Molecular Cardiology

Genetic Components of the Circadian Clock Regulate Thrombogenesis In Vivo

Elizabeth J. Westgate, PhD; Yan Cheng, MD, PhD; Dermot F. Reilly, PhD; Tom S. Price, PhD; Jacqueline A. Walisser, PhD; Christopher A. Bradfield, PhD; Garret A. FitzGerald, MD

Background — Myocardial infarction, stroke, and sudden death undergo diurnal variation. Although genes relevant to hemostasis and vascular integrity undergo circadian oscillation, the role of the molecular clock in thrombotic events remains to be established.

Methods and Results — A diurnal variation in the time to thrombotic vascular occlusion (TTVO) subsequent to a photochemical injury was observed in wild-type mice: TTVO varied from 24.6 ± 2.7 minutes at zeitgeber time (ZT) 2 to 40.3 ± 4.3 minutes at ZT8, 24.3 ± 2.3 minutes at ZT14, and 31.0 ± 4.4 minutes at ZT20. This pattern was disrupted or altered when core clock genes — BMAL1, CLOCK, and NPAS2 — were mutated or deleted. Mutation of CLOCK abolished the diurnal variation in TTVO, whereas deletion of NPAS2 altered its temporal pattern. NPAS2 deletion prolonged TTVO and reduced blood pressure irrespective of clock time. Global BMAL1 deletion shortened TTVO at ZT8, and the diurnal variation in TTVO, but not in systemic blood pressure, was disrupted in mice in which BMAL1 had been selectively deleted in endothelium.

Conclusions — Key components of the molecular clock regulate the response to a thrombogenic stimulus in vivo. Such a phenomenon may interact with environmental variables, and together with the influence of these genes on blood pressure may contribute to the diurnal variation in cardiovascular events observed in humans. (Circulation. 2008;117:2087-2095.)

Key Words: blood pressure ■ circadian rhythm ■ endothelium ■ thrombosis

The molecular clock is a well-conserved mechanism that permits biologically efficient circadian timing of physiology and behavior. In mammals, the master oscillator is located in the suprachiasmatic nucleus,1 and autonomous peripheral oscillators have been defined for most tissues, including the vasculature, heart, and kidney.2-4 Peripheral oscillators, which share many of their molecular components with the master oscillator, can also be distinguished by the expression of specific transcription factors (eg, NPAS2 and CLIF in the vasculature).5-8 The molecular oscillator is composed of interlocking positive and negative transcriptional and translational feedback loops that drive circadian gene expression.9 Heterodimers of the bHLH-PAS transcription factors CLOCK and/or NPAS2 with BMAL1 constitute the positive limb of this feedback loop,7 driving the cyclical expression of period (per) and cryptochrome (cry). PER and CRY proteins sequentially dimerize to repress their own transcription, forming the negative limb of this autoregulatory feedback loop.8,9 A second loop involves the circadian regulation of Bmal1 transcription by REV-ERBα9 and RORα.10

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Aspects of both cardiovascular physiology and the clinical manifestation of cardiovascular disease display diurnal variation. The early morning surge in blood pressure (BP) is accompanied by a decline in endothelial function; both phenomena coincide with the peak incidence in clinical thrombotic events.11,12 The timing of adverse cardiovascular events corresponds to oscillations in gene and protein expression of known regulators of hemostasis13,14; an example is plasminogen activator inhibitor-1 (PAI-1),15 a time-independent risk factor for cardiovascular disease.16 Cassettes of genes relevant to vascular injury and integrity have been shown to oscillate in mouse aorta, as does vascular leakage.2 We have recently implicated the molecular clock in regulating circadian variation in BP and the response to asynchronous stress.17 The present study sought to determine whether genes that are key components of the molecular clock might also regulate the response to a thrombogenic stimulus in vivo.

