Arteriosclerosis, Thrombosis, and Vascular Biology

American Heart Association®

Learn and Live℠

Daily and Circadian Rhythms of Tissue Factor Pathway Inhibitor and Factor VII Activity

Mirko Pinotti, Cristiano Bertolucci, Francesco Portaluppi, Ilaria Colognesi, Elena Frigato, Augusto Foà and Francesco Bernardi

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/3/646

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/02/27/01.ATV.0000153140.13148.e0.DC1.html

Daily and Circadian Rhythms of Tissue Factor Pathway Inhibitor and Factor VII Activity

Mirko Pinotti, Cristiano Bertolucci, Francesco Portaluppi, Ilaria Colognesi, Elena Frigato,
Augusto Foà, Francesco Bernardi

Objective—Diurnal variations in levels of factor VII (FVII), FVIII, proteins C and S, antithrombin, plasminogen activator inhibitor-1, prothrombin fragment F_{1+2}, and D-dimers in healthy humans point to the existence of circadian rhythms of coagulation factors. We sought for temporal fluctuations of tissue factor pathway inhibitor (TFPI) activity in human and mouse plasma.

Methods and Results—TFPI activity showed significant daily variations with highest levels in the morning in healthy men (+11%) and in mice at the light-to-dark transition (+63%), the beginning of the physically active period. Variations in FVII activity paralleled those in TFPI. In mice, the feeding schedule had a strong impact on these rhythms. Although restricted feeding and fasting shifted the peak of TFPI, the FVII peak disappeared. Investigation of temporal fluctuations in constant darkness indicated the existence of daily rhythms for TFPI and of true circadian rhythms for FVII.

Conclusions—For the first time, we report, both in humans and mice, temporal variations in TFPI activity. The coherent variations in FVII and TFPI activity could interplay to maintain the coagulation equilibrium. The chronobiological patterns should be considered to analyze activity levels of these factors. Moreover, the mouse model could be exploited to investigate modifiers of coagulation rhythms potentially associated to morning peaks of cardiovascular events. (***Arterioscler Thromb Vasc Biol***. 2005;25:646-649.)

Key Words: factor VII ■ TFPI ■ circadian ■ feeding schedule ■ mouse model

Frequencies of thromboembolic events in humans exhibit marked diurnal variations,[1–3] with peaks from morning to noon. Temporal variations in the occurrence of hemorrhagic events have also been reported.[4] Fluctuations in coagulation factor levels able to influence the hemostatic balance might contribute to these adverse outcomes. Diurnal rhythms in levels of factor VII (FVII),[5] FVIII,[6] proteins C and S,[7] antithrombin,[7] and plasminogen activator inhibitor (PAI)-1[8] have been described in healthy humans. Temporal oscillations in prothrombin fragment F_{1+2}[5–6] and D-dimer,[6] markers of thrombin generation and fibrinolysis, have been also described. These variations could reflect the existence of circadian rhythms of blood coagulation factors. Circadian rhythms are the overt expression of an internal timing mechanism measuring daily time, with the fundamental adaptive function of providing optimal temporal organization of physiological processes in relation to the environment.[9] Because formal assessment of circadian rhythms in coagulation factor levels is hardly feasible in humans, a circadian control has been so far demonstrated in a mouse model for PAI-1[10–11] and fibrinogen[12] mRNA expression.

Among factors interacting with circadian rhythms, daily availability of food represents a major component. Several studies suggested that postprandial and fasting lipoproteins are associated with plasma levels or activation state of coagulation factors, and particularly of FVII[13–17] that plays a key role in the initiation of the clotting cascade.[18]

The present investigation was aimed at testing the existence of diurnal variations of tissue factor pathway inhibitor (TFPI) in humans. We further tested for the presence of temporal variations of both TFPI and FVII activity levels in mice exposed to light-dark (LD) cycles and the potential effects of changing their schedules of feeding. Finally, sampling tests in mice were performed under constant darkness (DD) to establish whether the observed temporal variations of TFPI and FVII activity levels are truly circadian in nature.

Materials and Methods

Subjects

Thirteen healthy men (25 to 35 years old) recruited from the community were studied. Subjects were overnight fasted and after morning blood sampling ate a low-fat meal. Blood sampling was conducted at 8AM and 2PM.

