

# Diurnal Variation of the Fibrinolytic System

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## Key words

Diurnal – Fibrinolytic activity – PAI activity – t-PA – u-PA – PAI-1

## Summary

To elucidate which component(s) of the fibrinolytic system is (are) responsible for the diurnal variation of fibrinolytic activity we have studied several parameters of this system in 8 healthy male volunteers during a period of 24 h. Blood was collected at 8 a.m., 10 a.m., 12 a.m., 4 p.m., 8 p.m. and 8 a.m. next morning. The following tests were performed: euglobulin clot lysis time (ECLT), fibrinolytic activity of euglobulins on fibrin plates in the presence and absence of blocking antibodies to tissue-type plasminogen activator (t-PA) and/or urokinase (u-PA), overall plasminogen activator inhibitor (PAI) activity, antigen levels of t-PA, u-PA and PAI-1 and zymography of the euglobulin fraction after SDS-PAGE. From 8–10 a.m. to 4–8 p.m., total fibrinolytic activity increased by 113% ( $p < 0.01$ ) or 71% ( $p < 0.01$ ) when measured by ECLT or by fibrin plate assay, respectively. The immunoquenching experiments showed that this increase was entirely due to t-PA related activity whereas u-PA activity and t-PA/u-PA independent activity remained constant during the day. Average antigen levels of u-PA and t-PA in the afternoon were 6% and 25% lower than those measured in the morning. During this period, overall PAI activity and PAI-1 antigen decreased by 31% ( $p < 0.01$ ) and 52% ( $p < 0.01$ ) respectively. Electrophoretic-zymographic analysis of the euglobulins revealed that throughout the day the majority of t-PA was present in the form of the 110 kDa t-PA/PAI-1 complex. The intensity of this complex was lowest in the afternoon. Free t-PA was almost undetectable in morning samples, but constituted a significant proportion of total t-PA in the afternoon. The diurnal increase of fibrinolytic activity, therefore, is not due to an augmentation of antigen levels of t-PA and/or u-PA but to a decline of those of PAI-1.

## Introduction

The fibrinolytic system is a complex enzyme cascade system that leads to the formation of plasmin, a trypsin-like protease responsible for the degradation of fibrin and the dissolution of intravascular thrombi. In normal human plasma two distinct plasminogen activators (PAs), tissue-type PA (t-PA) and urokinase (u-PA), and a specific PA-inhibitor (PAI-1) have been identified (1–7).

The activity of the fibrinolytic system is subject to diurnal variation with highest values occurring late in the afternoon (8–10). A diurnal increase in t-PA activity concomitantly with a decrease of t-PA antigen and PAI activity was noted (9, 10). These studies, however, did not establish the cause of the reduced PAI activity. To answer this question and to determine whether other components might also contribute to this phenomenon we

studied in detail the diurnal variations of different components of the fibrinolytic system. To this end blood was collected from healthy volunteers at different times of the day and overall fibrinolytic activity of the euglobulins, as well as PAI activity and the plasma antigen levels of t-PA, u-PA and PAI-1 determined. Furthermore, the contribution of each of the two PAs to fibrinolytic activity early in the morning and late in the afternoon was established by immunoquenching experiments.

## Materials and Methods

### Plasmas

Eight healthy male volunteers, aged 18–30 years, of normal weight, were enrolled in the study. Blood samples were obtained at 8 a.m., 10 a.m., 12 a.m., 4 p.m., 8 p.m. and 8 a.m. the following day. The blood was mixed in precooled tubes with one tenth volume of 0.1 M citrate buffer, pH 4.5 (Behring-Hoechst, Marburg, Federal Republic of Germany) and the tubes were immediately transferred into ice and centrifuged for 20 min at 2,000 g and 4° C. The resulting platelet-poor plasma was frozen in aliquots at –70° C.

### Antisera

Antisera to t-PA and PAI-1 were obtained as described before (11, 12). Antiserum to u-PA was induced by repeated subcutaneous injections into rabbits of 200 µg of pure HM-u-PA (a gift of Dr. Sauser, Sero Laboratories, Coinsins, Switzerland). IgG was prepared by affinity chromatography on Protein A-Sepharose, aliquots of 0.5 ml of preimmune serum or antiserum were passed over a 0.5 ml column of protein A-Sepharose. After washing of the column with 10 ml of PBS, the IgG was eluted with 0.1 M glycine-HCl, pH 2.2. Immediately after elution the IgG solution was neutralized by the addition of 0.1 vol. of 1 M Tris.