Methods

Animals

All experimental protocols were reviewed and approved by the Institute for Animal Care and Use Committee at the University of Pennsylvania. Bmal1lox/lox mice were crossed with Crelox transgenic mice18 to generate either Bmal1lox/loxCrelox or Bmal1lox/lox littermate controls, kindly provided by Dr Chris Bradfield at the University of Pennsylvania.
Wisconsin; mice were backcrossed to C57BL/6j to the N4 generation. Global Bmal1 knockout mice (Bmal1−/−) were compared with Bmal1−/− (wild-type [WT]) littermate controls. Fully backcrossed CLOCKmut and NPAS2−/− mice were compared with the same group of C57BL/6j (WT) control mice. Mice were allowed to acclimatize to a 12-hour light/12-hour dark (LD) cycle, with lights on at 7 AM (zeitgeber time [ZT0]) and off at 7 PM (ZT12), for 2 weeks before surgical experimentation. All studies were performed in LD except for the Bmal1−/−Cre tk tissue harvest, in which animals were subjected to 18 hours of complete darkness (DD) before tissues were harvested at 4-hour intervals. Under DD, chronology is measured in circadian time (CT), with subjective day beginning at 7 AM (CT0) and subjective night beginning at 7 PM (CT12).

Photochemical Injury Model
Thrombotic vessel occlusion was produced in the femoral artery of male mice aged 8 to 14 weeks (CLOCKmut, NPAS2−/−, and WT, n=10 to 18; Bmal1−/− and Bmal1+/−, n=4 to 5) or 14 to 17 weeks (Bmal1+/+Cre tk and Bmal1+/+, n=10) with the use of a photochemical injury model described previously.19 Slight modifications in anesthesia (ketamine, 100 mg/kg; xylazine, 20 mg/kg) and transluminal laser (543-nm laser; 5 mm from vessel) were used. The time to vessel occlusion (TTVO) was measured as the time between rose bengal injection (Acrors Organics; 40 mg/kg) and blood flow cessation for at least 3 minutes.

Carotid Artery Implantation of Telemetry Probes
PA-C10 telemetry probes (Data Sciences Inc) were implanted into male mice aged 18 to 20 weeks (n=8 to 10) with the carotid artery placement described.20 Continuous 24-hour mean arterial pressure (MAP), heart rate (HR), and activity were monitored under LD in unrestricted animals 10 days after surgery with the Dataquest IV system (DSI) described.21 Circadian rhythm analysis of the individual hourly MAP and HR data was performed as described.17 Time-series analyses were conducted with the use of generalized estimating equations,22 as implemented in the package GEEpack version 1.0.10 for R-2.4.1 (www.cran.r-project.org/). Coefficients were estimated for the mean and interactive effects of strain and time. Orthogonal terms were estimated for animal-specific mean effects. A correlation structure for the residuals was assumed under which measurements at successive time points for each animal were autocorrelated with lag 1. Analyses of MAP and HR were conducted with a gaussian distribution assumed. Activity counts were assumed to be Poisson distributed.

Tissue Harvesting and Quantitative Real-Time Polymerase Chain Reaction
Tissues were harvested at prespecified times as previously described23 in male mice aged 10 to 15 weeks (CLOCKmut, NPAS2−/−, and WT, n=7 to 9) or 18 to 20 weeks (Bmal1+/+Cre tk and Bmal1+/+, n=3 to 6). Total RNA was isolated from tissues with an RNaseasy Mini-Kit (Qiagen) and from cells as described.23 Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR were performed as described,23 with compared samples run in the same RT-PCR and real-time PCR reactions. Primer sequences are described in supplemental Methods in the online-only Data Supplement.

Plasma Analysis
Total and active plasma PAI-1 antigen and total plasma tissue plasminogen activator (tPA) antigen were measured by commercially available enzyme-linked immunosorbent assay kits (Innovative Research, Southfield, Mich) (n=8 to 16). Plasma epinephrine and norepinephrine were measured by a commercially available enzyme immunoassay kit (Bi-CAT EIA, ALPCO Diagnostics, Salem, NH) (n=6 to 8). Total plasma nitrates were measured by a commercially available colorimetric assay kit (Nitrate/Nitrite Colorimetric Assay Kit, Caymen Chemical, Ann Arbor, Mich) (n=8 to 11).

