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ORIGINAL ARTICLE

Effect of warfarin treatment on thrombin activatable fibrinolysis inhibitor (TAFI) activation and TAFI-mediated inhibition of fibrinolysis

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Summary. *Background:* Severe clotting deficiencies are associated with enhanced *in vitro* fibrinolysis due to insufficient thrombin activatable fibrinolysis inhibitor (TAFI) activation. Because oral anticoagulant therapy (OAT) with warfarin causes a partial deficiency of vitamin K-dependent factors, its effect on clot lysability remains unclear. *Objectives*: To evaluate plasma and blood fibrinolytic capacity in patients under stable OAT ($n = 221$) as compared with controls ($n = 132$). *Methods*: Fibrinolysis resistance of plasma (turbidimetry) and blood (thromboelastography) clots was calculated as the lysis time of tissue factor-induced clots exposed to 30 and 100 ng mL^{-1} t-PA, respectively. *Results*: Plasma PAI-1 was similar in the two groups, whereas TAFI was slightly lower in patients. OAT plasma clots lysed faster than controls ($P = 0.001$). The addition of the TAFIa inhibitor PTCI reduced lysis time by 14% in OAT and 34% in controls, and the difference between the groups disappeared. Similar data were obtained with blood clots. Thrombin and TAFIa generation in OAT plasma amounted to roughly 50% of controls, supporting a reduced thrombin-dependent TAFI activation. Clot resistance of OAT plasma was normalized by Ba-citrate plasma eluate or prothrombin but not by $BaSO_4$ serum eluate, rFVIIa or FX. Surprisingly, circulating levels of TAFIa and its inactive derivative TAFIai were higher in warfarin patients ($P < 0.0001$) and correlated with plasmin-antiplasmin ($P = 0.0001$) but not with prothrombin F_{1+2}. *Conclusions*: OAT enhances both plasma and blood fibrinolysis by reducing thrombin-dependent TAFI activation, a phenomenon largely determined by low prothrombin levels. At variance with *in vitro* data, 'basal' *in vivo* TAFIa/ai levels seem related to plasmin rather than thrombin generation.

Keywords: clot resistance, clotting deficiency, TAFI, thrombin, warfarin.

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Introduction

Anticoagulants have been shown to promote *in vitro* fibrinolysis, mainly by reducing thrombin-induced activation of thrombin activatable fibrinolysis inhibitor (TAFI) [reviewed in ref. 1]. TAFI is a plasma procarboxypeptidase of liver origin (plasma procarboxypeptidase U or B) that, once converted into the active enzyme (TAFIa) by thrombin or other enzymes, removes the C-terminal lysines from partially degraded fibrin, thereby reducing the binding of tissue-type plasminogen activator (t-PA) and plasminogen to the clot and, as a consequence, plasmin formation [2,3]. However, anticoagulants may differ remarkably in their profibrinolytic effects, some of them (e.g. hirudin) being almost ineffective [4]. The reason for such different behavior is still unclear. Because TAFI-mediated inhibition of fibrinolysis seems to depend on 'late' thrombin [3], it has been hypothesized that for an anticoagulant to work as a profibrinolytic agent it must be able to efficiently inhibit thrombin generation and/or activity at the right time.

Oral anticoagulants such as warfarin have long been recognized as highly effective drugs for the prevention and treatment of numerous thromboembolic events [5]. Warfarin inhibits the production of functional vitamin

K-dependent blood procoagulant factors (i.e. prothrombin and factors VII, IX and X), as well as of protein C and protein S, two physiological anticoagulant proteins. At therapeutic dosages, warfarin administration results in reduced thrombin generation, as indicated by the reduction of plasma levels of prothrombin fragment 1 + 2 (F_{1+2}) and thrombin-antithrombin complex (TAT) [6,7]. Whether such a reduction in thrombin generation translates into a TAFI-dependent enhancement of fibrinolysis is unknown and difficult to predict. Some old studies showed that the removal of vitamin K-dependent factors from plasma by chemical means leads to an acceleration of fibrinolysis [8] because of insufficient TAFI activation. In these *in vitro* experiments, however, the levels of vitamin K-dependent clotting factors were far less than those achieved in warfarin-treated patients. On the other hand, studies in plasma with specific clotting deficiencies, such as factor (F) VIII and FXI, showed that as little as 1% of the missing factor was enough to restore a normal fibrinolysis [9,10], probably because of the threshold mechanism by which TAFIa inhibits fibrinolysis [11]. Therefore, considering that the level of vitamin K-dependent factors in patients on stable warfarin treatment is around 20%, it is really hard to foresee what impact this partial deficiency will have on the TAFI pathway. In this study we used a physiologically relevant clot lysis model [12] to assess the influence of warfarin therapy on TAFI-dependent fibrinolysis in plasma and blood *in vitro*. Moreover, we evaluated if the reduced *in vivo* thrombin formation in anticoagulated patients is paralleled by a decrease in the circulating levels of TAFIa and its inactive derivative TAFIai.

Patients and methods

Patients

Two hundred and twenty-one consecutive patients under oral anticoagulant therapy (OAT) with warfarin participated in the study. Patients receiving OAT for more than 3 months were eligible. The exclusion criteria were as follows: any acute illness, known cancer, hepatic or renal dysfunction, recent thromboembolic event (<3 months), autoimmune disease and steroid administration. One hundred and thirty-two healthy blood donors served as controls. The study was approved by the Ethics Committee at the University of Bari, and informed consent was obtained from each patient and control.