Mouse Model

We investigated C57BL/6JOlaHsd mice (n=46, males, 10 to 15 weeks old, Harlan Laboratory, Udine, Italy) kept under 12:12 LD (L: 150 1×; D: 0 1×) or in DD. Food and water were supplied ad libitum, except for restricted feeding (RF) and fasting (F) experiments. All treatments were conducted under the guidelines of the Italian Ministry of Health.

Sampling

Blood samples were collected in 0.38% trisodium citrate. After centrifugation at 2500*g* for 10 minutes, plasma was separated and stored at −80°C. In DD, a weak red light was used during the sampling.

Experimental Conditions

Sampling was performed on 2 groups of mice exposed to a LD cycle, with lights-on at 06:00 (n=12) or at 12:00 (n=8). It is conventional to divide the 24-hour LD cycle in 24 Zeitgeber hours (ZT) and indicate time of lights-on as ZT0 and time of lights-off as ZT12. Blood samples were withdrawn at ZT0, 6, 12, and 18. A third group of mice (n=10) was transferred to DD at ZT12. The 24-hour circadian cycle is divided in 24 circadian hours (CT). In DD it is conventional to use as reference point the phase that would normally (ie, in LD 12:12) coincide with the onset of darkness and to call this CT12. The part of the cycle covering CT0-CT12 is then called subjective day, and the part covering CT12–24, subjective night. Because the free running period of circadian rhythms in mice during the first days in DD did not change from 24 hours, ZTs and CTs marked the same phases and can be used to identify the same time points of sampling between the LD and DD tests. Sampling was performed after 3 days in DD at CT0, 6, 12, and 18.

In the RF and F experiments mice were exposed to LD cycle. In the RF schedule, after 1 entire day of fasting, mice (n=8) had access to food from ZT5 to ZT9 for 7 consecutive days. On day 7, blood samples were withdrawn at ZT0, 6, 12, and 18. In the F schedule, mice (n=8) were deprived of food for an entire day and the next day blood samples were withdrawn at ZT0, 6, 12, and 18. Water was freely available throughout all experiments.

TFPI and FVII Activity Assays

TFPI activity levels in human and mouse plasma were determined using the ACTICHROME TFPI Activity Assay (American Diagnostica) following the manufacturer's instructions.

FVII activity was evaluated by measuring the generation of activated factor X (FXa) in human and mouse plasma diluted 1:20 in human FVII-depleted plasma (Dade Behring). FXa generation assays were conducted essentially as previously described.[19–20] Coagulation in diluted plasma (1:40) was triggered by adding excess of Innovin (Dade Behring). Two hundred μmol/L FXa fluorogenic substrate (MeSO2-D-CHA-Gly-Arg-AMCAcOH; American Diagnostica) was added and relative fluorescence units (RFU) monitored over time. The initial rate of activity was expressed as RFU per seconds. Protocols were optimized by testing different dilutions of mouse plasma, and experimental conditions with comparable activities in human and mouse plasma were chosen. FVII coagulant activity in mouse plasma diluted 1:20 in human FVII-depleted plasma was evaluated using a single stage clotting assay (prothrombin time [PT]). The intra-assay coefficient of variation for the assays used in this study was <6%.

Statistical Analysis

Data were log transformed and results expressed as mean±SEM. Paired Student *t* test and 1-way repeated measures analysis of variance were used to determinate significant differences ($P<0.05$). Bonferroni test was applied for post hoc comparison. Data were analyzed using the software STATISTICA 5.5 (StatSoft Inc).
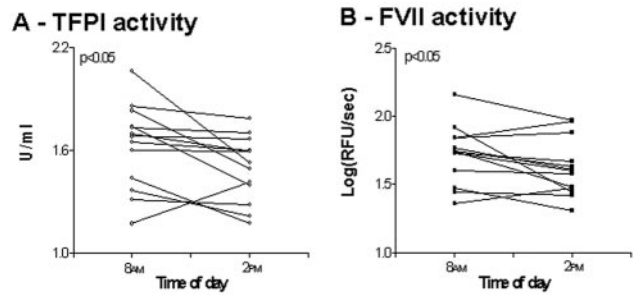

Figure 1. Individual variations of TFPI (A) and FVII (B) activity in healthy humans. TFPI and FVII activity is reported as U/mL and Log(RFU/sec), respectively.

