<tr>
<td>-------------</td>
</tr>
<tr>
<td>No.</td>
</tr>
<tr>
<td>Males/females</td>
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<tr>
<td>Median age, y (range)</td>
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<tr>
<td>Median age at the first thrombotic episode, y (range)</td>
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<td>Time elapsed since last episode, mo (range)</td>
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<td>Time elapsed since discontinuation of oral anticoagulant therapy, mo (range)</td>
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<tr>
<td>Type of first thrombotic episode</td>
</tr>
<tr>
<td>Deep vein thrombosis</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>Superficial vein thrombosis</td>
</tr>
<tr>
<td>Venous thrombosis of other sites</td>
</tr>
<tr>
<td>With 1 or more episodes</td>
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<td>With circumstantial risk factors* at first episode</td>
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*The following circumstantial risk factors were considered: surgery, trauma, immobilization, pregnancy/puerperium, and oral contraceptives.

**Plasma Hcy Assay**

Blood samples in K$_3$-EDTA were immediately placed on ice and centrifuged at 2000g, 4°C, for 15 minutes. The supernatant was stored in aliquots at −70°C until assay. The plasma levels of tHcy (free and protein bound) were determined by high-performance liquid chromatography (Waters Millipore 6000A pump, Millipore) and fluorescence detection (Waters 474) by the method of Ubbink et al., with slight modifications. Briefly, 100 µL of plasma was incubated with 10 µL of 10% tri-n-butylphosphine in dimethylformamide at 4°C for 30 minutes to reduce homocysteine and mixed disulfide and deconjugate Hcy from plasma proteins. Then, 100 µL of 10% trichloroacetic acid was added, and the mixture was centrifuged in an Eppendorf microcentrifuge at 13 000 rpm for 10 minutes. After centrifugation, the mixture was incubated with 1 mg/mL ABDF in borate buffer to derivatize the thiols. The mobile phase, pumped at 1 mL/min, consisted of 0.1 mol/L potassium dihydrogenophosphate, 0.06 mmol/L EDTA, and 12% acetonitrile (pH=2.1).

**Criteria for Diagnosis of HyperHcy**

HyperHcy was diagnosed when fasting plasma levels of tHcy or its postmethionine load absolute increments above fasting levels exceeded the 95th percentiles of distribution of values obtained in 388 healthy controls.

**Measurement of Plasma APC**

Plasma APC levels were measured with an enzyme capture assay, essentially as described by Gruber and Griffin. Blood samples were...
centrifuged within 60 minutes from collection at 1200g, 4°C, for 30 minutes to obtain platelet-poor plasma, which was frozen in aliquots at −70°C. A plasma pool from 30 healthy individuals (15 men, 15 women) was obtained in the same way and used to prepare the standards. Microtiter plate wells were incubated at 4°C overnight with 150 μg/mL of immunoaffinity-purified C3-Mab (a murine monoclonal antibody against the light chain of human protein C). After saturation of the wells with Super Block (Pierce) and treatment with the irreversible protease inhibitor APMSF at 4°C for 30 minutes, the plates were washed and kept at 4°C overnight with buffer. Serial dilutions of the pooled normal plasma and the samples were incubated in the empty wells at room temperature for 2 hours to capture the APC antigen. The plates were then rinsed 5 times to ensure the complete removal of benzamidine and plasma enzymes. The chromogenic substrate for APC S-2366 (0.46 mmol/L in Tris-buffered saline, pH 7.4) was then added to the wells. After incubation of the sealed plates at 4°C in wet chambers for 3 weeks, hydrolysis of the substrate was monitored at a dual wavelength setting of 405/655 nm. The concentration of APC in the unknown samples was calculated from the absorbance of each sample with the standard curve as a reference. Results were expressed as percentage of pooled normal plasma.

**Measurement of Plasma F1+2**

F1+2 was assayed by a commercial ELISA (Behringwerke), as previously described.21

**Statistical Analysis**

The two-tailed t test was used to compare VTE patients and healthy controls. ANOVA was used to compare VTE-HyperHcy, VTE controls, and healthy controls, followed by the Dunnett’s test for internal contrasts. The Pearson r value was calculated for correlations between the variables studied.

**Results**

The results obtained in all VTE patients and controls are presented, including those with the heterozygous G20210A mutation of the prothrombin gene. A subanalysis of the results obtained in the 40 patients and 98 controls, in whom the mutation was looked for, revealed that exclusion of the subjects heterozygous for the mutation did not significantly affect the results.