Proteins and reagents

The following reagents were used: single chain human recombinant tissue-type plasminogen activator (rt-PA, Actilyse) from Boehringer Ingelheim (Florence, Italy), thromboplastin S from Dasit (Milan, Italy), rabbit thrombomodulin (TM) and reptilase ST from American Diagnostica Inc. (Stamford, CT, USA), carboxypeptidase inhibitor from potato tuber (PTCI) and bovine fibrinogen from Sigma (Milan, Italy). Unless otherwise specified, reagents were dissolved and diluted in Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.4). Human prothrombin and FX were from Haematologic Technologies Inc (Essex Junction, VT, USA); recombinant factor VIIa (NovoSeven) was from NovoNordisk (Bagsværd, Denmark). Vitamin K-dependent clotting factors were isolated from plasma and serum of healthy donors by Ba-citrate and $BaSO_4$ adsorption, respectively, as previously described [13,14]. Final preparations were dissolved in half the starting volume and dialyzed overnight against 0.02 M Tris buffer (pH 7.4) containing 0.1% sodium citrate. The concentration of vitamin K-dependent factors in plasma and serum eluates was assessed by clotting assays using commercially available FVII-, FIX-, FX- or prothrombin-depleted plasmas (Instrumentation Laboratory, Milan, Italy). Plasma eluates contained 80–110% of vitamin K-dependent clotting factors whereas serum eluates contained 78–125% of FVII, FIX and FX and less than 1% of prothrombin.

Blood collection and plasma preparation

Blood was collected under fasting conditions between 08.00 and 09.00 into vacutainer tubes (Greiner Bio-one, Kremsmuenster, Austria) containing 3.8% trisodium citrate. An aliquot was used for whole blood clot lysis assay, while the remainder was first centrifuged for 15 min at 1 000 ***g***, and then for 10 min at 12 000 ***g*** at room temperature, to obtain platelet-poor plasma (PPP), which was immediately used for clot lysis assay or stored frozen (−80 °C) until tested. Some assays required defibrinated plasma, which was obtained by incubation with the thrombin-like enzyme reptilase (final dilution 1:50) for 1 h at 37 °C.

Plasma clot lysis assay

The lysis of tissue factor (TF)-induced plasma clots exposed to exogenous rt-PA was studied using a turbidimetric assay [15] modified as follows: 100 μL PPP, 10 μL thromboplastin (1:1000, final dilution, corresponding to approximately 6 pM TF), 10 μL t-PA (30 ng mL^{-1}, final concentration, f.c.), 30 μL buffer and 100 μL $CaCl_2$ (8.3 mM, f.c.) were added to microplate wells. The plate was incubated at 37 °C and the changes in OD at 405 nm were measured every minute up to 3 h in a microplate reader (Multiskan Ascent, American Instrument Exchange, Haverhill, MA, USA). Clotting time was defined as the time to the midpoint of clear to maximum turbid transition. Clot lysis time was defined as the interval between the clotting time and the midpoint of the maximum turbid to clear transition. In some experiments, clotting was induced by replacing thromboplastin and

$CaCl_2$ with thrombin (50 IU mL^{-1}, f.c.) in order to make TAFI activation independent of endogenous thrombin. To evaluate the contribution of TAFI, samples were tested in the presence of PTCI (25 μg mL^{-1}, f.c.), a specific inhibitor of TAFIa [3], which was added to the clot lysis mixture in place of the buffer. The contribution of TAFI was calculated as the difference in lysis time between samples without and with PTCI, referred to as PTCI difference. PTCI addition had a negligible effect on clotting time (<7% change). In some experiments, the influence of vitamin K-dependent factors on clot lysis was evaluated by supplementing OAT plasma with a plasma or serum eluate. In this case, to compensate for the volume of the eluate (100 μL), 10 μL of a 10-fold concentrated $CaCl_2$ solution was used. Whenever other reagents were to be tested they were added directly to the microplate well, and the volume of buffer was reduced accordingly.

Whole blood clot lysis assay

The resistance of blood clots to t-PA-induced fibrinolysis was evaluated by thromboelastography (TEG® 5000, Medival, Padua, Italy), as reported [16] with slight modifications. Briefly, 330 μL citrated blood and 30 μL of a mixture consisting of thromboplastin, t-PA and $CaCl_2$ were added to the cup of the thromboelastograph and the changes in the viscoelastic properties of blood were monitored until complete lysis. Final concentrations/dilutions were as follows: thromboplastin, 1:1000; t-PA, 100 ng mL^{-1}; $CaCl_2$, 20 mM. Whenever other reagents were to be tested, they were included in the 30-μL mixture. Clot lysis time was calculated as the time between the midpoints of clot formation and clot lysis, as previously reported [17].

Evaluation of thrombin and TAFIa generation

Tissue factor-induced thrombin generation in plasma was assessed by a commercial kit (Innovance® ETP assay on a BCS-XP analyzer, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) [18] according to the manufacturer's instructions.