Results

TFPI and FVII Variations in Humans

Temporal variations of TFPI and FVII activity were measured in humans exposed to natural conditions. Plasma samples from 13 healthy volunteers were withdrawn at 8AM and 2PM and assayed. TFPI levels were significantly higher (+11%; $t_{12}=2.4$, $P<0.05$) in the morning than in the afternoon (Figure 1A). FVII was also higher (+6%; $t_{12}=2.28$, $P<0.05$) in the morning than in the afternoon (Figure 1B).

TFPI and FVII Variations in Mice

Intravascular TFPI activity was monitored in mice exposed to LD cycles. The capability of the assay to measure TFPI inhibition on FXa generation in mouse plasma was preliminarily tested by adding purified human TFPI (Figure I, available online at http://atvb.ahajournals.org). TFPI activity showed a significant variation during the 24 hours ($F_{(3,12)}=7.4$, $P<0.005$; Figure 2A), with a peak (63% over the baseline; $P<0.05$) around the light-to-dark transition (ZT12).

Daily variations in the activity of TFPI prompted us to investigate those in its target serine protease FVII. FVII activity was assessed by means of a fluorogenic FXa generation assay in mouse plasma diluted in human FVII-depleted plasma. In mice maintained under LD cycles, FVII activity showed a clear daily rhythm ($F_{(3,24)}=3.8$, $P<0.03$; Figure 2B) with a peak at ZT12 (16% over the baseline; $P<0.05$).

FVII activity was also assessed by PT to further support these findings. FXa generation levels strongly correlated with PT clotting time ($r=-0.85$, $P<0.01$; Figure I).
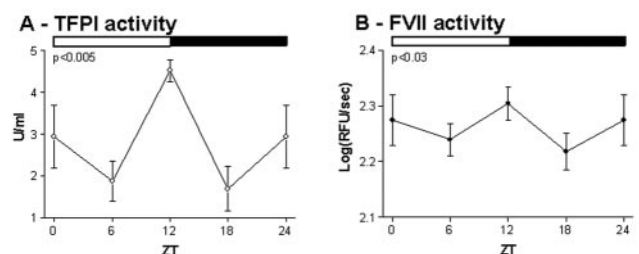

Figure 2. TFPI (A) and FVII (B) activity levels in mice exposed to LD. TFPI and FVII activity is reported as U/mL and Log(RFU/sec), respectively. Mean±SEM at each time point is shown. White and black bars indicate the duration of light and dark phases. RFU indicates relative fluorescence units; ZT, Zeitgeber time.
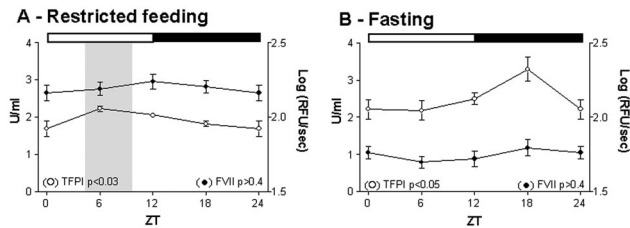
Figure 3. TFPI and FVII activity levels in mice subjected to restricted feeding (A) and fasting (B). TFPI (○) and FVII (●) activity is reported as U/mL and Log(RFU/sec), respectively. Mean±SEM at each time point is shown. The shaded area indicates time of food availability. White and black bars indicate the duration of light and dark phases. RFU indicates relative fluorescence units; ZT, Zeitgeber time.

Effects of Changing Feeding Schedule

To elucidate the effects of feeding on temporal variations of TFPI and FVII activity levels, mice were subjected to either RF or F schedule. Twenty-four–hour rhythms of TFPI activity were observed both in the RF and F treatments (RF: $F_{(3,21)}=3.7$, $P<0.03$; F: $F_{(3,21)}=2.9$, $P<0.05$; Figure 3A), which shifted the peak with respect to the ad libitum conditions (Figure 2A, ZT12). In the detail, the peak in RF changed from ZT12 to ZT6, whereas the peak in F changed from ZT12 to ZT18. Twenty-four–hour rhythms of FVII were abolished both in RF and F (RF: $F_{(3,21)}=0.84$, $P>0.4$; F: $F_{(3,21)}=0.74$, $P>0.4$; Figure 3).

