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Activated Protein C Plasma Levels in the Fasting and Postprandial States among Patients with Previous Unprovoked Venous Thromboembolism

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Abstract

Introduction—The protein C anticoagulant system is of major importance in the regulation of thrombotic risk, but it is not known whether low plasma levels of activated protein C (APC) *in vivo* reflect a compromised anticoagulant situation with increased thrombotic risk. Previous studies have reported low, normal or increased plasma APC levels in unselected patients with venous thromboembolism (VTE).

Materials and Methods—We performed a population-based, case-control study in patients with a previous history of unprovoked VTE and subjected the participants to a standard fat tolerance test (1 g fat/kg body weight) in order to promote physiological coagulation activation.

Results: VTE patients had higher BMI (28.3 ± 4.4 kg/m² versus 26.3 ± 3.9 kg/m², $p=0.045$) and greater waist circumference (98.2 ± 12.5 cm versus 93.4 ± 13.4 cm, $p=0.041$) than age and sex matched controls. APC levels were equal in fasting plasma (3.00 ± 0.74 ng/ml and 2.99 ± 0.60 ng/ml, $p=0.66$) but higher in postprandial plasma (3.18 ± 0.57 ng/ml and 2.81 ± 0.38 ng/ml, $p=0.008$) collected from VTE patients and controls, respectively. Endogenous thrombin generation in plasma following a standardized meal, assessed by thrombin-antithrombin complex (TAT), increased similarly in both groups, whereas APC increased only among the VTE patients during the postprandial state. Plasma levels of APC increased linearly with TAT in the postprandial state (p for linear trend=0.012).

Conclusions—Our findings fail to support the hypothesis that low APC levels are linked to increased thrombotic risk in unprovoked VTE, and they suggest that plasma APC is a biomarker of thrombin generation.

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Keywords

Activated protein C; venous thromboembolism; postprandial lipemia; coagulation activation

The protein C anticoagulant system is of major importance in the regulation of haemostasis [1] and thrombosis [2]. Activated protein C (APC) is a normal plasma component [3,4] measurable with specific and sensitive methods [5,6], indicating that the protein C anticoagulant pathway is continuously activated *in vivo*. Generation of APC is dependent on the assembly of protein C, thrombin, and their respective endothelial receptors, endothelial protein C receptor and thrombomodulin [7,8], and its function is known to be modified by protein S [9] and lipid cofactors such as high density lipoprotein (HDL) [10], glucosylceramide [11], cardiolipin [12], and phosphatidylethanolamine [13].

It is conceivable that any disturbance in this assembly, including lipid and haemostatic changes during the postprandial phase, could influence APC generation *in vivo*. However, it is not clear whether plasma levels of APC activity reflects increased thrombin generation in the circulation or whether low levels of APC activity *in vivo* represent an attenuated anticoagulant situation with increased thrombotic risk. Both low [14] and high [15] plasma levels of APC activity have been reported in patients with a previous history of venous thromboembolism (VTE) [14,15] and superficial vein thrombosis [15] without congenital or acquired thrombophilic states. The latter studies included patients with both unprovoked and provoked VTE with incident and recurrent events.

Previous studies in healthy individuals and patients with combined hyperlipemia have reported increased coagulation activation, assessed by plasma levels of activated factor VII (FVIIa) [16] and endogenous thrombin generation [17,18], during postprandial lipemia. We wanted to investigate plasma levels of APC in patients with previous unprovoked VTE under fasting and postprandial conditions. We hypothesized that patients with unprovoked VTE would have higher APC activity than healthy controls, and that this difference would be augmented during the postprandial state. To address this question, we performed a case-control study in patients with a previous history of VTE and population-based controls who underwent a standard fat tolerance test (1 g fat/kg body weight).