### Fibrinolytic Activity Determinations

Euglobulins were prepared as follows: 0.8 ml of plasma were diluted 10-fold with ice-cold deionized water and the pH adjusted to 5.9 by addition of 0.6 ml of 0.25% (v/v) acetic acid. The tubes were kept on ice for 30 min, after which they were centrifuged for 10 min at 1,300 g and 4° C. The supernatant was discarded and the tube walls carefully dried with an absorbent paper. The euglobulin precipitate was redissolved in 0.4 ml of 0.1 M Tris-HCl buffer, pH 7.5.

**Euglobulin clot lysis time assay.** 100 µl of the euglobulin solution were mixed with 100 µl of 0.1 M Tris-HCl, pH 7.5, containing 0.2% Tween 80, and clotted with 100 µl of a solution of 10 NIH U/ml thrombin and 0.025 M CaCl<sub>2</sub> followed by incubation at 37° C. Lysis times were recorded in minutes. Activity was expressed in arbitrary units (AU) using the formula  $AU = 300/\text{lysis time in minutes}$ .

**Fibrin plate assay.** Fibrin plates (using bovine plasminogen-rich fibrinogen from Poviet, Bostel, The Netherlands) were prepared exactly as described previously (13). Thirty µl of the euglobulin solution and 5 µl of 17.5 mM sodium flufenamate (Aldrich, Beerse, Belgium) were applied in quadruplicate on the plates. After 24 h of incubation at 37° C, lysis zones were measured and converted to international units of t-PA by comparison of the lysis diameters with those of a laboratory standard preparation of pure human t-PA (11) that had been standardized against the International Reference Preparation for t-PA (NIBSC 83/517, National Institute for Biological Standards and Controls, South Mimms, Hertfordshire, UK).

t-PA and u-PA related activities were derived from the difference in fibrinolytic activity on fibrin plate of euglobulins preincubated for 1 h with

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Table 1 Diurnal variation of fibrinolytic parameters

		8 a.m.	10 a.m.	12 a.m.	4 p.m.	8 p.m.	8 a.m.	Significance (Friedman's test)
ECLT	M $\pm$ 95% C.L. (AU/ml)	1.0 $\pm$ 0.2	1.2 $\pm$ 0.4	1.6 $\pm$ 0.6	2.3 $\pm$ 1.1	2.5 $\pm$ 0.9	1.2 $\pm$ 0.3	p < 0.01
	Range (AU/ml)	0.8–0.5	0.8–2.0	0.8–2.9	0.8–4.6	0.9–4.6	0.8–1.6	
	Median (AU/ml)	0.91	0.95	1.58	2.16	2.31	1.20	
Act. on FP	M $\pm$ 95% C.L. (IU/ml)	2.3 $\pm$ 0.4	2.5 $\pm$ 0.4	3.0 $\pm$ 0.7	4.2 $\pm$ 1.2	4.7 $\pm$ 0.9	2.3 $\pm$ 0.3	p < 0.01
	Range (IU/ml)	1.7–3.0	1.8–3.1	1.8–4.3	2.4–6.9	3.0–6.5	1.7–2.7	
	Median (IU/ml)	2.28	2.46	3.17	3.95	4.8	2.37	
t-PA Ag	M $\pm$ 95% C.L. (ng/ml)	5.0 $\pm$ 0.7	3.7 $\pm$ 1.1	3.9 $\pm$ 1.3	3.4 $\pm$ 1.5	2.9 $\pm$ 1.3	4.2 $\pm$ 1.2	p = 0.05
	Range (ng/ml)	3.9–6.5	2.2–6.3	1.9–5.8	1.2–5.9	0.5–4.5	2.3–5.6	
	Median (ng/ml)	5.05	3.75	4.10	2.75	3.15	4.55	
u-PA Ag	M $\pm$ 95% C.L. (ng/ml)	8.1 $\pm$ 1.1	8.0 $\pm$ 0.8	8.2 $\pm$ 1.1	7.3 $\pm$ 0.8	7.1 $\pm$ 0.6	8.1 $\pm$ 1.2	p < 0.01
	Range (ng/ml)	6.2–10.2	6.7–9.2	6.2–9.7	5.5–8.0	6.2–8.0	6.7–10.2	
	Median (ng/ml)	8.25	7.87	8.37	7.75	7.00	7.87	
PAI Act.	M $\pm$ 95% C.L. (IU/ml)	8.7 $\pm$ 3.2	8.5 $\pm$ 3.3	7.0 $\pm$ 2.9	5.5 $\pm$ 1.6	7.0 $\pm$ 6.1	10.0 $\pm$ 5.4	p < 0.01
	Range (IU/ml)	6.2–17.5	4.7–16.5	4.2–14.5	3.9–9.5	3.4–23.7	5.7–21.7	
	Median (IU/ml)	8.06	7.37	6.37	5.25	5.00	6.81	
PAI-1 Ag	M $\pm$ 95% C.L. (ng/ml)	21.9 $\pm$ 11.3	23.5 $\pm$ 6.8	20.7 $\pm$ 10.9	12.3 $\pm$ 4.6	8.8 $\pm$ 2.3	24.5 $\pm$ 22.4	p < 0.01
	Range (ng/ml)	11–47	12–31	8–42	6–21	6–14	5–82	
	Median (ng/ml)	18.7	25.5	17.7	13.1	7.8	13.8	