Circadian Nature of Rhythms

To assess the circadian nature of the TFPI and FVII rhythms, variations in the activity of these factors was also investigated in mice kept in DD. Mice exposed to DD did not show any TFPI level variation across the 24 hours ($F_{(3,12)}=0.07$, $P>0.9$; Figure 4A). On the contrary, FVII activity continued to express a robust rhythm ($F_{(3,24)}=3.4$, $P<0.04$; Figure 4B) in DD, with a peak at CT18 ($P<0.05$), the middle of the subjective night.

Discussion

Variations in coagulation factor levels could potentially influence the hemostatic balance both in physiological and pathological conditions. For the first time, we report clear experimental evidence, both in healthy humans and mice, for the existence of temporal variations in the activity levels of TFPI in plasma. TFPI, the direct inhibitor of the FXa/TF/FVIIa complex,[21] has a complex biology with respect to both
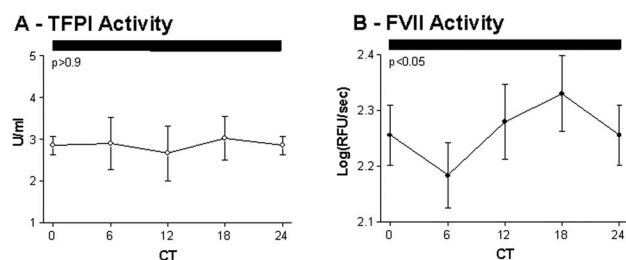

Figure 4. TFPI (A) and FVII (B) activity levels in mice exposed to DD. TFPI and FVII activity is reported as U/mL and Log(RFU/sec), respectively. Mean±SEM at each time point is shown. RFU indicates relative fluorescence units; CT, circadian time.

mechanism of action and distribution. A major portion of TFPI in blood is normally bound to vascular endothelium, and a minor fraction (20% to 30%) is intravascular.[22] Our investigation in healthy men showed that intravascular TFPI activity levels in humans were higher in the morning, and their variations paralleled that observed in FVII activity. Although the physiological role of circulating TFPI has not yet been clearly elucidated,[22] its variations could interplay with those of FVII and other coagulation factors[5–8] to influence the coagulation equilibrium,[23] overall producing the morning hypercoagulability suggested by the increased levels of prothrombin fragment F_{1+2}[5–6] and D-dimer.[6] The morning increase in TFPI levels observed in angina patients after coronary spasms[24] might represent a further, albeit insufficient, response aimed at maintaining the coagulation equilibrium.

To formally characterize temporal variations of TFPI activity, we exploited the mouse model. TFPI and FVII levels showed large 24-hour variations, with peaks at the light-to-dark transition. Taking into account the mouse nocturnal habit, peaks of TFPI and FVII activity at the beginning of the dark phase in mice would correspond to that observed in humans during the first hours of the light phase.

Our findings provide clear evidence that TFPI and FVII daily variations are strongly influenced by the feeding schedules, in accordance with the notion that food is a major determinant of coagulation function.[25] The shift of TFPI activity peaks was similar to that reported for PAI-1,[11] which like TFPI[22] is of endothelial origin.

Feeding also had a profound impact on FVII rhythms that were markedly attenuated by RF and completely abolished by fasting. Accordingly, recent data showed that 1 to 3 days of fasting does not affect the phase of circadian rhythms but alters the expression levels of some clock genes (ie, *Per1*, *Per2*, and *Dbp*) in the mouse liver.[26] Hence, the abolition of daily rhythms of FVII, mainly synthesized by liver,[27–28] could depend either on food-related changes in FVII synthesis or indirectly on alterations of clock genes expression.

The expression in liver and endothelium of a variety of clock controlled genes prompted us to investigate the true circadian nature of daily variation in levels of TFPI and FVII activity, formally feasible only in the mouse model.

Data in DD excluded the presence of true circadian rhythms for TFPI activity which resulted strongly entrained by light. It is tentative to speculate that the marked light-dependence of TFPI levels could make their variations particularly prone to lifestyle components, such as shift-work.

