Diurnal Variation of Tissue-Type Plasminogen Activator and Its Rapid Inhibitor (PAI-1)

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Previous studies have shown that overall fibrinolytic activity in blood follows a diurnal rhythm with a peak in the morning and a trough in the evening. The purpose of this study was to determine which fibrinolytic factor(s) was responsible for this diurnal rhythm. Resting and postvenous occlusion tissue-type plasminogen activator (t-PA) activity, resting t-PA antigen, and resting plasminogen activator inhibitor 1 (PAI-1) activity were measured in the morning and evening in 33 healthy men (mean age, 31 years) and in 15 patients (mean age, 57 years) with previous myocardial infarction or unstable angina. PAI-1 activity and t-PA antigen were significantly higher ($p < 0.01$) in the morning compared with the evening in controls and patients. In contrast, resting t-PA activity was significantly lower in the morning ($p < 0.01$) in both groups and was inversely correlated with PAI-1 activity ($r = -0.57, p < 0.0001$). Postvenous occlusion t-PA activity and t-PA capacity were not significantly different between morning and evening in either group. Because t-PA antigen levels and PAI-1 activity were highest in the morning, the variation in t-PA activity was probably due to decreased secretion of t-PA but instead to changes in the secretion of PAI-1. Our findings indicate that diurnal variations in PAI-1 activity may reduce fibrinolytic activity in the morning in healthy individuals and in patients with coronary artery disease. (*Circulation* 1989;79:101-106)

Incidence of acute myocardial infarction follows a circadian rhythm with a peak in the morning and a trough in the evening. Muller et al. found a threefold increase in the frequency of myocardial infarction at 9 AM compared with 11 PM. A similar diurnal variation in the time of onset has been found for a number of other disorders associated with arterial thrombosis, including sudden cardiac death, angina at rest, and stroke.2-4

A number of different factors may play a role in the circadian variation of arterial thrombosis. Heart rate, arterial blood pressure, and catecholamine levels have been shown to peak in the morning hours, indicating that coronary vasoconstriction may be increased in the morning as well.5-7 Another approach has been to study factors that may increase the risk of initiating thrombus formation. Platelet aggregability has been found to increase in the morning after arising.8,9 A concurrent increase in plasma epinephrine and norepinephrine were also found.

These studies have concentrated on factors that may increase the risk of vasoconstriction or thrombus initiation. Another possible explanation would be alterations in blood components that depress the rate of thrombus removal. In vivo, newly forming thrombi are removed by the fibrinolytic system. Fibrinolysis is initiated by tissue-type plasminogen activator (t-PA), an enzyme secreted by endothelial cells that, in the presence of fibrin, converts the proenzyme plasminogen into its active form, plasmin.10 In turn, plasmin proteolytically degrades the fibrin that holds the thrombus together. The level of t-PA activity in blood is regulated by a specific fast-acting plasminogen activator inhibitor (PAI-1).11 Circadian variations in blood fibrinolytic activity may affect the rate of endogenous thrombolysis and, thus, vascular occlusion.

The purpose of this study was to determine whether diurnal variations exist for the principal activator (t-PA) and inhibitor (PAI-1) of the fibrinolytic system in healthy individuals and in patients with coronary artery disease. Basal activator and inhibitor levels can be estimated by measuring resting t-PA activity, t-PA antigen, and PAI-1 activity in plasma. The ability of endothelial cells to release t-PA can be determined by measuring plasma t-PA activity after a release stimulus such as venous occlusion.12
Subjects and Methods

Subjects

The control group consisted of 33 healthy men (mean age, 31 years; range, 21–92 years) with no history of angina, myocardial infarction, or other heart disease. The patient group consisted of 15 men (mean age, 57 years; range, 41–75 years) with angiographically documented atherosclerotic coronary artery disease (>70% stenosis of one or more coronary arteries). Eleven of the 15 patients had a myocardial infarction 5–84 months (mean, 27 months) before blood sampling for this study. None of the four patients had unstable angina.13 Potential subjects were rejected if they had a history of irregular sleep habits, shift work, or recent short-term illness. To eliminate potential variations due to menstruation, menopause, oral contraceptives, and pregnancy, only men were studied. None of the individuals was on anticoagulants or corticosteroids at the time of the study. Informed consent was obtained from all subjects.