ECLT: euglobulin clot lysis time, AU: arbitrary units (see Methods), Act. on FP: fibrinolytic activity of euglobulins on fibrin plate (see Methods), t-PA Ag: tissue-type plasminogen activator antigen, u-PA Ag: urokinase plasminogen activator antigen, PAI Act.: plasminogen activator inhibitor activity, PAI-1 Ag: plasminogen activator inhibitor 1 antigen. For each time mean  $\pm$  95% confidence limits (M  $\pm$  95% C.L.; n = 8), range and median values are shown

50  $\mu$ g/ml of preimmune IgG or anti t-PA IgG and/or anti u-PA IgG. The concentrations of the anti t-PA or anti u-PA IgG were such that in the fibrin plate assay over 95% of 250 IU of t-PA or 50 IU of u-PA was quenched, without affecting the activity of a similar amount of the other PA. t-PA/u-PA independent activity was defined as the activity that could not be blocked by a combination of anti t-PA and anti u-PA IgG.

#### PAI Activity

PAI activity was measured by titration with single chain t-PA as described by Verheijen et al. (6).

#### t-PA, u-PA and PAI-1 Antigen Levels

Antigen levels of t-PA in plasma were determined using the IMULYSE 5 t-PA ELISA kit of Biopool (Umeå, Sweden).

Plasma u-PA and PAI-1 antigen concentrations were determined by a radioimmunoassay as described before (12, 14).

#### Electrophoretic-Zymographic Analysis of PA Activity

Polyacrylamide gel (7.5%) electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (15) and electrophoretic-zymographic analysis of PA activity was performed as described by Granelli-Piperno and Reich (16). Fifteen  $\mu$ l of the euglobulins or different concentrations of standard t-PA dissolved in SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.8, 12% SDS, 18% Ficoll, 0.05% bromophenol blue) were applied on the gel. After 4 h of electrophoresis at 35 mA per gel, the gels were washed for 1 h in 2.5% Triton X-100 and briefly rinsed in deionized water. Bands of free t-PA and of t-PA complexed to inhibitor were zymographically revealed by placing the gels on a plasminogen-rich fibrin-agarose underlay (16).

#### Statistical Analysis

The significance of the diurnal variations of the components of the fibrinolytic system was established by the Friedman's test. Differences between paired data were tested for significance with the Wilcoxon's signed rank test (17).