Statistical Analysis
Data are expressed as mean±SEM. Statistical analysis was performed with ANOVA or nonparametric Mann-Whitney test, unless otherwise noted. Data analyzed by 2-way ANOVAs were reported as having a significant effect of CT or genotype. Probability values of <0.05 were considered statistically significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
CLOCK and NPAS2 in the Diurnal Variation in Thrombogenesis
With the use of a photochemical injury model in the mouse femoral artery, mean TTVO was recorded at 4 ZTs: ZT2, ZT8, ZT14, and ZT20 in CLOCKmut and NPAS2−/− mice and WT controls. A diurnal variation in TTVO was observed in WT mice (Figure 1A and 1B; P=0.02), with significantly faster TTVO observed when injury was performed at either ZT2 or ZT14 (24.6±2.7 and 24.3±2.3 minutes, respectively) compared with injury performed at ZT8 (40.3±4.3 minutes; P<0.01 versus ZT2, P<0.05 versus ZT14). Similar TTVO was observed at both the start of the light cycle (inactive period) and 12 hours later at the start of the dark cycle (active period), reminiscent of the secondary peak in human adverse cardiovascular events at ~7 to 9 PM.13,24-25 TTVO at ZT20 (31.0±4.4 minutes) was not significantly different from any other time of day. The diurnal variation in TTVO in WT mice was not correlated with a diurnal variation in platelet reactivity ex vivo; whole blood platelet activation and aggregation were similar at ZT2, ZT8, and ZT14 (Data Supplement Figure I).

In contrast to WT animals, the diurnal variation in TTVO was completely abolished in CLOCKmut mice (P=0.273; Figure 1B), with similar TTVO observed at ZT2, ZT8, ZT14, and ZT20 (37.4±5.6, 30.8±4.9, 36.1±3.7, and 41.9±4.7 minutes, respectively). Interestingly, NPAS2−/− mice displayed a diurnal variation (P=0.035) with a significantly faster occlusion time observed at ZT20 (36.4±7.2 minutes) in comparison to ZT2, ZT8, and ZT14 (59.2±5.2, 63.7±5.5, and 56.5±5.9 minutes, respectively; P<0.05). These data are consistent with a role for CLOCK, but not NPAS2, in maintaining a diurnal variation in thrombogenesis.

We also found apparent contributions of these molecular clock components to the overall TTVO (P<0.0001 for genotype). NPAS2 had a greater effect independent of time on this parameter compared with its homolog: CLOCKmut mice had a small but significant increase in TTVO at ZT14 compared with WT (P<0.05), whereas NPAS2−/− mice had significantly longer TTVO at ZT2, ZT8, and ZT14 (P<0.0001) but not at ZT20 (Figure 1B). We also noted that these animals had significantly lower baseline blood flow in comparison with WT controls (0.162±0.004 versus 0.184±0.005 mL/min; P<0.05) (Figure 1C), a phenotype that may more directly affect TTVO. CLOCKmut mice, on the other hand, retained normal baseline blood flow, in accordance with their normotensive phenotype17 and their more subtle effect on absolute TTVO.
ECs are present in almost all tissues, and because TekCre is (data supplement figure III and data not shown). Given that tissue clock genes were previously determined to be transcribed rhythmically in this tissue (2 clock genes were altered in Bmal1flox/CreTek and Bmal1flox mice for the presence of both the Bmal1flox, unexcised and Bmal1flox-excised alleles, as described previously.27 In the absence of CreTek, the Bmal1flox/mice revealed only the Bmal1flox-unexcised allele in all tissues examined (data supplement figure III and data not shown). Given that ECs are present in almost all tissues, and because TekCre is also expressed in cells of hematopoietic lineage,18 we found the presence of both the Bmal1flox-unexcised and Bmal1flox, excised alleles in all organs examined from Bmal1flox/CreTek mice. However, the Bmal1flox-excised allele was more prominent in the vasculature (aorta) and highly vascularized tissues such as the heart and kidney in comparison to the liver. In contrast to Bmal1-null mice,28 Bmal1flox/CreTek mice are of normal body weight and have normal complete blood counts (data not shown); they appear to be otherwise healthy.