TAFIa generation was assessed in defibrinated plasma by a two-stage functional assay. A mixture similar to that used for clot lysis assay, consisting of 300 μL defibrinated plasma, 30 μL thromboplastin (1:1000 final dilution), 30 μL t-PA (30 ng mL^{-1}, f.c.), 30 μL buffer or PTCI (25 μg mL^{-1}, f.c.) and 300 μL $CaCl_2$ (8.3 mM, f.c.), was prepared in a test tube and incubated at 37 °C. At intervals, aliquots of 55 μL were withdrawn and mixed with 5 μL hirudin (200 U mL^{-1}, f.c.; Knoll, Ludwigshafen, Germany) to stop TAFI activation, and kept on melting ice until tested. TAFIa activity was then evaluated as the ability to prolong the lysis time of purified fibrin clots. Fifty μL of sample was added to a microplate well along with 25 μL bovine fibrinogen (830 μg mL^{-1}, f.c.) and 5 μL t-PA (30 ng mL^{-1}, f.c.), after which clot formation was induced by the addition of 10 μL reptilase (1:50, final dilution). The plate was read every minute at 405 nm at room temperature (in order to reduce the temperature-dependent TAFIa decay) and lysis times were calculated as described above. Results were expressed as the prolongation of lysis time over the PTCI-containing sample. For each plasma sample a calibration curve was constructed as follows. In the first step, t-PA was omitted and thromboplastin was replaced by a solution containing thrombin (5 U mL^{-1}, f.c.) and TM (1 nM, f.c.), in order to completely activate TAFI. After 5 min at 37°, the reaction was stopped with hirudin (200 U mL^{-1}, f.c.) and the sample was serially diluted in the same plasma (not activated by thrombin/TM) containing 200 U mL^{-1} hirudin. The samples with decreasing TAFIa concentrations were then tested by the microplate clot lysis assay exactly as reported above. The calibration curve was used to calculate the per cent of TAFI activation in our test samples.

In some experiments, TAFIa generation was assessed by an ELISA (Asserachrom TAFIa/ai) specific for TAFIa and its inactive derivative (TAFIai), as described elsewhere [17].

Other assays

PT-INR, fibrinogen and blood cell counts were determined by standard methods. The following biomarkers were measured in plasma by commercially available ELISAs as described by the manufacturer: TAFI antigen (Asserachrom TAFI), activated TAFI (Asserachrom TAFIa/ai) and factor VIIa/antithrombin complex (Asserachrom VIIa/AT) from Diagnostica Stago; prothrombin F_{1+2} (Enzygnost F_{1+2}) from Dade Behring (Marburg, Germany); PAI-1 (Imubind PAI-1) and plasmin/$alpha_2$-antiplasmin complex (Imuclone PAP) from American Diagnostica Inc. Because some assays could not be performed in all samples, the number of tests ranged between 92 and 132 in controls and between 152 and 221 in patients.

Statistical analysis

Data distribution was assessed by the Kolmogorov-Smirnov test, and the variables that did not show a normal distribution were log-transformed. Because OAT patients and controls were not age-matched, differences between groups were assessed by ANCOVA, including age as covariate. Adjustment for age was also made in regression analyses. Differences in clotting time and lysis time in samples supplemented with vitamin K-dependent factors were assessed by repeated measures ANOVA. For simplicity, all data are expressed as mean ± SD regardless of their distribution. Statistical analyses were carried

out with the MEDCALC software (Mariakerke, Belgium, USA).

Results

Characteristics of patients on oral anticoagulation

The mean age of OAT patients was 67.1 ± 13.1 years as compared with 53.0 ± 11.6 years in controls ($P < 0.0001$). There was no difference in gender between patients and controls (males, 69% and 72%, respectively). Patients suffered from atrial fibrillation ($n = 92$), prosthetic heart valves ($n = 56$), venous thromboembolism ($n = 39$), dilated cardiomyopathy ($n = 25$), or other diseases ($n = 9$).

Laboratory biomarkers

Mean PT-INR in OAT patients was 2.78 as compared with 1.07 in controls (Table 1). Leukocyte count, red cell count and hematocrit were similar in the two groups, whereas platelet concentration was lower in the OAT group. OAT patients displayed higher levels of fibrinogen, slightly lower levels of TAFI and similar levels of PAI-1 as compared with controls. The circulating levels of prothrombin F_{1+2}, a marker of thrombin generation, and VIIa-AT complexes, a marker of TF-mediated clotting activation, were significantly lower in the OAT group than in controls. In contrast, PAP levels, an index of plasmin generation, were higher in OAT patients, but the difference was no longer significant after age adjustment. Surprisingly, the concentration of TAFIa/ai was appreciably higher in patients than in controls. TAFIa/ai levels did not correlate with prothrombin F_{1+2} nor with VIIa-AT complex in either group ($r < 0.1$, $P > 0.1$), whereas they did correlate with PAP complex both in OAT patients ($r = 0.53$, $P < 0.0001$) and controls ($r = 0.30$, $P = 0.004$).

Fibrinolytic resistance of plasma clots

Fibrinolysis time of tissue factor-induced plasma clots exposed to exogenous t-PA was significantly shorter in OAT patients than in controls ($P = 0.001$, Fig. 1A), whereas, as expected, the clotting time was markedly longer (291 ± 138 vs. 138 ± 48, $P < 0.0001$). Moreover, maximum absorbance was higher in patients than in controls (0.84 ± 0.24 vs. 0.66 ± 0.16), probably because of a higher fibrinogen concentration in the former.

To quantify the impact of TAFI on fibrinolysis, we performed the clot lysis assay in the presence of PTCI, a specific inhibitor of TAFIa [3], and calculated the shortening of lysis time (referred to as PTCI difference). As shown in Fig. 1(B), the PTCI difference in the OAT group was markedly lower than in controls ($P < 0.0001$), indicating that TAFI had little impact on fibrinolysis in warfarin-treated patients. Moreover, the fibrinolysis time recorded in the presence of PTCI, reflecting TAFI-independent fibrinolysis, was slightly longer in OAT patients than in controls ($P = 0.017$, Fig. 1C). These findings indicate that the increase in fibrinolytic capacity in OAT patients was almost entirely TAFI mediated, and likely to be due to a reduction of thrombin-induced TAFI activation. To support this hypothesis, we evaluated the fibrinolytic resistance of clots induced by a high concentration of thrombin (50 IU mL^{-1}) in order to make TAFI activation independent of endogenous thrombin. Under this condition (Fig. 2A) both the lysis time and the effect of PTCI were similar in OAT patients and controls.