## Results

### Diurnal Variation of Euglobulin Fibrinolytic Activity, of Plasma PAI Activity and of Plasma Antigen Concentrations of t-PA, u-PA and PAI-1

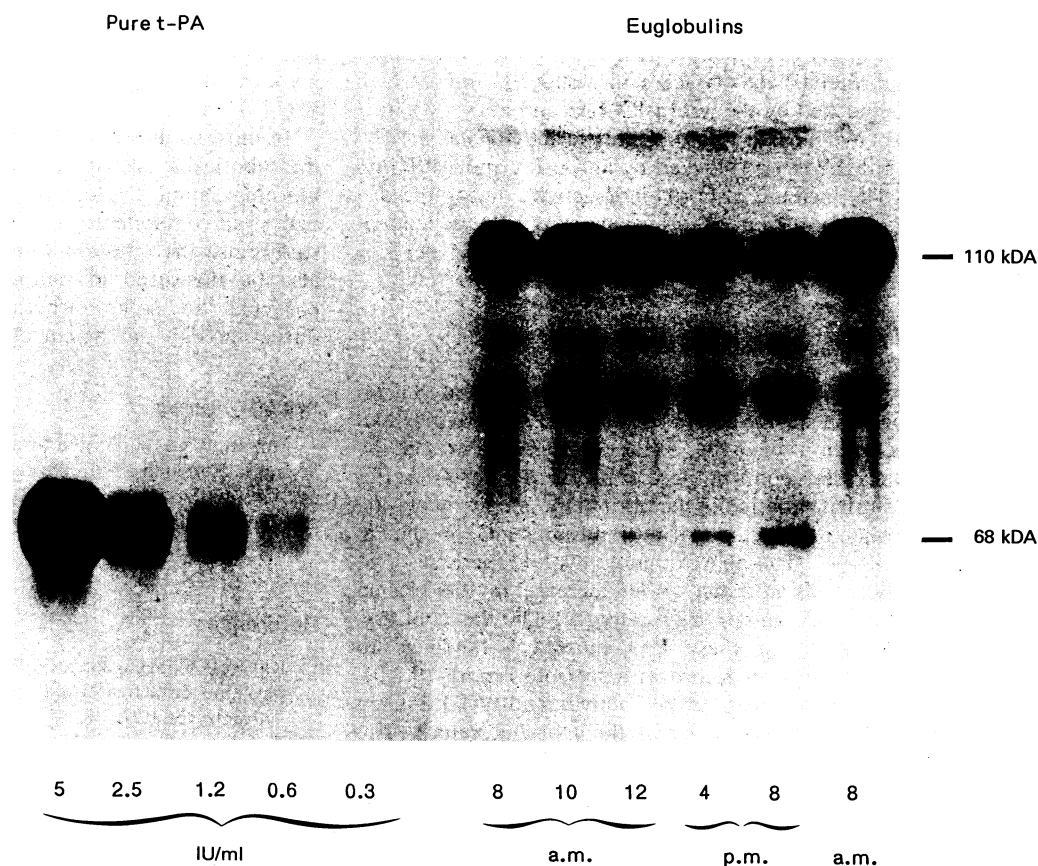
Analysis of the data (Friedman's test) indicated that all fibrinolytic parameters varied significantly in the course of a day (p < 0.01; for t-PA p = 0.05) (Table 1). Extreme values always occurred at 8–10 a.m. and at 4–8 p.m. For all parameters tested, 8 a.m. values of the following day were not significantly different from those at 8 a.m. on the first day. To assess the extent of the diurnal variations we compared morning values (= average of 8 and 10 a.m.) with afternoon values (= average of 4 and 8 p.m.) for each of the assays.

**Fibrinolytic activity and PAI activity.** During the day a significant increase of the fibrinolytic activity of the euglobulins was observed. Maximal activity in the afternoon was 2.1-fold (euglobulin clot lysis time assay) and 1.7-fold (fibrin plate assay) higher than that in the morning (p < 0.01, Wilcoxon's signed rank test).

During the same period, PAI activity decreased by 31% (p < 0.01, Wilcoxon's signed rank test).

**t-PA, u-PA and PAI-1 antigen levels.** The increased fibrinolytic activity and decreased PAI activity may have been caused by a shift in the balance between PAs and PAI-1. We, therefore, also determined the antigen concentrations of t-PA, u-PA and PAI-1. During the day, PAI-1 concentrations decreased by 52% (p < 0.01, Wilcoxon's signed rank test) whereas t-PA and u-PA showed a tendency to decrease with afternoon values 25% and 6%, respectively, lower than those in the morning.