MATERIAL AND METHODS

Study population

Patients with unprovoked VTE were recruited from the registry of VTE patients in the municipality of Tromsø [19]. A VTE event was recorded in the registry when each and all of the following criteria were fulfilled; (i) objectively confirmed by diagnostic procedures, (ii) the medical record indicated that a physician had made a diagnosis of deep venous thrombosis (DVT) or pulmonary embolism (PE), (iii) signs and symptoms consistent with DVT or PE were present, and (iv) treatment with anticoagulants (heparin, warfarin, or a similar agent), thrombolytics, or vascular surgery was required. Unprovoked VTE was defined as complete absence of provoking factors at the time of diagnosis. The following were regarded as provoking factors; recent surgery or trauma (within eight weeks before the event), acute medical conditions (acute myocardial infarction (MI), acute ischemic stroke, major infectious disease), cancer, marked immobilization (bed rest > 3 days, wheelchair patients, long distance travel > 4 hours within the last 14 days), pregnancy or puerperium, estrogen supplementation, or other potential provoking factor specifically described by a physician in the medical record (e.g. intravascular catheter). Subjects were eligible for the VTE group if they were aged 20-80 years, had an unprovoked VTE event without recurrence 1-5 years prior to the investigation, and had stopped anticoagulant treatment at least 3

months prior to the investigation. Patients who responded positive to our invitation letter were invited to a screening visit. At the screening visit, a complete medical history, physical examination and blood samples were taken with special emphasis on exclusion criteria. A detailed interview on the occurrence of cardiovascular events (prior or present transient ischemic attacks (TIA), stroke, angina pectoris, and MI), recurrent venous thrombosis, diabetes mellitus and other concurrent diseases was obtained with a self-administrated questionnaire which also included dietary habits, physical exercise, and alcohol consumption. Exclusion criteria were any of the following conditions: regular use of lipid-lowering drugs (HMG-CoA reductase inhibitors, resins or nicotinic acid derivatives) sex hormones or oral anticoagulants, cancer or other serious life-threatening medical conditions, present or previous cardiovascular diseases, recurrent venous thrombosis, diabetes mellitus, hypothyroidism, renal, hepatic, or psychiatric disease, and current abuse of alcohol or drugs. Hypertension was defined as self-reported (in a health questionnaire) use of antihypertensive medication, or systolic pressure above 160 mmHg and/or diastolic pressure above 95 mmHg. Height and weight were measured with the participants in light clothing without shoes, and waist circumference was measured in centimeters at the umbilical line. Blood pressure was recorded in seated position. Three recordings were made at 1-minute intervals, and the mean of the two last values is used in this report. For each VTE patient, one apparently healthy person matched for age and gender was recruited from a health survey (the sixth Tromsø study) and underwent the same screening visit as the VTE patients. In total, 24 cases and 24 controls were eligible according to inclusion and exclusion criteria, and were invited to a second visit in which they were subjected to a fat tolerance test. Informed written consent was obtained from the participants, and the regional ethical committee approved the study. The study was performed at the Clinical Research Unit at the University Hospital of North-Norway.

Fat tolerance test

A fat-tolerance test was conducted using a test meal prepared from standard porridge cream containing 70% of calories from fat of which 66% saturated fat, 32% monounsaturated fat and 2% polyunsaturated fat. The test meals were served with two teaspoons of sugar, cinnamon, and two glasses (150 ml each) of sugar-free juice. The test meals were freshly prepared each morning. A weight-adjusted meal (1 gram fat per kg body weight) was served at 8:00 a.m. and consumed over a 15-min. period. The participants were allowed to drink 350 ml calorie-free beverages and eat an apple during the following 8 hrs. Blood samples for lipoprotein isolation, and serum- and plasma preparations were collected before the meal, and every second hour during the next 8 hrs.

Blood collection and storage

Blood was drawn from an antecubital vein in the morning at 7:45 a.m. after 12 hours overnight fasting and 48 hrs refrain of exhaustive physical exercise and alcohol consumption, and then 2, 4, 6, and 8 hrs after the meal, using a 19-gauge needle in a vacutainer system with minimal stasis. Serum was prepared by clotting whole blood in a glass tube at room temperature for 1 hour. Blood for plasma preparation was collected into 4.5 ml vacutainers (Becton Dickinson, Meylan Cedex, France) containing 0.129 M sodium citrate (1 vol anticoagulant and 9 vol whole blood) as anticoagulant without and with addition of 100 μ l 1.65 M benzamidine (Sigma Chemicals Co, St.Louis, MO, USA) and protected from light. Serum and plasma was prepared by centrifugation at 2000 g for 15 minutes at 22°C, transferred into cryovials (Greiner laboratechnik, Nürtingen, Germany) in aliquots of 1 ml and stored at -70°C until further analysis.