Gene expression in mouse aortas harvested from Bmal1flox/CreTek and Bmal1flox control mice every 4 hours for 16 hours in DD was analyzed by real-time PCR for transcripts previously determined to be transcribed rhythmically in this tissue.2 Clock genes Per2 and Bmal1 were both rhythmically expressed in Bmal1flox and Bmal1flox/CreTek aortas (P<0.0001; figure 2A), with oscillations antiphase to one another, likely reflecting conserved expression of these genes in aortic cells other than endothelium. In contrast, genes associated specifically with ECs (Claudin-5, Tie-1) displayed rhythm in Bmal1flox (P=0.02 and P=0.03, respectively) but not Bmal1flox/CreTek mice (P=0.22 and P=0.32, respectively; figure 2B and 2C). Tek, which is also specifically expressed by ECs, was not significantly rhythmic in either Bmal1flox (P=0.09) or Bmal1flox/CreTek mice (P=0.9; figure 2D). Some genes associated with both ECs and VSMCs (Pai-1, Icam2) were altered in Bmal1flox/CreTek mice, whereas the 2 VSMC-specific transcripts analyzed (Titin-Cap and Calponin-3) were not rhythmically or differentially expressed in either Bmal1flox or Bmal1flox/CreTek animals (data supplement figure IIIID and data not shown).

Figure 1. CLOCK and NPAS2 affect diurnal variation of and/or absolute TTVO. A, Representative Doppler flow tracings after photochemical injury of WT femoral arteries at either ZT2 (top panel) or ZT8 (bottom panel). Rose bengal (RB) injections and vessel occlusion are marked with arrows. B, Mean TTVO for all groups (2-way ANOVA, P<0.0001 for genotype; 1-way ANOVA, P=0.024 [WT, n=16 to 18], P=0.273 [CLOCKmut, n=10 to 14], and P=0.035 [NPAS2-/-, n=12 to 15]). P<0.05 and *P<0.01 for comparisons within genotype, tP<0.05 and tP<0.01 for comparisons across genotype. C, Combined baseline Doppler blood flow (BF) for all time points (P<0.0001). NPAS2-/- have significantly lower baseline blood flow compared with WT controls (0.154±0.003 and 0.177±0.005 mL/min, n=83 and n=64, respectively; *P<0.01), whereas there was no difference between WT and CLOCKmut (0.180±0.005 mL/min, n=63). No diurnal variation in blood flow across the 4 time points was evident (data not shown).

Depression of Endothelial Bmal1 Affects Thrombogenesis
An augmented thrombotic response to vascular injury at ZT14 compared with ZT8 was confirmed in the Bmal1flox control mice used for this study (36.4±2.6 versus 66.8±7.5 minutes, respectively; P<0.05; figure 3A and 3B). Interest-
ingly, this phenomenon was lost in the Bmal1^fx/fxCreTek animals: Similar TTVO was recorded at ZT8 and ZT14 (32.5 ± 5.3 and 28.9 ± 3.8 minutes, respectively). Moreover, although disruption of either NPAS2 or CLOCK expression led to significantly longer TTVO (Figure 1B), disruption of endothelial BMAL1 expression led to a significantly shorter TTVO at ZT8 (P < 0.01; Figure 3B). Importantly, a shorter TTVO was also observed in global BMAL1 knockout mice (Bmal1^fx/fx) in comparison to Bmal1^fx/fx littermate controls (26.6 ± 4.8 versus 57.2 ± 3.3 minutes, respectively; P < 0.05; Figure 3D). The observed difference in TTVO does not appear to be due to alterations in arterial blood flow because overall baseline blood flow was not significantly different between Bmal1^fx/fx and Bmal1^fx/fxCreTek (Figure 3C).

Depression of Endothelial BMAL1 Alters BP, HR, and Activity Levels but Not Their Diurnal Variation

With the use of telemetry, MAP, HR, and activity were recorded every 5 minutes over 72 hours in Bmal1^fx/fxCreTek and Bmal1^fx/fx littermate controls. A robust diurnal variation in MAP, HR, and activity was apparent in Bmal1^fx/fxCreTek and Bmal1^fx/fx mice (Figure 4A to 4C). MAP data gathered over 3 days was fitted to a 24-hour period harmonic; a percent rhythm to this harmonic was scored as described.30 Bmal1^fx/fx and Bmal1^fx/fxCreTek mice had similar 24-hour period harmonics for MAP (30.1 ± 3.5% versus 28.9 ± 2.3%), HR (26.1 ± 2.9% versus 29.0 ± 3.3%), and activity (25.3 ± 3.4% versus 25.1 ± 1.4%).
MAP, HR, and activity were also increased during the dark cycle relative to the light cycle both in Bmal1fx/fx and Bmal1fx/fxCreTek mice (Figure 4D to 4F; \( P < 0.0001 \)). Bmal1fx/fx CreTek mice had significantly lower MAP at distinct time points within the active phase versus Bmal1fx/fx mice (Figure 4A; \( P < 0.05 \)). MAP was also lower in Bmal1fx/fxCreTek mice during the active phase with a 12-hour average (Figure 4D; \( P < 0.0001 \)). HR was significantly higher in Bmal1fx/fxCreTek mice during both the rest and active phases versus Bmal1fx/fx controls (Figure 4B and 4E; \( P < 0.0001 \)).