To assess whether a variable release of PAI-1 from platelets may have contributed to the diurnal variation of PAI-1, we have also determined  $\beta$ -thromboglobulin ( $\beta$ TG) levels.  $\beta$ TG concentrations in 8–10 a.m. samples (median 155 ng/ml) were not significantly different from those in 4–8 p.m. samples (median 181 ng/ml). Assuming that 100 ng/ml of  $\beta$ TG correspond to 2.2 ng/ml of PAI-1 released from platelets (12), the average



**Fig. 1** Analysis of the euglobulins by SDS-PAGE followed by fibrin-zymography. At the left: pure t-PA at the concentrations of 5, 2.5, 1.3, 0.6, and 0.3 IU/ml. At the right: euglobulins of plasma samples collected at the specified times of a representative volunteer

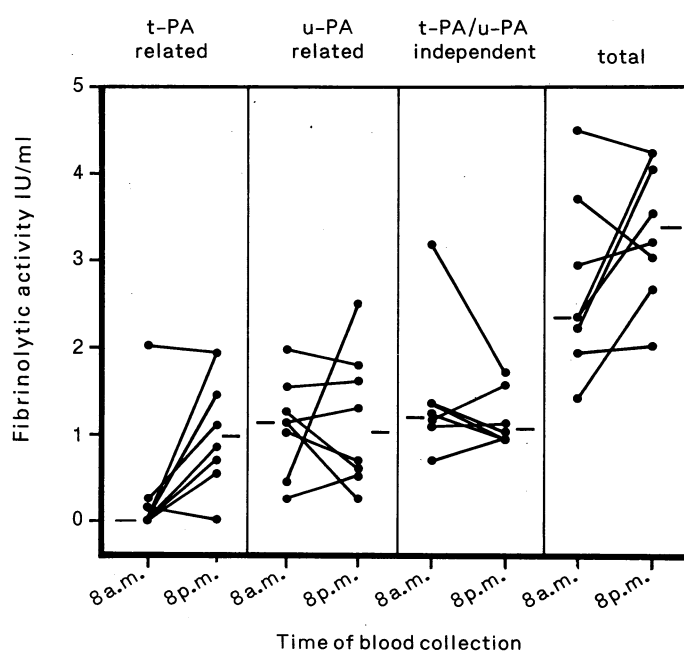
contribution of platelets to plasma PAI-1 in our samples was estimated to be 3.4 ng/ml in the 8–10 a.m. samples and 4 ng/ml in the 4–8 p.m. samples. After correction of each PAI-1 value for the corresponding platelet contribution, afternoon PAI-1 concentrations (median 6.5 ng/ml) still were significantly decreased ( $p < 0.01$ , Wilcoxon's signed rank test) with respect to those in the morning (median 16.8 ng/ml).

#### Analysis of Fibrinolytic Activity by Fibrin-Zymography

To perform a semi-quantitative determination of the proportion of t-PA bound to inhibitor and present in a free form, we subjected the euglobulins to SDS-PAGE and fibrin-zymography. The zymographies (a representative example is shown in Fig. 1) revealed that the intensity of the 110 kDa lysis zone (corresponding to t-PA/PAI-1 complex) was minimal in the afternoon, whereas the 68 kDa lysis zone of free t-PA, almost undetectable in the morning, reached a maximum in the afternoon (estimated activity: 0.5–1 IU/ml).

#### Immunoquenching Studies on Fibrinolytic Activity

To quantify the respective contribution of t-PA and u-PA to fibrinolytic activity in morning and afternoon samples, we performed immunoquenching experiments by preincubation of the euglobulins with blocking concentrations of anti t-PA and/or anti u-PA IgG followed by the determination of remaining activity in the fibrin plate assay. Fig. 2 shows the results of experiments on plasmas obtained from eight volunteers at 8 a.m. and 8 p.m. During the day, median t-PA activity increased from undetectable