Because TM was reported to normalize the enhanced lysability of hemophilic plasma [9], we tested its effect on clot lysis of OAT samples. At a concentration of 1 nM, TM caused a similar prolongation of lysis time in the two groups (prolongation in min, 47.4 ± 13.1 and 43.7 ± 14.6 in OAT patients and controls, respectively). As a result, the difference between OAT patients and

Table 1 Blood variables in warfarin-treated patients (oral anticoagulant therapy, OAT) and controls

Variable	OAT	Controls	Age-adjusted difference	Age-adjusted *P*
PT-INR	2.78 ± 0.71	1.07 ± 0.12	1.74 ± 0.14	< 0.0001
Red cells (10^6 μL^{-1})	4.43 ± 0.56	4.56 ± 0.32	−0.03 ± 0.08	0.72
Leukocytes (10^3 μL^{-1})	5.92 ± 1.43	5.69 ± 1.46	0.39 ± 0.25	0.121
HCT (%)	37.9 ± 3.56	38.5 ± 2.59	−1.04 ± 0.60	0.086
Platelets (10^3 μL^{-1})	125.4 ± 41.7	154.3 ± 32.1	−16.3 ± 6.58	0.014
Fibrinogen (mg dl^{-1})	355 ± 75.9	258 ± 55.1	70.4 ± 9.8	< 0.0001
PAI-1 (ng mL^{-1})	52.4 ± 22.7	55.6 ± 19.2	−3.5 ± 3.1	0.255
TAFI (μg mL^{-1})	10.0 ± 1.60	10.7 ± 1.86	−0.64 ± 0.25	0.012
TAFIa/ai (ng mL^{-1})	28.7 ± 9.2	22.2 ± 5.1	4.8 ± 1.2	0.0001
TAFIa/ai/TAFI ratio (ng μg^{-1})	3.0 ± 1.1	2.14 ± 0.55	0.63 ± 0.14	< 0.0001
Prothrombin F_{1+2} (pM)	87.9 ± 51.0	242.4 ± 113.2	162 ± 16.2	< 0.0001
FVIIa/AT complex (pM)	74.7 ± 10.3	110.3 ± 39.5	−36.5 ± 5.3	< 0.0001
PAP complex (ng mL^{-1})	27.9 ± 11.2	23.7 ± 7.8	0.86 ± 2.05	0.67

HCT, hematocrit; PAI-1, plasminogen activator inhibitor 1; TAFI, thrombin activatable fibrinolysis inhibitor; TAFIa/ai, activated TAFI (TAFIa) and its inactive derivative (TAFIai) generated by spontaneous conformational change; AT, antithrombin; PAP, plasmin/alpha$_2$-antiplasmin. Data are the mean ± SD.

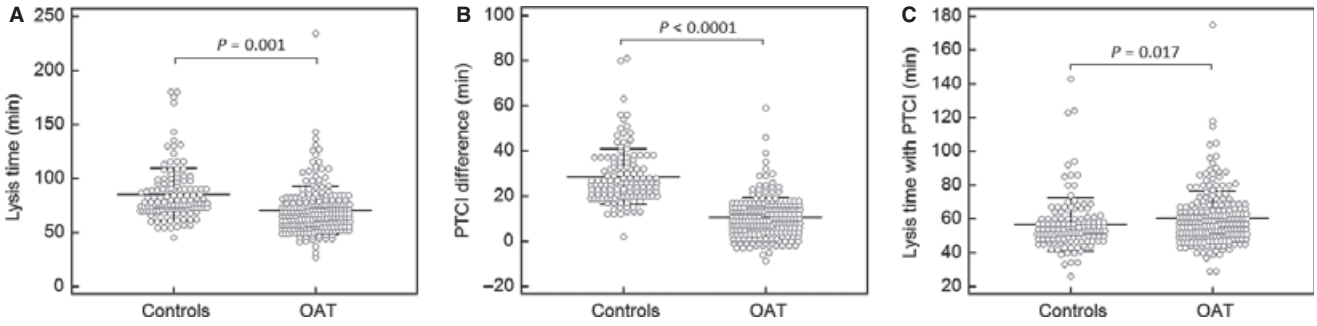

Fig. 1. Fibrinolytic resistance of plasma clots from warfarin-treated patients (oral anticoagulant therapy, OAT) and controls. Fibrinolytic resistance was evaluated by a turbidimetric assay as the lysis time of tissue factor-induced plasma clots containing 30 ng mL^{-1} t-PA (panel A). The contribution of thrombin activatable fibrinolysis inhibitor (TAFI) was calculated as the difference in lysis times in the absence and in the presence of carboxypeptidase inhibitor from potato tuber (PTCI) (PTCI difference, panel B). The lysis time in the presence of PTCI, representing the TAFI-independent fibrinolytic resistance, is shown in panel C. *P* values are age-adjusted.