**Plasma tHcy Levels**

The mean (±SD) fasting levels of plasma tHcy were significantly higher in VTE-HyperHcy (28.8±19.5 μmol/L) than in VTE-NormoHcy (12.0±5.2, P<0.001) and healthy controls (11.0±5.3, P<0.001). The mean postmethionine load increments of tHcy above fasting levels were also higher in VTE-HyperHcy (32.9±13.5 μmol/L) than in VTE-NormoHcy (19.8±7.5, P<0.001) and healthy controls (16.1±7.6, P<0.001). Differences between VTE-NormoHcy and healthy controls were not statistically significant. Six healthy controls (6.3%) had HyperHcy, according to the diagnostic criteria previously outlined.

**Plasma Levels of APC**

**Healthy Controls**

The mean plasma level of APC in healthy controls was 116±20%. There was a statistically significant correlation between the plasma levels of APC and those of protein C (r=0.48, P<0.001) (Figure 1). Therefore, because APC levels are influenced by the concentration of their zymogen, both the absolute APC levels and the activated protein C/protein C (APC/PC) ratios were used for subsequent analysis. The mean value of the APC/PC ratio in healthy controls was 1.01±0.2.

There was no correlation between the plasma levels of APC (not shown) or the APC/PC ratios and the fasting plasma levels of tHcy (Figure 2) or its postmethionine load increments above fasting levels (not shown). The mean APC plasma levels and APC/PC ratios were similar in healthy controls whose tHcy plasma levels fell within the first (115 and 1.0), second (118 and 0.96), or third (115 and 1.01) tertiles of distribution.

The mean fasting plasma levels of APC and the APC/PC ratios of 10 healthy controls did not significantly differ from those measured in the same subjects 4 hours after an oral methionine load, which increased the concentration of tHcy by more than 2-fold (Table 2).

**VTE Patients**

The mean plasma levels of APC and APC/PC ratios were higher in VTE patients than in healthy controls (124±32 versus 116±20, P=0.03 and 1.12±0.32 versus 0.99±0.19, P=0.0004). This difference was mostly due to VTE-HyperHcy patients whose plasma APC levels and APC/PC ratios were significantly higher than those of healthy controls (Table 3). In contrast, differences between VTE-NormoHcy and healthy controls and between VTE-HyperHcy and VTE-NormoHcy did not reach statistical significance (Table 3). Results did not change substantially when we excluded patients with thrombosis of the superficial veins (APC levels, 124±26 in VTE-HyperHcy and 121±31 in VTE-NormoHcy; APC/PC ratio, 1.17±0.25 in VTE-HyperHcy and 1.09±0.3

![Figure 1. Correlation between the plasma levels of protein C and APC in 98 healthy volunteers. Values are expressed as percentage of the concentrations measured in pooled normal plasma from 30 healthy blood donors.](image)

![Figure 2. Correlation between the fasting plasma levels of tHcy and APC/PC ratios of 98 healthy volunteers.](image)
TABLE 2. Plasma Levels tHcy, APC, and APC/PC Ratios in 10 Healthy Controls Before and 4 Hours After Methionine Loading (PML)

<table>
<thead>
<tr>
<th></th>
<th>tHcy, μmol/L</th>
<th>APC, %</th>
<th>APC/PC Ratio</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>10.5±3.8</td>
<td>118±43</td>
<td>0.98±0.2</td>
</tr>
<tr>
<td>4 h PML*</td>
<td>29.5±7.6</td>
<td>113±32</td>
<td>0.95±0.1</td>
</tr>
<tr>
<td>P†</td>
<td>0.0001</td>
<td>0.57</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Data are mean±SD.

*Methionine was given orally at a dose of 3.8 g/m² body surface area.

†t test for paired samples.

in VTE-NormoHcy) or women taking oral contraceptives (APC levels, 115±19 in controls, 130±29 in VTE-HyperHcy, and 121±33 in VTE-NormoHcy; APC/PC ratio, 0.98±0.23 in controls, 1.13±0.4 in VTE-HyperHcy, and 1.08±0.3 in VTE-NormoHcy).

The prevalence of high APC/PC ratios was significantly higher in VTE patients than in controls, independent of the tHcy levels in their plasma (Table 4), whereas that of high plasma APC levels was significantly increased in VTE-HyperHcy patients only (Table 4).