FVII exhibited true circadian rhythms, further supported by the presence in mouse[29] (-483, -1054) and human[30] (-140) FVII 5′ flanking regions of E-box sequences, hallmarks of putative regulation by clock proteins.[31]

Our data suggest that the chronobiological features of these coagulation factors, and particularly their variation patterns, have to be taken into account in the analysis of FVII and TFPI plasma levels. In humans, increased FVII activity levels are considered an independent risk factor for myocardial infarction,[32–33] and recent studies indicated that intravascular TFPI activity levels are associated with thrombotic risk.[34–35]

The characterization in a mouse model of daily and circadian rhythms in coagulation activity provides a powerful tool to investigate natural or pharmacological modifiers of coagulation rhythms and could help to interpret the temporal variations in the frequency of cardiovascular events.[1–4]
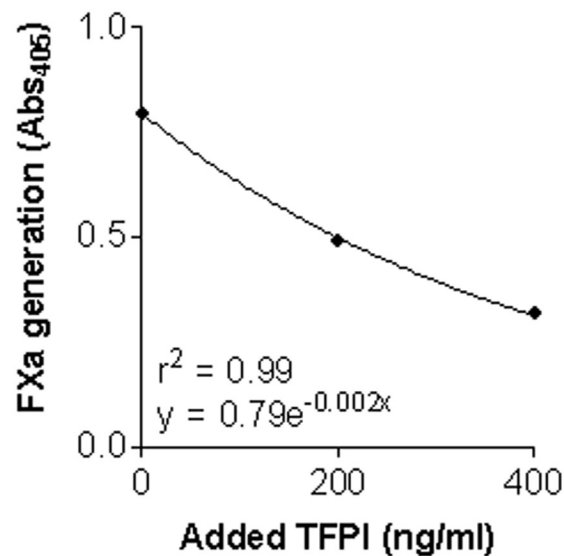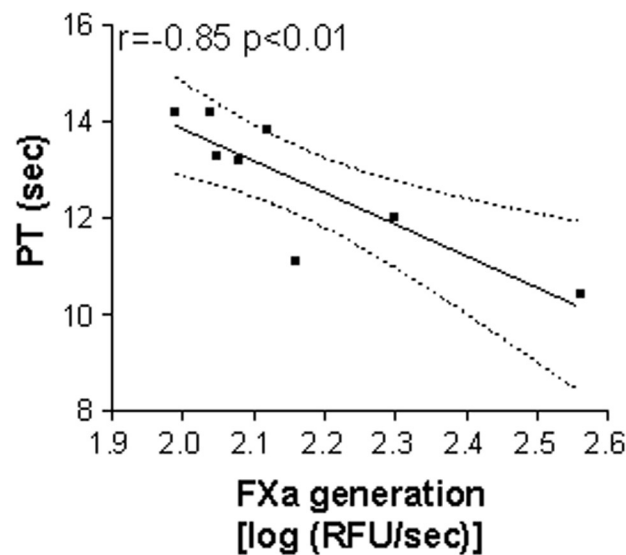
References