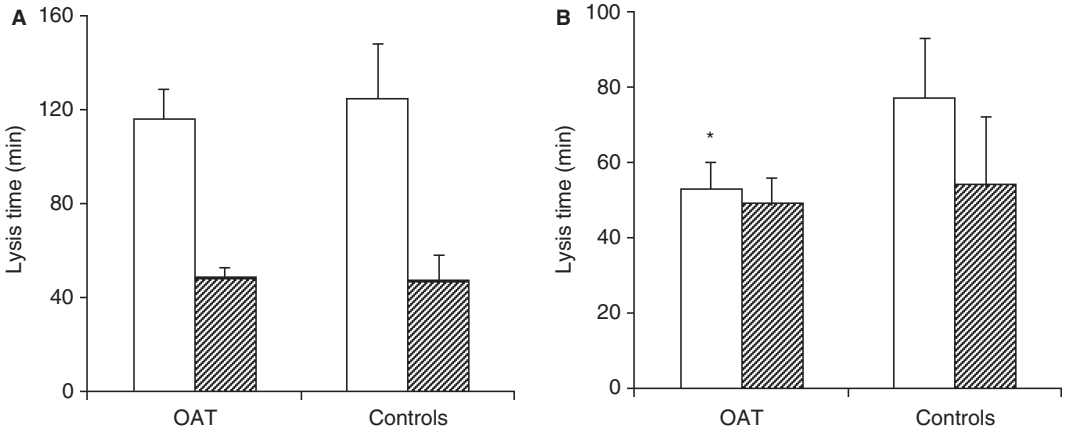

Fig. 2. Fibrinolytic resistance of plasma clots generated by exogenous thrombin. Fibrinolytic resistance was evaluated by a turbidimetric assay as the lysis time of clots containing 30 ng mL^{-1} t-PA. Two different experimental conditions were used. In panel A, plasma samples were clotted by adding human thrombin (50 IU mL^{-1}, final concentration) instead of tissue factor (TF)-$CaCl_2$ in order to obtain a comparable activation of thrombin activatable fibrinolysis inhibitor in oral anticoagulant therapy (OAT) and control samples. In panel B the clots were induced by TF-$CaCl_2$ as reported in the legend of Fig. 1. Experiments were carried out in the absence (white bars) and in the presence of carboxypeptidase inhibitor from potato tuber (dashed bars). Results are the mean ± SD of 10 samples per group. *, $P = 0.005$ as compared with controls.

controls remained virtually unchanged (116 ± 17.5 vs. 128 ± 20.8 min, $P = 0.001$).

Fibrinolytic resistance of blood clots

Blood fibrinolysis was investigated by thromboelastography, using freshly collected and minimally diluted blood. In agreement with the plasma results, the clotting time was longer (4.8 ± 1.9 vs. 2.9 ± 1.1 min, $P < 0.0001$) and the lysis time was shorter (Fig. 3A) in the OAT group as compared with controls. The differences remained highly significant ($P \leq 0.002$) after correction for both age and platelet concentration. There was no difference in maximal amplitude between the two groups (42.3 ± 16.9 vs. 44.4 ± 16.1 mm), suggesting that OAT did not affect clot strength.

The addition of PTCI to blood resulted in a very modest shortening of the lysis time in OAT blood, which was markedly and significantly lower than in control blood (Fig. 3B). In this case too, the lysis time recorded in the presence of PTCI was slightly longer in the OAT group (Fig. 3C).

Thrombin generation and TAFI activation

As expected, thrombin generation in the OAT group was markedly delayed and severely impaired, with a > 50% reduction in ETP (Table 2). Accordingly, the generation of TAFIa activity, as assessed by a two-stage functional assay, was visibly lower in OAT patients than in controls (Fig. 4). Qualitatively similar results were obtained when TAFI activation was assayed by an ELISA specific for TAFIa and its inactive derivative TAFIai (TAFIa/ai concentration after 15 min, 67.7 ± 8.4 and 125 ± 23.6 ng mL^{-1}, respectively; $n = 10$ per group, $P < 0.0001$).

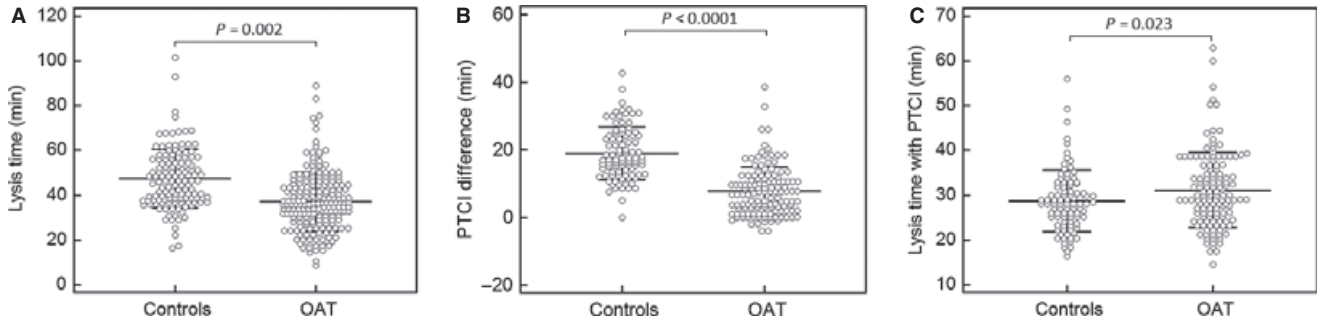

Fig. 3. Fibrinolytic resistance of blood clots from warfarin-treated patients (oral anticoagulant therapy, OAT) and controls. Fibrinolytic resistance was evaluated by thromboelastography as the lysis time of tissue factor-induced clots containing 100 ng mL^{-1} t-PA (panel A). The contribution of thrombin activatable fibrinolysis inhibitor (TAFI) was calculated as the difference in lysis times in the absence and in the presence of carboxypeptidase inhibitor from potato tuber (PTCI) (panel B). The lysis time in the presence of PTCI, representing the TAFI-independent fibrinolytic resistance, is shown in panel C. Experiments with PTCI were performed in 91 controls and 131 patients, respectively. *P* values are age-adjusted.