Norepinephrine (\( P < 0.05 \)) and epinephrine (\( P < 0.05 \)) were unaltered in Bmal1fx/fxCreTek mice (Table). Although a diurnal variation in total plasma nitrates was evident, no difference was detected between Bmal1fx/fx and Bmal1fx/fxCreTek mice (Table). Activity was significantly reduced in Bmal1fx/fxCreTek mice versus Bmal1fx/fx during both the rest (\( P < 0.05 \)) and active (\( P < 0.0001 \)) phases (Figure 4C and 4F).

**CLOCK Mutation, but Not Endothelial BMAL1 Deficiency, Alters the Fibrinolytic System**

Previous studies have shown an impact of CLOCK31–33 and CRY34 on the circadian oscillation of plasma PAI-1 and its mRNA.15,35,36 Plasma levels of PAI-1 and tPA were measured to determine whether fluctuations in fibrinolytic activity related to the diurnal variation in the thrombotic response to injury. Plasma levels of total PAI-1 and tPA in WT mice displayed diurnal variation (\( P = 0.02 \) and 0.01, respectively) with the opposite phase (Figure 5A and 5B), as previously described.14 Total PAI-1 was significantly increased at ZT8 (0.59±0.10 ng/mL) and ZT14 (1.00±0.14 ng/mL) in comparison to ZT2 (0.17±0.04 ng/mL; \( P < 0.01 \) and 0.001, respectively). Active PAI-1 displayed a similar trend, with significantly higher levels at ZT8 and ZT14, in comparison to ZT2 (Data Supplement Figure IV). tPA, on the other hand, was significantly decreased at ZT14 in comparison to ZT2 (1.81±0.05 versus 2.28±0.16 ng/mL; \( P < 0.05 \)). Oscillations in plasma levels of these enzymes were also assayed in Bmal1fx/fx mice under free-running conditions (Figure 5C and 5D). Although total PAI-1 continued to show a significant rhythm (\( P = 0.004 \)), with increasing levels into late subjective day, tPA failed to attain a significant circadian rhythm (\( P = 0.33 \)). Similar to CLOCKmut, NPAS2+/− mice also had significantly lower plasma PAI-1 levels at ZT14 (\( P < 0.01 \); Figure 5A), although this effect was not reflected in their Pai-1 mRNA expression in aorta, heart, or liver (Data Supplement Figure IV and data not shown). NPAS2+/− also displayed normal plasma tPA at ZT14. Depression of BMAL1 in ECs had no effect on either total plasma PAI-1 or tPA levels (Figure 5C and 5D), whereas global Bmal-deficient animals had significantly lower plasma PAI-1 levels versus control at ZT14 (data not shown).
We report a diurnal variation in response to a thrombogenic stimulus in vivo. The susceptibility to thrombogenesis at both the start of the dark (active) phase and the start of the light (rest) phase is reminiscent in these rodents of the early morning rise in the incidence of thrombotic events in humans, with a secondary rise occurring 12 hours later. Ideally, a diurnal rhythm would be defined by 4 measurements over repeated days in an experimental paradigm, whereas here the observations were made over a single 24-hour period. Although the rhythm contains 1 peak value at ZT8, a corresponding trough at ZT20 was not evident. This may be due in part to the limit of detection for this particular model. Despite these limitations, the data presented here represent the first evidence for a diurnal variability in thrombogenicity in vivo in a model system; reassuringly, it mimics the temporal incidence of thrombotic cardiovascular events in humans.