Blood Sampling

Early studies based on clot lysis assays have demonstrated a diurnal variation in overall fibrinolytic activity.14 These studies indicated that fibrinolytic activity was lowest in the morning with a trough at approximately 8 AM. Previous studies have also indicated that repeated stimulation of t-PA release by venous occlusion lessens the fibrinolytic response, presumably because of depletion of endothelial cell t-PA.15 To prevent variations in t-PA activity due to endothelial cell depletion and alteration of basal PAI-1 activity due to repeated t-PA release, we sampled blood at only two times during the day: morning, which corresponds to the trough in overall fibrinolytic activity and the peak incidence of myocardial infarction, and evening, which corresponds to the peak in overall fibrinolytic activity and the trough in the incidence of myocardial infarction.

Blood samples were drawn in the morning (8:20 AM, ± 60 minutes) and in the evening (7:30 PM, ± 70 minutes) from each subject as described previously.16 t-PA release was stimulated experimentally by venous stasis produced by a tourniquet applied to the upper arm at 100 mm Hg (between systolic and diastolic pressures) for 10 minutes. Blood was obtained before and after venous occlusion from the antecubital vein. The first 5 ml blood was discarded; then, 5 ml venous blood was drawn into an evacuated tube containing ethylenediamine tetraacetic acid (EDTA) (for determination of the hematocrit level); lastly, 4.5 ml venous blood was drawn into an evacuated glass tube containing 0.5 ml sodium citrate (0.13 mol/l, pH 7.5). After mixing, 1 ml citrated whole blood was withdrawn and combined with 0.5 ml 1 M sodium acetate buffer (pH 3.9) and immediately centrifuged at 1,000g for 5 minutes at room temperature. Both acidified and citrated plasma samples were stored at −60°C until analyzed.

Reagents

Two-chain human melanoma-cell t-PA with a specific activity of 500,000 IU/mg17 (compared with National Institute for Biologic Standards and Controls t-PA standard 83/517), goat-anti-t-PA (with and without conjugated horseradish peroxidase), and human glu-plasminogen were obtained from American Diagnostica (Greenwich, Connecticut). The plasmin substrate 3-Val-Leu-Lys-p-nitroanilide (S-2251) was obtained from Helena Diagnostics (Beaumont, Texas). Soluble cyanogen bromide fibrinogen fragments were prepared as previously described with plasminogen free fibrinogen from Sigma Chemical (St. Louis, Missouri).18 Immunoassay microtiter plates were obtained from Beckman (Palo Alto, California).

Determination of Tissue-Type Plasminogen Activator Activity

The t-PA activity was measured in acidified plasma samples by means of a spectrophotometric parabolic rate assay.18 To measure t-PA activity, 10 μl acidified plasma or t-PA standard was mixed with 400 μl plasminogen-chromogenic substrate reagent that consisted of 120 mM Tris (pH 8.5 at 25°C), 0.1% Triton-X-100, 0.35 mM S-2251, 0.4 μM human plasminogen, and 100 μg/ml CNBr-cleaved fibrinogen. Immediately after mixing, a 50-μl aliquot of the reaction solution was removed and mixed with 100 μl 25% acetic acid. The absorbance of the solution was then measured with a Cobas bio centrifugal analyzer (Roche Analytical Instruments, Nutley, New Jersey). The remaining reaction solution was incubated for 90 minutes at 37°C. At the end of the incubation, the reaction was again stopped by the addition of 50 μl reaction solution to 100 μl 25% acetic acid, and the final absorbance was measured. For the measurement of t-PA activity, the intra-assay and interassay coefficients of variation were 4% and 6%, respectively, using an acidified plasma sample with a mean activity of 1.0 IU/ml (SD=0.04 and 0.06 IU/ml, respectively).

Spun capillary hematocrit levels (11,000g for 5 minutes) were determined for each patient. Plasma t-PA activity was corrected for aceate buffer dilution and hematocrit (Hct) level as follows: Plasma t-PA = measured t-PA * (1.5−Hct)/(1−Hct).

t-PA capacity was defined as the difference in activity between prevenous and postvenous occlusion samples.19

Determination of Plasminogen Activator Inhibitor-1 Activity

PAI-1 activity in citrated plasma obtained before venous occlusion was measured as previously described.11,17 Plasma was initially diluted from 1:2 to 1:8 in Tris buffer (120 mM Tris, pH 8.5 at 25°C; 0.1% Triton-X-100). Equal volumes of diluted plasma and 10 IU/ml t-PA in Tris buffer were mixed and
incubated for 15 minutes at room temperature (final plasma dilutions from 1:4 to 1:16). At the end of the incubation, the reaction was stopped by addition of 1 M sodium acetate, pH 3.9. Residual t-PA activity was then measured as described above. One arbitrary unit (AU) of PAI-1 activity was defined as the amount inhibiting 1 IU t-PA activity. Inhibition of t-PA activity was linear down to a final plasma dilution of 1:4 when the sample contained less than 10 AU/ml PAI-1 activity. Samples with higher PAI-1 activities were diluted further (final dilution, from 1:16 to 1:64) and repeated. For the PAI-1 activity assay, the intra-assay and interassay coefficients of variation were 5% and 10%, respectively, using a sample with a PAI-1 activity of 8 AU/ml (SD = 0.4 and 0.8 AU/ml, respectively).