Measurements

Serum lipids were analyzed on an ABX Pentra 400 (Horiba ABX Diagnostics, Montpellier, France) with reagents from Horiba ABX Diagnostics (Montpellier, France). Total cholesterol (CHOD-PAP) and triglycerides (GPO-PAP) were measured using enzymatic photometric methods provided by Horiba ABX Diagnostics, Montpellier, France.

Plasma levels of antithrombin amidolytic activity (STA-STACHROM AT III), protein S cofactor activity (STA-STACLOT PROTEIN S), protein C amidolytic activity (STA-STACHROM PROTEIN C), activated protein C-resistance (Coatest APC Resistance VS kit, ChromogenixAB, Möndal, Sweden), and lupus inhibitors (STA StacLOT DRV Screen F) was determined by commercially available assays obtained from Diagnostica Stago (Asnieres-Sur-Seine, France) on an automatic analyzer (STA-R Evolution, Diagnostica Stago). Screening for factor V Leiden and factor II G20210A mutations was carried out on genomic DNA as previously described [20,21]. Plasma concentration of thrombin-antithrombin (TAT) complexes was measured with a commercial assay (Enzygnost TATmicro, Dade Behring Marburg GmbH, Marburg, Germany) according to the protocol given by the manufacturer.

The concentration of endogenous circulating APC present in the plasma at the time of sampling was measured by a previously published enzyme capture assay method [5]. Briefly, patient blood was collected directly into an anticoagulant cocktail containing citrate and the reversible active site inhibitor, benzamidine. A monoclonal antibody against the protein C light chain was used to capture the APC from the plasma. The monoclonal antibody selected does not inhibit enzyme activity. After removal of benzamidine by washing, the amount of captured APC was determined by its hydrolysis of the chromogenic substrate S-2366. The normal range of APC determined from 41 donors was 70% to 143% with a mean of $104.9\% \pm 19.6\%$ (SD). The mean value corresponded to an actual APC concentration of 2.26 ± 0.20 ng/mL.

Statistical analysis

The primary hypothesis in the study was that patients with a previous history of VTE would have 20% higher plasma levels of APC than control subjects at peak concentration of postprandial lipemia. Power calculations with a two-tailed significance level of 0.05 (alpha), expected average APC level of 2.40 with a standard deviation of 0.40, revealed that 15 subjects were need in each group to achieve a statistical power of at least 90% (Power and Precision™, Biostat Inc., Englewood, NJ, USA).

All variables were checked for frequency distribution. The TAT and endogenous APC response during the postprandial phase was determined as the average of the two highest postprandial TAT and APC concentrations minus baseline concentrations, respectively. Deficiency of antithrombin, protein S and total protein C was defined by a plasma level 65% of normal plasma levels. Unpaired t-test was used to compare differences between groups on continuous variables, whereas χ^2 (chi-square) test was used to compare differences between groups on categorical variables. General linear models for univariate analysis of variance were used for adjustments. Pearson's correlation coefficients (r) were used to examine correlations between continuous variables. Linear regression models were used to test linear trends. All analyses were performed using SPSS (SPSS Inc. Chicago, Illinois, USA) for windows software, version 17.0. Two-sided p-values < 0.05 were considered statistically significant. Values are means $\pm$ 1 standard deviation (SD) if not otherwise stated.

RESULTS

Characteristics of cases and controls with regard to age, anthropometric measures of obesity, traditional cardiovascular risk factors and inherited thrombophilia are shown in table 1. VTE patients had significantly higher BMI (28.3 ± 4.4 kg/m² versus 26.3 ± 3.9 kg/m², $p=0.045$) and greater waist circumference (98.2 ± 12.5 cm versus 93.4 ± 13.4 cm, $p=0.041$) than age and gender matched controls. There was no difference between groups with regard to smoking habits, blood pressure and serum lipids. Although FV-Leiden was more frequent among VTE patients, no significant differences in inherited thrombophilia (VTE group; 3 FV-Leiden, 1 FII G20210A, 2 protein C deficiency and 1 protein S deficiency. Control group; 1 FV-Leiden and 2 protein S deficiency) and family history of VTE was observed between groups. No individuals were found to have lupus anticoagulants (data not shown). No significant associations were found between APC and plasma levels of protein C, protein S or antithrombin (data not shown).