Plasma Levels of F1+2

The mean plasma level of F1+2 in VTE patients (1.6±0.5 nmol/L) did not significantly differ from that measured in healthy controls (1.5±0.6 nmol/L). There was no statistically significant difference between plasma levels of F1+2 in VTE-HyperHcy (1.6±0.6 nmol/L), VTE-NormoHcy (1.6±0.6 nmol/L), and healthy controls. The mean F1+2 plasma levels were similar in healthy controls whose plasma levels of tHcy fell within the first, second, or third tertiles of distribution (not shown). F1+2 levels and APC/PC ratios were significantly correlated in controls (r=0.28, P=0.005) but not in VTE-HyperHcy (r=-0.03, P>0.05) or VTE-NormoHcy (r=0.08, P>0.05).

Discussion

This study shows that patients with previous episodes of VTE have higher circulating plasma levels of APC than healthy controls, particularly if they have HyperHcy. The patients studied had none of the known congenital or acquired thrombophilic states, in which the circulating levels of markers of activation of the coagulation system may be increased.23–24 Even though the recently described G20210A mutation of the prothrombin gene77 could be looked for retrospectively in only approximately one third of the patients, also those patients in whom the prothrombin mutation was ruled out had high APC levels, excluding that they were mainly due to the presence of the mutation. APC is generated from its plasma precursor, protein C, on activation by thrombin-thrombomodulin complex on the endothelial cell surface, probably acting in concert with the endothelial cell protein C receptor.1 Subcoagulant amounts of thrombin in the circulation may increase the plasma levels of endogenous APC, which can therefore be considered markers of a hypercoagulable state.4 Accordingly, the high APC plasma levels that we measured in patients with previous episodes of VTE may be interpreted as an index of ongoing thrombin formation, despite the fact that at least 4 months (and a median of 14 months) elapsed since their last thrombotic episode. However, the plasma concentrations of F1+2, a marker of thrombin generation, were not increased significantly in the same VTE patients and were not correlated with APC levels or APC/PC ratios. In contrast to VTE patients, a statistically significant correlation between APC and F1+2 plasma levels was found in healthy controls. On the basis of these data, we hypothesize that the increased plasma levels of APC found in patients with previous episodes of VTE are not caused by heightened thrombin generation but by alternative mechanisms. Although we did not measure markers of activation of the fibrinolytic system, the possibility that high plasma levels of plasmin could be responsible for protein C activation25 in these patients should be considered.

The greatest increase of APC plasma levels in VTE patients was observed in subjects with fasting and/or postmethionine-loading HyperHcy. VTE patients with normal plasma levels of tHcy had lower concentrations of APC than patients with HyperHcy, but this difference could be due to chance alone, because it was not statistically significant. These results contrast with the alleged inhibitory effect of Hcy on protein C activation that was shown in vitro studies.8–10 Our data obtained in healthy individuals support the view that Hcy does not affect protein C activation in vivo, because the mean plasma levels of APC of subjects in the highest tertile of distribution of tHcy levels were not different from those of subjects in the lowest tertile. Moreover, the rapid increase in plasma tHcy brought about by an oral methionine load did not affect the concentration of circulating
APC. Therefore, the results of our study suggest that Hcy does not negatively influence the plasma APC levels and argue against the hypothesis that it inhibits the activation of protein C in vivo by interfering with the activity of thrombomodulin.

Recently, Lentz et al., in an experimental study of monkeys with diet-induced moderate HyperHcy, showed that the thrombin-stimulated endothelium of aortas from hyperhomocysteinemic animals activated protein C in vitro less effectively than that of control animals. This study, which supports the hypothesis that Hcy interferes with protein C activation, is in apparent contradiction with our results. At least two possible explanations for their different results can be proposed. First, Hcy would not affect protein C activation that is ongoing in vivo under physiological conditions, whereas it would interfere with its activation at sites at which atherogenic or thrombogenic stimuli injured the endothelium and increased the local concentration of thrombin. Second, due to the different relative densities of endothelial cell protein C receptor and thrombomodulin on the endothelium of large vessels and capillaries, the regulation of protein C activation may differ in the two vascular districts. Although Lentz et al measured protein C activation by the endothelium of the aorta, we measured circulating APC, which mostly reflects protein C activation occurring in the microcirculation. On the basis of the considerations above, we speculate that Hcy does not interfere with protein C activation ongoing in the microcirculation under physiological conditions, whereas it could inhibit protein C activation on large, injured vessels.

In conclusion, our study shows that APC plasma levels are high in patients with previous episodes of VTE in whom the plasma levels of F1+2 are normal. Therefore, APC plasma levels represent a sensitive marker of activation of the hemostatic system. In addition, the study showed that high Hcy levels are not associated with heightened thrombin generation and do not interfere with the activation of protein C under physiological conditions in vivo. Further studies are needed to unravel the mechanism(s) by which HyperHcy increases the risks for atherosclerosis and thrombosis.

References