**Determination of Total Tissue-Type Plasminogen Activator Antigen**

Total t-PA antigen was determined as described previously with an enzyme linked immunosorbent assay. Briefly, the wells of a microtiter plate were coated with 200 μl goat-anti-t-PA (10 μg/ml) in 0.1 M NaHCO₃ for 3 hours at 25°C. The wells were then washed four times with 0.02 M phosphate, pH 7.4, containing 0.14 M NaCl, 0.01 M EDTA, and 0.5 g/l Tween 20 (PBS-EDTA-Tween).

Purified melanoma t-PA was used as a standard. The t-PA was diluted into human citrate-anticoagulated plasma containing less than 2 ng/ml t-PA. Coated wells were first filled with 200 μl PBS-EDTA-Tween followed by 10 μl standard, control, or patient plasma. When all the plasma samples had been added, the plate was covered and incubated for 3 hours at 25°C on a microplate shaker. After incubation, the wells were emptied and washed as described above. The wells were then filled with 200 μl 10 μg/ml goat-anti-t-PA peroxidase conjugate diluted in PBS-EDTA-Tween, incubated for 2 hours at 25°C on a microplate shaker, and again emptied and washed.

Finally, the wells were filled with 200 μl 0.4 mg/ml o-phenylene diamine substrate containing 0.01% H₂O₂ and incubated for 30 minutes at 25°C in the dark. The reaction was stopped by the addition of 50 μl 4.5 H₂SO₄, and the absorbance was read at 492 nm. The assay was linear to 30 ng/ml t-PA antigen. The intra-assay and interassay imprecisions were 7.9±0.2 and 5.8±0.5 ng/ml (mean±SD), respectively.

**Statistical Analysis**

Group distributions were tested for normality with the Wilk-Shapiro test and by calculation of the coefficients of skewness and kurtosis. Variables not normally distributed were logarithmically transformed before calculation of the mean. Group dispersions were given as 5–95th percentile ranges. Differences between groups were tested for significance with a two-tailed Mann-Whitney test. Linear regressions were calculated by conventional methods. Nonlinear regressions were calculated by an iterative least-squares curve-fitting method.

**Results**

**Plasminogen Activator Inhibitor-1 Activity**

Mean PAI-1 activity was significantly higher in the morning compared with the evening in controls and patients (Table 1). As shown in Figure 1, PAI-1 activity in plasma decreased between morning and evening in the majority of individuals in both groups. Among controls, PAI-1 activity decreased in 70%,
did not change significantly in 24%, and increased in 6% between morning and evening. In the patient group, PAI-1 activity decreased in 66%, did not change in 27%, and increased in 7%. The mean decrease in PAI-1 activity was 3.7 AU/ml (44%) in controls and 7.7 AU/ml (48%) in patients between morning and evening.

There was a wide distribution of PAI-1 activity in the morning in both groups (overall range, 2.8–49.8 AU/ml). The distribution of PAI-1 activity narrowed considerably in the evening. Excluding two outliers from the patient group, overall PAI-1 activity ranged from 0 to 10.8 AU/ml in the evening.

**Tissue-Type Plasminogen Activator Activity**

In contrast to the PAI-1 results, resting t-PA activity was significantly lower in the morning compared with the evening (Table 1). Among controls, resting t-PA activity increased in 61% between morning and evening, showed no significant change in 33%, and decreased in 6%. The corresponding values for the patient group were 67%, 33%, and 0%. On average, resting t-PA activity increased 0.8 IU/ml (80%) in controls and 0.9 IU/ml (113%) in patients between morning and evening.

The average level of t-PA activity postvenous occlusion was not significantly different between morning and evening. All of the individuals studied showed a t-PA capacity of at least 1.0 IU/ml during at least one of the two sampling periods (morning or evening). This indicated that all the individuals studied could release t-PA when challenged. Of the controls, 9% and 0% showed less than 1.0 IU/ml t-PA capacity during the morning and evening, respectively. For the patients, 40% showed t-PA capacities of less than 1.0 IU/ml during one of the two sampling periods.

**Tissue-Type Plasminogen Activator Antigen**

On average, t-PA antigen levels were significantly higher in the morning compared with the evening in controls and patients (Table 1). Among controls, t-PA antigen levels decreased during the day in 30%, did not change significantly in 67%, and increased in 3%. In the patient group, 53% showed a decrease in t-PA antigen during the day, whereas 47% showed no significant change. The mean decrease in t-PA antigen between morning and evening was 0.7 ng/ml in controls and 2.1 ng/ml in patients.