1. Muller JE, Tofler GH, Stone PH. Circadian variation and triggers of onset of acute cardiovascular disease. *Circulation*. 1989;79:733–743.
2. Muller JE, Stone PH, Turi ZG, Turi ZG, Rutherford JD, Czeisler CA, Parker C, Poole WK, Passamani E, Roberts R, Robertson T, et al. Circadian variation in the frequency of onset of acute myocardial infarction. *N Engl J Med*. 1985;313:1315–1322.
3. Marler JR, Price TR, Clark GL, Muller JE, Robertson T, Mohr JP, Hier DB, Wolf PA, Caplan LR, Foulkes MA. Morning increase in onset of ischemic stroke. *Stroke*. 1989;20:473–476.
4. Casetta I, Granieri E, Portaluppi F, Manfredini R. Circadian variability in hemorrhagic stroke. *JAMA*. 2002;287:1266–1267.
5. Kapiotis S, Jilma B, Quehenberger P, Ruzicka K, Handler S, Speiser W. Morning hypercoagulability and hypofibrinolysis. Diurnal variations in circulating activated factor VII, prothrombin fragment F1+2, and plasmin-plasmin inhibitor complex. *Circulation*. 1997;96:19–21.
6. Iversen PO, Groot PD, Hjeltnes N, Andersen TO, Mowinckel MC, Sandset PM. Impaired circadian variations of haemostatic and fibrinolytic parameters in tetraplegia. *Br J Haematol*. 2002;119:1011–1016.
7. Undar L, Ertugrul C, Altunbas H, Akca S. Circadian variations in natural coagulation inhibitors protein C, protein S and antithrombin in healthy men: a possible association with interleukin-6. *Thromb Haemost*. 1999;81:571–575.
8. Angleton P, Chandler WL, Schmer G. Diurnal variation of tissue-type plasminogen activator and its rapid inhibitor (PAI-1). *Circulation*. 1989;79:101–106.
9. Daan S, Aschoff J. Circadian contribution to survival. In: Aschoff J, Daan S, Groos G, eds. *Vertebrate Circadian System*. Berlin, Germany: Springer-Verlag; 1982;305–321.
10. Maemura K, de la Monte SM, Chin MT, Layne MD, Hsieh CM, Yet SF, Perrella MA, Lee ME. CLIF, a novel cycle-like factor, regulates the circadian oscillation of plasminogen activator inhibitor-1 gene expression. *J Biol Chem*. 2000;275:36847–36851.
11. Minami Y, Horikawa K, Akiyama M, Shibata S. Restricted feeding induces daily expression of clock genes and Pai-1 mRNA in the heart of Clock mutant mice. *FEBS Lett*. 2002;526:115–118.
12. Sakao E, Ishihara A, Horikawa K, Akiyama M, Arai M, Kato M, Seki N, Fukunaga K, Shimizu-Yabe A, Iwase K, Ohtsuka S, Sato T, Kohno Y, Shibata S, Takiguchi M. Two-peaked synchronization in day/night expression rhythms of the fibrinogen gene cluster in the mouse liver. *J Biol Chem*. 2003;278:30450–30457.
13. Humphries SE, Lane A, Green FR, Cooper J, Miller GJ. Factor VII coagulant activity and antigen levels in healthy men are determined by interaction between factor VII genotype and plasma triglyceride concentration. *Arterioscler Thromb Vasc Biol*. 1994;14:193–198.
14. Miller GJ, Stirling Y, Howarth DJ, Cooper JC, Green FR, Lane A, Humphries S. Dietary fat intake and plasma factor VII antigen concentration. *Thromb Haemost*. 1995;73:893.
15. Mennen LI, Schouten EG, Grobbee DE, Kluft C. Coagulation factor VII, dietary fat and blood lipids: a review. *Thromb Haemost*. 1996;76:492–499.
16. Mariani G, Bernardi F, Bertina R, Vicente VV, Prydz H, Samama M, Sandset PM, Di Nucci GD, Testa MG, Bendz B, Chiarotti F, Ciarla MV, Strom R. Serum phospholipids are the main environmental determinants of activated factor VII in the most common FVII genotype. European Union Concerted Action "Clotart." *Haematologica*. 1999;84:620–626.
17. Kjalke M, Silveira A, Hamsten A, Hedner U, Ezban M. Plasma Lipoproteins Enhance Tissue Factor–Independent Factor VII Activation. *Arterioscler Thromb Vasc Biol*. 2000;20:1835–1841.
18. Hedner U, Davie EW. Introduction to hemostasis and the vitamin K-dependent coagulation factors. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic Basis of Inherited Disease*. New York, NY: McGraw-Hill Inc; 1989:2107–2134.