Table 2 Thrombin generation in oral anticoagulant therapy (OAT) patients and controls

Parameter	OAT patients	Controls	*P*
Lag time (s)	51.2 ± 14.1	25.0 ± 3.8	< 0.0001
Cmax (mA min^{-1})	42.4 ± 10.3	101 ± 12.3	< 0.0001
Time to peak (s)	99.2 ± 20.5	67.7 ± 12.0	< 0.0001
ETP (mA)	168 ± 36.6	386 ± 56.4	< 0.0001

Thrombin generation in TF-activated plasma (see Methods section for details) was monitored with an automated chromogenic assay. Data are the mean ± SD (n = 165 and 95, for OAT patients and controls, respectively).

Role of vitamin K-dependent factors in plasma fibrinolysis

When OAT plasma was supplemented with a plasma eluate, the clotting time was reduced (Fig. 5A) and the fibrinolysis time was prolonged to a level comparable to controls (Fig. 5B). In contrast, when OAT plasma was supplemented with a serum eluate (devoid of prothrombin), the clotting time was visibly shortened whereas the fibrinolysis time was only slightly prolonged. Accordingly, the addition of human prothrombin prolonged fibrinolysis time to an appreciable extent, whereas FX and recombinant FVIIa were practically ineffectual despite their ability to shorten the clotting time as efficiently as prothrombin (Fig. 6).

Correlations between clot lysis and blood biomarkers

Tables 3 and 4 illustrate the correlations of relevant biomarkers with plasma and blood clot lysis, respectively. Concerning plasma fibrinolysis, the variables displaying a

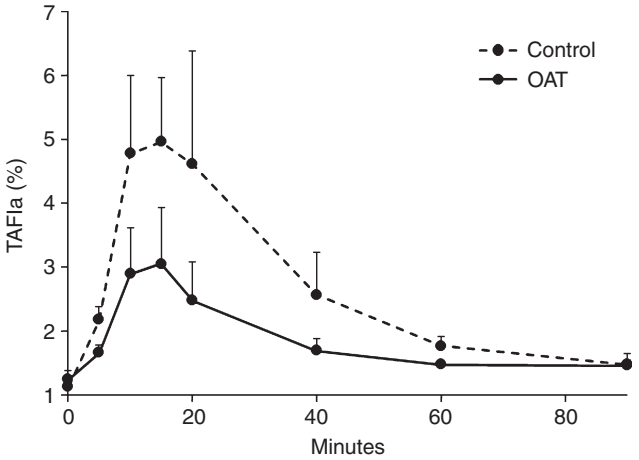

Fig. 4. Generation of thrombin activatable fibrinolysis inhibitor (TAFI)a activity in tissue factor-activated oral anticoagulant therapy (OAT) and control plasma. The time-course of TAFIa activity was evaluated by a two-stage functional assay as reported in the Methods section. Experimental conditions were similar to those used for clot lysis assay except that defibrinated plasma was used instead of normal plasma. At intervals, aliquots were taken from the reaction mixture and TAFIa activity was assayed as the ability to prolong the lysis time of clots made from purified fibrinogen. Data are expressed as per cent of total TAFI by reference to a calibration curve constructed by testing serial dilutions of the same plasma after complete activation of TAFI by thrombin-thrombomodulin (see Methods for details). Results are the mean + SD (n = 6 per group). P = 0.0016 by comparing the area under the TAFIa curves.

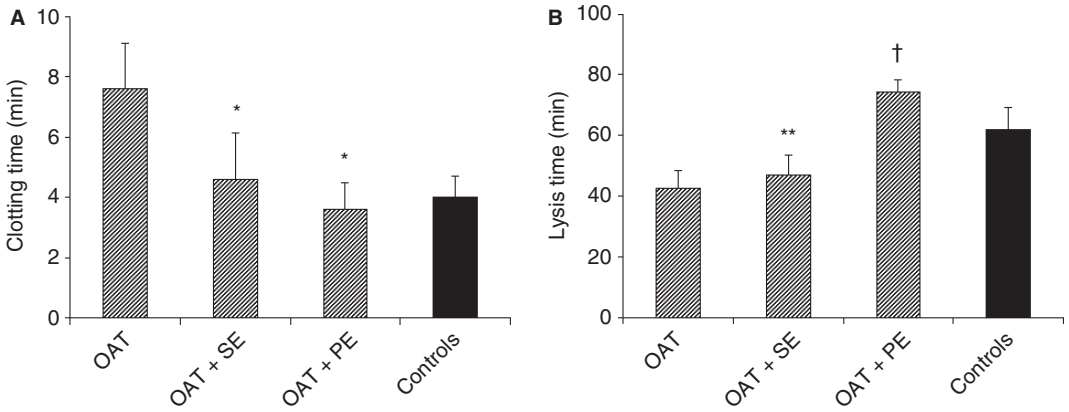

Fig. 5. Effect of vitamin K-dependent factors on clot formation and fibrinolysis in plasma from warfarin-treated patients (oral anticoagulant therapy, OAT). Plasma samples (n = 6), randomly selected from warfarin-treated patients, were supplemented with $BaSO_4$ serum eluate (SE) or with Ba-citrate plasma eluate (PE) in order to bring the concentration of vitamin K-dependent factors to between 90 and 110%. Tissue factor-induced clotting time (panel A) and t-PA-induced fibrinolysis time (panel B) were determined by a turbidimetric assay as reported in the Methods section. Data are the mean + SD. *, $P < 0.01$ vs. OAT; **, $P = 0.012$ vs. OAT; †, $P < 0.01$ vs. OAT and OAT+SE (repeated measures ANOVA). For reference the results from six control plasmas tested in parallel are shown (black columns).