VTE patients and controls underwent a standard fat tolerance test (1 gram fat/kg body weight), and serum concentrations of total triglycerides was measured every second hour during 8 hrs in the postprandial phase (figure 1). Serum triglycerides, assessed by incremental area under the triglyceride curve (AUCi), increased significantly in both groups ($p<0.001$). Peak levels of serum triglycerides were registered 4 hrs after ingestion of the meal, and there was no difference in postprandial lipemia assessed neither by peak response of serum triglycerides (1.06 ± 0.68 mmol/l and 1.10 ± 0.75 mmol/l, $p=0.84$) nor by AUCi (4.82 ± 3.58 mmol/l*h and 5.24 ± 4.32 mmol/l*h, $p=0.71$) between VTE patients and controls, respectively.

Plasma levels of endogenous thrombin formation, assessed by TAT (figure 2A) (4.67 ± 3.08 µg/ml versus 3.68 ± 1.98 µg/ml, respectively, $p=0.24$), and of APC (figure 3A) (3.00 ± 0.74 ng/ml versus 2.99 ± 0.60 ng/ml, respectively, $p=0.66$) were not significantly different in cases and controls (statistical analysis adjusted for BMI, smoking, serum lipids, blood pressure and inherited thrombophilia). Plasma levels of TAT increased significantly, assessed by TAT response ($p<0.001$) and incremental area under the TAT curve ($p<0.001$) in both groups during postprandial lipemia (figure 2B). Peak levels of plasma TAT were observed 4-6 hrs after ingestion of the meal, and there was no difference neither in peak response of plasma TAT (14.1 ± 9.1 µg/ml versus 12.6 ± 9.7 µg/ml, $p=0.65$) nor in AUCi (53.9 ± 36.1 µg/ml*h versus 52.1 ± 45.5 µg/ml*h, $p=0.90$) between VTE patients and controls, respectively, during the postprandial phase.

Postprandial lipemia was associated with a transient decrease in plasma levels of APC ($p=0.026$ in VTE patients and 0.043 in controls) followed by a significant increase in VTE patients ($p=0.039$) and no further change in control subjects (figure 3B). Plasma levels of APC were significantly higher in VTE patients than controls at 4 hrs (3.18 ± 0.57 ng/ml versus 2.81 ± 0.38 ng/ml, $p=0.008$) and at 6 hrs (3.12 ± 0.51 ng/ml versus 2.79 ± 0.35 ng/ml, $p=0.009$) after ingestion of the standard meal. The differences in APC levels during the postprandial phase remained significant after adjustment for BMI, smoking, serum lipids, blood pressure and inherited thrombophilia ($p=0.018$ at 4 hrs and $p=0.021$ at 6 hrs).

Serum triglycerides was neither correlated with plasma levels of TAT ($r=-0.17$, $p=0.23$) and APC ($r=-0.22$, $p=0.14$) under fasting conditions, nor during postprandial lipemia (data not shown). To investigate the relation between plasma TAT and APC under fasting and postprandial conditions, linear regression analysis was conducted for APC across tertiles of TAT under fasting conditions (figure 4A), and the APC response across tertiles of the TAT response during postprandial lipemia (figure 4B). Plasma levels of TAT and APC showed a non-significant correlation under fasting conditions ($r=0.24$, $p=0.08$) and linear trend

analysis (figure 4A). However, the responses (mean of two highest postprandial values minus baseline) for TAT and APC during the postprandial phase was strongly correlated ($r=0.43$, $p=0.003$). Moreover, the APC response increased linearly ($p=0.012$) across tertiles of the TAT response in regression analysis (figure 4B).