**Relation Between Plasminogen Activator Inhibitor-1 and Tissue-Type Plasminogen Activator**

As shown in Figure 2 (all data combined), PAI-1 activity was inversely correlated with resting t-PA activity \((r = -0.57, p < 0.0001)\). The best fit between the variables was found with an exponential relation \((r = 0.93)\). PAI-1 activity was also inversely correlated with t-PA capacity (Figure 3, \(r = -0.26, p = 0.012\)). The best fit between PAI-1 activity and t-PA capacity was found with an exponential relation \((r = 0.71)\).

In contrast, t-PA antigen was positively correlated with PAI-1 activity \((r = 0.50, p < 0.001)\). There was no significant correlation between t-PA antigen levels and resting t-PA activity. In essence, t-PA antigen levels followed the pattern of PAI-1 activity, indicating that total t-PA antigen in the blood is actually highest in the morning when t-PA activity is at its nadir.

There was no significant correlation \((p > 0.05)\) between age and any of the variables measured for either the morning or the evening samples in either the control group or the patient group studied separately (Figure 4). In examining the controls, there was no difference in any of the factors between controls less than 30 years of age (mean age, 24
years) and those 30 years and over (mean age, 43 years). The only significant correlation with age was found between PAI-1 activity and age in the evening samples when all the data (controls and patients) were combined (r=0.39, p=0.01).

The control group was, on average, younger than the patients (mean ages, 31 and 57 years, respectively). The patient group had significantly higher PAI-1 activities in the morning (p=0.05) and evening (p=0.01) compared with the controls. Similarly, t-PA antigen levels were higher in the patients compared with the controls in the morning and evening (p=0.01). Postvenous occlusion t-PA activity was significantly lower in the evening in patients compared with controls. There was no difference in any of the other factors.

**Discussion**

Previous studies have shown that overall fibrinolytic activity follows a diurnal variation with the lowest activities in the morning. This is supported by our data showing that t-PA activity was lowest in the morning in healthy controls and patients with coronary artery disease. We found that total t-PA antigen levels were on average higher in the morning, indicating that decreased release or availability of t-PA was probably not causing the variation. Furthermore, there was no change in the ability of cells to release t-PA activity after venous occlusion between morning and evening.

In contrast, PAI-1 activity was highest in the morning. This suggests that circadian variations in PAI-1 activity cause both the diurnal rhythm for t-PA activity and overall fibrinolytic activity. This is supported by the magnitude of the variation in the various factors. PAI-1 activity varied about twofold during the day in controls and patients. In contrast, t-PA antigen levels varied on average only 15–25%, showing no change in two thirds of the controls and half of the patients.

Previous studies of the circadian variation in fibrinolytic activity have included only healthy controls. All agree with our findings that t-PA activity is lowest and that PAI-1 activity and t-PA antigen are highest in the morning. Our data strengthens these findings in healthy individuals and extends it to patients with coronary artery disease.

The effect of age on fibrinolysis varies depending on the study. Although most studies show that t-PA and PAI-1 antigen appear to increase with age, whether or not PAI-1 activity increases with age in a healthy population group is not clear. We found a weak positive correlation between PAI-1 activity and age only in the evening samples and only after combining the control and patient data.

Increased levels of PAI-1 activity in plasma has been suggested to result in an increased risk of coronary artery thrombosis. Several studies have shown that individuals with a history of thrombotic coronary artery disease or myocardial infarction or both have elevated levels of PAI-1 activity compared with healthy controls. Further, it has been shown that PAI-1 activity increases during acute myocardial infarction. Elevated PAI-1 activity does not appear to affect the initial levels of circulating t-PA activity after infusion or the effectiveness of t-PA thrombolysis. After the infusion of t-PA is complete though, elevated PAI-1 levels may cause a decline in t-PA activity, possibly predisposing the individual to coronary reocclusion.

We hypothesize that individuals who show substantial variations in the level of PAI-1 activity during the day may have an increased probability of developing an arterial thrombus when PAI-1 activity is at its peak, which is usually in the morning. This theory could, in part, explain the increased incidence of onset of myocardial infarction in the morning.

Other explanations for the circadian rhythm associated with myocardial infarction have included rhythmic increases in coronary artery tone or spasm, arterial pressure, catecholamines, and the tendency of platelets to aggregate. Which of these possibilities are clinically important is not clear at present. More studies are needed to determine those variables that best predict the time of onset of thrombosis for individual patients.

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