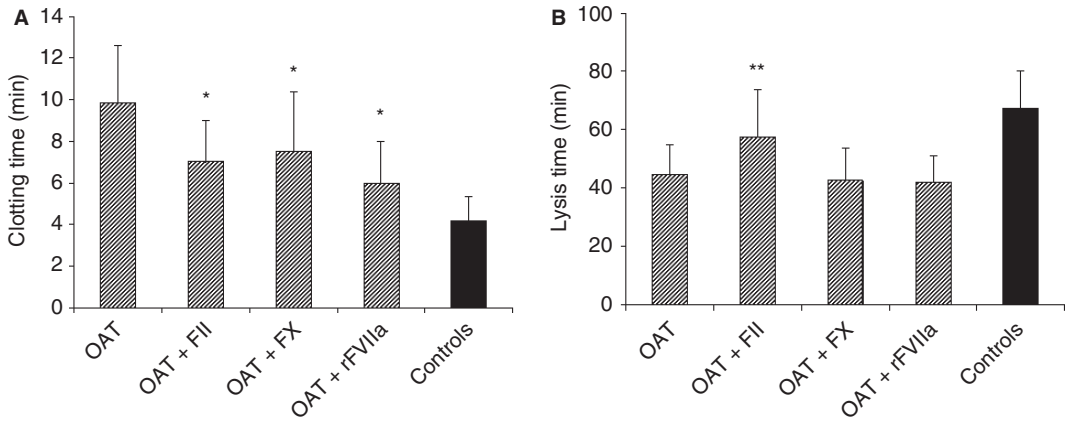

Fig. 6. Effect of single vitamin K-dependent factors on clot formation and fibrinolysis in plasma from warfarin-treated patients (oral anticoagulant therapy, OAT). Plasma samples (n = 6), randomly selected from warfarin-treated patients, were supplemented with factor (F) II (1 U mL^{-1}), FX (1 U mL^{-1}) or rFVIIa (100 U mL^{-1}). Tissue factor-induced clotting time (panel A) and t-PA-induced fibrinolysis time (panel B) were determined by a turbidimetric assay as reported in the Methods section. Data are the mean + SD. *, $P < 0.02$ vs. OAT; **, $P < 0.015$ vs. OAT, OAT + FX and OAT + rFVIIa (repeated measures ANOVA). For reference the results from six control plasmas tested in parallel are shown (black columns).

Table 3 Correlations between plasma clot lysis and blood biomarkers

	OAT patients		Controls	
Biomarker	r	P	r	*P*
PT-INR	−0.08	0.169	−0.172	0.051
Thrombin (ETP)	0.202	0.022	0.016	0.89
Thrombin (Cmax)	0.199	0.014	−0.074	0.45
PAI-1	0.478	< 0.0001	0.335	0.0001
TAFI	0.227	0.0015	0.292	0.0010
Fibrinogen	0.128	0.006	0.170	0.053

All correlations were age-adjusted. OAT, oral anticoagulant therapy.

Table 4 Correlations between blood clot lysis and blood biomarkers

	Controls		OAT patients	
Biomarker	r	*P*	r	*P*
PT-INR	0.177	0.040	−0.098	0.152
Thrombin (ETP)	−0.164	0.13	0.092	0.396
Thrombin (Cmax)	−0.152	0.18	0.087	0.342
PAI-1	0.084	0.30	0.095	0.204
TAFI	0.087	0.37	0.173	0.016
Fibrinogen	0.073	0.56	0.193	0.0007
Platelets	0.141	0.12	0.178	0.076
Red cells	−0.255	0.008	−0.120	0.058
Leukocytes	0.054	0.57	0.005	0.88

All correlations were age-adjusted. OAT, oral anticoagulant therapy.

significant correlation in both groups were PAI-1 and TAFI. Thrombin generation parameters and fibrinogen correlated with lysis time only in OAT patients whereas PT-INR did not correlate with lysis time in either group. In the blood clot lysis model (Table 4) the results were somewhat different. In particular, PAI-1 was no longer correlated with lysis time, probably because of the high t-PA concentration added to blood [17], whereas TAFI showed an association only in patients. Moreover, thrombin generation parameters lost their correlation with blood fibrinolysis in OAT patients. Concerning blood cells, only red cells showed a significant correlation with lysis time in controls.

Discussion

This study shows that clots from warfarin-treated patients are more susceptible to lysis, due to a reduced thrombin-mediated TAFI activation. Both plasma and blood clots from OAT patients lysed significantly faster than control clots when exposed to exogenous t-PA. This increased lysability was no longer visible if TAFIa was quenched by PTCI addition, under which condition (reflecting TAFI-independent fibrinolysis) the lysis time was slightly but significantly longer in OAT patients, probably because of their older age and/or other changes caused by the underlying diseases. The contribution of TAFI to fibrinolysis, calculated as the shortening of lysis time induced by PTCI, was very weak in OAT patients (approximately 14% and 16% shortening in plasma and blood, respectively) as compared with controls (approximately 34% and 39% shortening), which is in agreement with the finding that the amount of TAFIa generated in OAT plasma was markedly lower than in control plasma, probably because of a reduction in thrombin generation (i.e. the main activator of TAFI in our fibrinolysis model). Indeed, if we restored thrombin generation by adding a Ba-citrate plasma eluate, or if we clotted the plasma by adding a high concentration of thrombin, clot resistance of OAT samples was comparable to controls.