DISCUSSION

The present study was conducted to investigate plasma levels of APC in patients with a previous history of unprovoked VTE under fasting and postprandial conditions. We found no difference in plasma levels of APC between VTE patients and healthy controls under fasting conditions, but plasma levels of APC exhibited a modest increase among VTE patients in the postprandial state which differed significantly from controls. The postprandial changes in APC were significantly associated with increased endogenous thrombin generation assessed by TAT, consistent with the assumption that thrombin generates APC. Our findings suggest that fasting plasma levels of APC are not associated with thrombotic risk in patients with unprovoked venous thrombosis, but that APC levels in the postprandial state fluctuate with physiological increases in endogenous thrombin generation.

Even though provoking factors such as surgery, trauma, acute medical conditions, hospitalization, malignancy, immobilization, pregnancy and puerperium, and use of estrogens are most often associated with VTE, still 30-50% of the events have no obvious predisposing factors [22] and are likely influenced by unrecognized prothrombotic conditions. Thus, we expected that the chance of finding changes in fasting plasma APC activity would be highest among patients with a previous history of unprovoked VTE. However, we found no difference in fasting plasma APC levels between patients with unprovoked VTE and healthy age- and sex-matched controls. In contrast, both low [14] and high [15] plasma levels of APC have previously been reported in VTE patients without congenital inherited thrombophilia, whereas unchanged APC activity, but higher APC/PC ratios have been reported in unselected VTE patients with some congenital inherited thrombophilia [23]. In fact, Cattaneo et al. [15] found higher APC levels only among VTE patients with hyperhomocysteinemia, and not among patients with normal homocysteine levels compared to healthy controls. The reason(s) for the apparent controversial findings among studies on plasma APC in VTE patients are unknown, but may include differences in selection of patients and controls and methods used for determination of APC in plasma. Previous studies [14,15] included non-thrombophilic patients with a mixture of incident and recurrent VTE, provoked and unprovoked VTE, whereas our population consisted of incident cases of unprovoked VTE and thrombophilia was not a selection criteria. In the study reporting low APC levels in VTE patients, APC was determined indirectly by calculating the difference in circulating APC:PCI complexes in heparin-treated plasma [to promote APC:PCI complex formation] and in benzamidine-treated plasma (to avoid APC:PCI formation ex vivo) [14]. Along with the other studies [15,23], we determined APC levels by an enzyme capture assay method using a monoclonal antibody against the protein C light chain [5].

Analyses of protein C activation, reflecting the endogenous levels of APC:PCI, reported an association with acute thrombotic events such as arterial and venous thrombosis [24,25] and disseminated intravascular coagulation [26]. Furthermore, significantly increased plasma concentrations of the APC:PCI complex were reported in acute deep venous thrombosis (DVT) patients [27] and for patients with a previous history of DVT who carried FV-Leiden [28] using a sensitive sandwich immunofluorimetric assay for measurement of APC:PCI complex [29]. The plasma concentrations of the APC:PCI complex were strongly associated with markers of endogenous thrombin generation, such as TAT [27], suggesting that plasma concentration of APC:PCI complex may be a sensitive marker of coagulation activation.

Although a significant association between plasma APC and fragment 1+2 was reported in healthy individuals [32], our finding of a non-significant association between endogenous thrombin generation, assessed by TAT, and APC activity under fasting and resting conditions is in agreement with previous studies in patients with coronary artery disease and venous thrombosis [15,32]. However, acute inflammatory stress due to surgery [32,33] or serious infection [34] have been associated with increased APC activity and related to an accompanying increase in endogenous thrombin generation [32]. Most of diurnal time is spent in a postprandial state due to successive meal intakes during the day. The postprandial state is a dynamic, non-steady-state condition, with rapid remodeling of lipoproteins by intestinal and hepatic synthesis of triglyceride-rich lipoproteins, lipid and apolipoprotein exchange between lipoproteins by carrier molecules, and subsequent receptor-mediated clearance of these lipoprotein particles from the circulation [30]. Thus, the postprandial phase is a physiological condition with expected increased coagulation activation [14,16-18,31]. In our study, VTE patients and healthy controls experienced a similar increase in endogenous thrombin generation during the postprandial phase. We found a modest increase in plasma APC activity during the postprandial phase among VTE patients which differed significantly from healthy controls at four and six hours after ingestion of the meal. The postprandial changes in plasma APC were associated with changes in endogenous thrombin generation but not associated with changes in serum triglycerides. Our findings may suggest that endogenous thrombin generation is an important regulator of plasma APC under physiological conditions in order to avoid overt coagulation activation.