19. Pinotti M, Etro D, Bindini D, Papa ML, Rodorigo G, Rocino A, Mariani G, Ciavarella N, Bernardi F, Pinotti M, Etro D, Mariani G. Residual factor VII activity and different hemorrhagic phenotypes in CRM(+) factor VII deficiencies (Gly331Ser and Gly283Ser). *Blood*. 2002;99:1495–1497.
20. Pinotti M, Marchetti G, Baroni M, Cinotti F, Morfini M, Bernardi F. Reduced activation of the Gla19Ala FX variant via the extrinsic coagulation pathway results in symptomatic CRMred FX deficiency. *Thromb Haemost*. 2002;88:236–241.
21. Broze GJ Jr. Tissue factor pathway inhibitor and the revised theory of coagulation. *Annu Rev Med*. 1995;46:103–112.
22. Kato H. Regulation of functions of vascular wall cells by tissue factor pathway inhibitor: basic and clinical aspects. *Arterioscler Thromb Vasc Biol*. 2002;22:539–548.
23. Butenas S, van't Veer C, Mann KG. "Normal" thrombin generation. *Blood*. 1999;94:2169–2178.
24. Misumi K, Ogawa H, Yasue H, Soejima H, Suefuji H, Nishiyama K, Takazoe K, Kugiyama K, Tsuji I, Kumeda K. Circadian variation in plasma levels of free-form tissue factor pathway inhibitor antigen in patients with coronary spastic angina. *Jpn Circ J*. 1998;62:419–424.
25. Tracy RP. Diet and hemostatic factors. *Curr Atheroscler Rep*. 1999;1:243–248.
26. Kobayashi H, Oishi K, Hanai S, Ishida N. Effect of feeding on peripheral circadian rhythms and behaviour in mammals. *Genes Cells*. 2004;9:857–864.
27. Cooper DN, Millar DS, Wacey A, Banner DW, Tuddenham EG. Inherited factor VII deficiency: molecular genetics and pathophysiology. *Thromb Haemost*. 1997;78:151–160.
28. Furie B, Furie BC. Molecular and cellular biology of blood coagulation. *N Engl J Med*. 1992;326:800–806.
29. Idusogie E, Rosen ED, Carmeliet P, Collen D, Castellino FJ. Nucleotide structure and characterization of the murine blood coagulation factor VII gene. *Thromb Haemost*. 1996;76:957–964.
30. O'Hara PJ, Grant FJ, Haldeman BA, Gray CL, Insley MY, Hagen FS, Murray MJ. Nucleotide sequence of the gene coding for human factor VII, a vitamin K-dependent protein participating in blood coagulation. *Proc Natl Acad Sci U S A*. 1987;84:5158–5162.
31. Cardone L, Sassone-Corsi P. Timing the cell cycle. *Nat Cell Biol*. 2003;5:859–861.
32. Meade TW, Mellows S, Brozovic M, Miller GJ, Chakrabarti RR, North WR, Haines AP, Stirling Y, Imeson JD, Thompson SG. Haemostatic function and ischaemic heart disease: principal results of the Northwick Park Heart Study. *Lancet*. 1986;2:533–537.
33. Girelli D, Russo C, Ferraresi P, Olivieri O, Pinotti M, Friso S, Manzato F, Mazzucco A, Bernardi F, Corrocher R. Polymorphisms in the factor VII gene and the risk of myocardial infarction in patients with coronary artery disease. *N Engl J Med*. 2000;343:774–780.
34. Dahm A, Van Hylckama Vlieg A, Bendz B, Rosendaal F, Bertina RM, Sandset PM. Low levels of tissue factor pathway inhibitor (TFPI) increase the risk of venous thrombosis. *Blood*. 2003;101:4387–4392.
35. Soejima H, Ogawa H, Yasue H, Kaikita K, Nishiyama K, Misumi K, Takazoe K, Miyao Y, Yoshimura M, Kugiyama K, Nakamura S, Tsuji I, Kumeda K. Heightened tissue factor associated with tissue factor pathway inhibitor and prognosis in patients with unstable angina. *Circulation*. 1999;99:2908–2913.

Data supplements

A

Plot A: FXa generation (Abs$_{405}$) vs Added TFPI (ng/ml). Y-axis from 0.0 to 1.0, X-axis from 0 to 400.

$r^2 = 0.99$

$y = 0.79e^{-0.002x}$

B

Plot B: PT (sec) vs FXa generation [log (RFU/sec)]. Y-axis from 8 to 16, X-axis from 1.9 to 2.6.

r=-0.85 p<0.01

A. TFPI inhibition on FXa generation in mouse plasma tested by adding increasing concentration of purified human TFPI

B. Correlation between FXa generation activity and prothrombin time. Plasma samples were assayed in parallel for FXa and Prothrombin Time. Prothrombin time was measured in mouse plasma using a single-stage clotting assay.