The impact of thrombin on the lysability of clots and the ability of anticoagulants to promote fibrinolysis have been known for a long time [1–4]. However, the mechanism of action of warfarin is different to that of all other currently used anticoagulants, for it down-regulates blood coagulation by decreasing the functional level of vitamin K-dependent factors. The influence of clotting deficiencies on fibrinolysis is not straightforward. In severe hemophilia, for example, plasma clots are very susceptible to lysis because of inadequate TAFI activation. However, as little as 1% FVIII was enough to restore a normal clot resistance [9]. In the case of FXI deficiency, the level of FXI needed to normalize fibrinolysis was even lower than 1% [10]. In warfarin-treated patients the functional levels of vitamin K-dependent factors are around 20% [6,7]. This suggests that the reduced clot resistance of OAT plasma is either the result of multiple deficiencies or the consequence of a single factor deficiency whose threshold concentration for efficient TAFI activation is much higher than that of FVIII or FXI. The finding that the addition of a serum eluate (containing FVII, FIX and FX) to OAT plasma had virtually no effect on clot lysability supports the second hypothesis and underscores the prominent role of prothrombin. This assumption is further reinforced by the observation that prothrombin replenishment, contrary to FVIIa or FX addition, increases clot resistance of OAT plasma to a biologically relevant extent. This is in agreement with Xi *et al*. [19], who showed that the major determinant of thrombin formation in orally anticoagulated patients was the prothrombin level.

TM greatly enhances the efficiency of thrombin as a TAFI activator [2,3]. However, the addition of TM, while prolonging the lysis time of both groups, did not abolish the difference between OAT and controls, suggesting that the availability of TM on the endothelial surface is unlikely to normalize the fibrinolytic resistance in OAT patients. However, in view of the duplicitous role of TM in TAFI and protein C activation [1,3], further studies are needed to settle this point.

The possibility that other factors had contributed appreciably to the reduced fibrinolytic resistance associated with OAT is unlikely. The PAI-1 level, which is one of the major determinants of clot resistance [20], was similar in OAT patients and controls. The fibrinogen level was higher in OAT patients and, thus, it might at best have partially masked the profibrinolytic effect of warfarin treatment, given that the increase in fibrinogen is expected to enhance fibrinolysis resistance [21]. Platelets, which are known to inhibit fibrinolysis through different mechanisms [22,23], were slightly lower in warfarin-treated patients. However, seeing that the effect of OAT was equally visible in (platelet-poor) plasma it is highly unlikely that the reduction in platelet number had contributed significantly to the profibrinolytic effect of oral anticoagulation.

The intensity of anticoagulation during warfarin treatment is measured by the prothrombin time. PT-INR, however, did not correlate with fibrinolysis time of OAT patients, implying that it is unsuitable for predicting the changes in fibrinolytic resistance induced by warfarin. On the other hand, thrombin generation parameters (ETP, Cmax) correlated with plasma but not with blood fibrinolysis of OAT samples, suggesting that even these variables may not accurately reflect the decrease in clot resistance associated with OAT.

An intriguing finding of our study is that the circulating levels of TAFIa and its inactive derivative TAFIai are higher in OAT patients than in controls, implying an increased *in vivo* activation. This is surprising in the light of the purported role of thrombin as a TAFI activator

[1–3], and is in sharp contrast with our *in vitro* results (Table 2). Apparently, the *in vivo* 'basal' levels of TAFIa/ai are unrelated to thrombin generation, as witnessed by the lack of correlation with prothrombin F_{1+2} and with VIIa-AT complex. Conversely, TAFIa/ai levels did correlate with PAP complex, especially in OAT patients, suggesting that plasmin might contribute to *in vivo* TAFI activation, at least under resting conditions.

One limitation of our study is that the patients were significantly older than controls. We do not believe, however, this age difference has biased our results. Firstly, all analyses were adjusted for age; secondly, plasma fibrinolysis time has been reported to increase with age [24]; thirdly, the restoration of a normal fibrinolytic resistance upon replenishment of vitamin K-dependent factors indicates that the fibrinolytic changes were indeed the consequence of the clotting deficiency determined by warfarin treatment. The latter finding also rules out that the diminished clot resistance observed in our patients was due to the underlying diseases rather than to warfarin treatment.

In conclusion, our data show that warfarin treatment, besides inhibiting fibrin formation, promotes fibrinolysis by reducing clot resistance to lysis through a TAFI-mediated mechanism. This profibrinolytic effect is largely determined by the impairment of thrombin formation resulting from the reduction of prothrombin functional levels, and is maintained in the presence of thrombomodulin. Clinical studies are warranted to establish if the assay of fibrinolytic resistance might help in getting a better picture of actual antithrombotic effect associated with warfarin treatment and if it adds to the currently used clotting tests in tailoring anticoagulant therapy, particularly in complicated patients. Finally, the finding that the basal levels of circulating TAFIa/ai were correlated with plasmin but not thrombin generation suggests that the *in vivo* activation of TAFI may occur through different mechanisms.

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Disclosure of Conflict of Interests

BW is an employee of Diagnostica Stago. The other authors state that they have no conflict of interests.

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