In conclusion, plasma levels of APC were similar among patients with previous unprovoked VTE and healthy controls under fasting conditions, and they increased modestly, and in parallel with endogenous thrombin generation, among VTE patients during the postprandial phase. Our data suggest that increased levels of APC in plasma during postprandial lipemia are a biomarker for thrombin generation in the circulation.

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Abbreviations

APC	Activated protein C
AUCi	incremental area under the curve
BMI	Body mass index
DVT	Deep vein thrombosis
FVIIa	Activated factor VII
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
MI	Myocardial infarction
PE	pulmonary embolism
PC	Protein C
SD	Standard deviation
TAT	thrombin-antithrombin
TIA	transient ischemic attacks

VTE venous thromboembolism

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Figure 1.

Line graph showing serum concentrations of triglycerides before and after intake of a standard meal (1 gram fat/ kg bodyweight) in patients with a previous history of unprovoked venous thromboembolism and age- and gender matched controls. Values are mean $\pm$ SEM.

Figure 2.

Plasma levels of thrombin-antithrombin complexes (TAT) in patients with a previous history of unprovoked venous thromboembolism and age- and gender matched controls under fasting conditions (panel A) and during postprandial lipemia (panel B). Values are mean $\pm\text{SEM}$.

Figure 3.

Plasma levels of endogenous activated protein C in patients with a previous history of unprovoked venous thromboembolism and age- and sex matched controls under fasting conditions (panel A) and during postprandial lipemia (panel B). Values are mean $\pm$ SEM. ** indicates $p < 0.01$ for difference between groups.

Figure 4.

Relation between endogenous TAT and APC levels in plasma. Line graphs showing plasma levels of APC across tertiles of TAT under fasting conditions (panel A) and peak responses of APC across increasing tertiles of TAT responses during postprandial lipemia (panel B).

panel A: T1: 1.90 (0.81-2.43)(mean and range), T2: 3.55 (2.44-4.31) and T3: 7.08 (4.43-20.95)

panel B; T1: 3.01 (-0.14-6.24), T2: 10.83 (7.46-15.28), T3: 26.28 (15.84-47.92).

Table 1

Characteristics of patients with a previous history of unprovoked VTE and healthy age- and sex matched controls recruited from a general population. Values are means $\pm$ 1 SD or percentage with numbers in brackets.

Variables	VTE patients (n=24)	Controls (n=24)	p-values
Age (yrs)	53.9 $\pm$ 16.1	53.7 $\pm$ 16.0	0.98
Sex (% women)	58 (14)	58 (14)	1.00
BMI (kg/m ²)	28.3 $\pm$ 4.4	26.3 $\pm$ 3.9	0.045
Waist circumference (cm)	98.2 $\pm$ 12.5	93.4 $\pm$ 13.4	0.041
Smoking (%)	33 (8)	16 (4)	0.32
Systolic blood pressure (mmHg)	141 $\pm$ 21	137 $\pm$ 27	0.62
Diastolic blood pressure (mmHg)	87 $\pm$ 14	84 $\pm$ 14	0.58
Total Cholesterol (mmol/l)	5.60 $\pm$ 1.44	5.48 $\pm$ 1.21	0.77
Triglycerides (mmol/l)	1.29 $\pm$ 0.87	1.23 $\pm$ 0.69	0.80
Inherited Thrombophilia FV-Leiden (heteroz.) (%)	13 (3)	4 (1)	0.35
FII G20210A (heteroz.) (%)	4 (1)	0 (0)	0.49
Protein-C (%)	108 $\pm$ 22	103 $\pm$ 21	0.48
Protein-S (%)	101 $\pm$ 18	104 $\pm$ 18	0.63
Antithrombin (%)	102 $\pm$ 9	104 $\pm$ 8	0.37
Family history of VTE (%)	21 (5)	13 (3)	0.54