**Fig. 2** Immunoquenching studies on fibrinolytic activity of plasma collected at 8 a.m. and 8 p.m. The figure shows the individual values of 8 volunteers of total fibrinolytic activity, t-PA related activity, u-PA related activity and of t-PA/u-PA independent activity of 8 a.m. and 8 p.m. These activities were derived from the difference in fibrinolytic activity on fibrin plates of euglobulins preincubated for 1 h with 50 µg/ml of preimmune IgG or anti t-PA and/or anti u-PA IgG respectively (see Methods section). The bars indicate median values

to 1.0 IU/ml ( $p < 0.05$ ; Wilcoxon's signed rank test), whereas u-PA activity and u-PA/t-PA independent activity did not change. Median total fibrinolytic activity at 8 a. m. was 2.3 IU/ml of which 1.1 IU/ml was blocked by the anti u-PA IgG and none by anti t-PA IgG; at 8 p. m., median total fibrinolytic activity was 3.4 IU/ml, of which 1.0 IU/ml was blocked by anti u-PA and 1.0 IU/ml by anti t-PA. The increase in fibrinolytic activity during the day thus can entirely be attributed to an increased t-PA dependent activity.

## Discussion

In the present study we measured in detail the diurnal (8 a. m. to 8 p. m.) variation of fibrinolytic as well as PAI activities and antigen concentration of t-PA, u-PA and PAI-1 in healthy volunteers. Our results confirm those of previous reports describing a marked diurnal variation of the fibrinolytic activity in plasma with minimal value early in the morning and a maximal one late in the afternoon (8–10). The approximately twofold increase of fibrinolytic activity was not due to an increase in the plasma concentration of t-PA and/or u-PA antigen. On the contrary, plasma concentrations of these PAs showed a tendency to decrease. For t-PA this is in accord with previous reports (9, 10). Most striking was the decrease of PAI activity and PAI-1 antigen concentrations to approximately half the morning values. The PAI-1 decline still was significant after correction for the portion of PAI-1 released by platelets as estimated from  $\beta$ TG levels in morning and afternoon samples. Since t-PA and u-PA show a tendency to decrease, it is tempting to ascribe the twofold augmentation of fibrinolytic activity to the decline in PAI-1 concentrations. However, global euglobulin fibrinolytic activity is composed of several different activities (18) that may not all be equally sensitive to PAI-1 (12). To determine which of these different activities is more directly responsible for the increased fibrinolytic activity, it is essential to study specifically the changes of each of the activities separately. To this end we also performed zymographic and immunoquenching experiments. The zymographies revealed that the intensity of the lysis zone of the t-PA/inhibitor complex decreased during the day, while that of free t-PA increased reaching approximately 0.5–1 IU/ml in the afternoon. The immunoquenching experiments indicated that plasma euglobulins contain three separately measurable activities, two that can be blocked by antibodies to t-PA (1) and u-PA, respectively (2–4, 19), and a third, at present still ill-characterized, that is refractory to these antibodies. The u-PA related and the t-PA/u-PA independent activity, which in morning samples were approximately equal, remained constant during the day, whereas the t-PA related activity increased from below detection limit ( $< 0.2$  IU/ml) to approximately 1.0 IU/ml. The increase in fibrinolytic activity thus can be ascribed to an augmented t-PA dependent activity, occurring despite the decrease of total t-PA antigen concentrations. This result is, most likely, due to the twofold decrease of PAI-1 concentrations, leading to an increased fraction of free t-PA as demonstrated by the zymography.

The observation that u-PA related and t-PA/u-PA independent activity do not change much during the day while PAI-1 levels vary strongly, implies that these activities are relatively insensitive to PAI-1. This is in accord with previous suggestions based upon the poor correlation of fibrinolytic activity of morning samples and PAI-1 concentrations (12).

The diurnal variations of the fibrinolytic system may have clinical consequences. A 3-year follow-up study in young survivors of myocardial infarction demonstrated that increased PAI activity constitutes a risk factor for a recurrence of myocardial infarction (20, 21). The higher PAI activities observed in the

morning, possibly in combination with an increased platelet aggregability (22), may thus contribute to the higher incidence of myocardial infarction and sudden death occurring in the morning (23, 24).

In most studies on fibrinolysis concerned with risk factors for thrombotic disease fibrinolytic parameters have been measured in morning samples. However, the profound diurnal changes of almost all components of the fibrinolytic system observed in this study raise the question whether fibrinolytic parameters should also be measured in samples collected in the afternoon, in particular, to explore whether a failure of PAI-1 to decrease during the day may be correlated with thrombotic disease.

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