Further experiments revealed the importance of CLOCK and BMAL1 in the maintenance of this diurnal variation in thrombogenesis. Mutation or depression of these core clock components in either the whole animal (CLOCKmut) or endothelium alone (Bmal1fx/fxCreTek) resulted in loss of the temporal pattern in susceptibility to thrombotic vascular occlusion, whereas loss of NPAS2 had no effect on diurnal variation in TTVO. Surprisingly, we observed a differential impact of the clock components on the functional response: The TTVO was prolonged in CLOCKmut at a single time point and at almost all time points in NPAS2−/− mice, whereas it was shortened when BMAL1 was

**Discussion**

We report a diurnal variation in response to a thrombogenic stimulus in vivo. The susceptibility to thrombogenesis at both the start of the dark (active) phase and the start of the light (rest) phase is reminiscent in these rodents of the early morning rise in the incidence of thrombotic events in humans, with a secondary rise occurring ~12 hours later. Ideally, a diurnal rhythm would be defined by >4 measurements over repeated days in an experimental paradigm, whereas here the observations were made over a single 24-hour period. Although the rhythm contains 1 peak value at ZT8, a corresponding trough at ZT20 was not evident. This may be due in part to the limit of detection for this particular model. Despite these limitations, the data presented here represent the first evidence for a diurnal variability in thrombogenicity in vivo in a model system; reassuringly, it mimics the temporal incidence of thrombotic cardiovascular events in humans.

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deleted either selectively in ECs or globally. Interestingly, shortened TTVO in Bmal1<sup>fx/fx</sup>CreTek or Bmal1-null mice corresponded with enhanced Npas2 expression in vascular cells. Previously, we have observed distinct time-dependent and -independent influences of clock components on BP. Further experiments will elucidate the mechanism by which these discrete transcription factors impinge on thrombogenesis and to what extent their effects, either direct or indirect, may be distinct from their roles within the circadian oscillator.

The elements of an oscillatory network are widely expressed in peripheral tissues, and here we document their presence in ECs. Challenging ECs in culture with serum derived predominantly from platelets under physiological conditions, possibly the liver, because we extend these observations by implicating Npas2 along with CLOCK as a regulator of PAI-1. The dominant source of platelet count have been reported to oscillate in some but not all studies. Given that platelets are anucleate fragments of megakaryocytes, they may not contain the necessary components for a transcriptionally dependent molecular clock. Neither platelet activation nor platelet aggregation in WT mouse whole blood ex vivo displayed diurnal variation in the present studies. However, a limitation of this and the similar approaches applied in humans is that ex vivo analysis of platelet function may not mimic platelet behavior in vivo. Parenthetically, excretion of the major 11-dehydro metabolite of thromboxane B<sub>2</sub>, which derives predominantly from platelets under physiological conditions in vivo, does not vary in a circadian fashion in healthy volunteers (G.A. FitzGerald, MD, unpublished data, 2008).

Time-dependent oscillation in elements of the fibrinolytic system has been reported previously; both PAI-1 and, to a lesser extent, tPA are subject to diurnal variation. Our data extend these observations by implicating Npas2 along with CLOCK as a regulator of PAI-1. The dominant source of circadian regulation of fibrinolysis appears to reside in a tissue other than the endothelium, possibly the liver, because we found that depression of endothelial Bmal1 failed to affect

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**Table.** Depression of Endothelial Bmal1 Does Not Alter Sympathoadrenal Function or Nitric Oxide Biosynthesis

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Values are mean±SEM in ng/mL (norepinephrine and epinephrine; n=6 to 8) or μmol/L (nitrate + nitrite; n=8 to 11).

*P<0.001 vs corresponding ZT3 value.

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**Figure 5.** The effect of circadian clock components CLOCK, Npas2, and Bmal1 on plasma levels of PAI-1 and tPA. A. Mean plasma PAI-1 (P<0.001, n=8 to 16).

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levels of either PAI-1 or tPA. Oscillations in the fibrinolytic system were not fully concordant with the diurnal variation in thrombogenesis observed in the present study or, indeed, in humans.\textsuperscript{11,12} Although the changes in PAI-1 may predispose to the increased thrombogenesis at the start of the dark cycle, corresponding to the early morning in humans, they fail to replicate the secondary predisposition to thrombogenesis 12 hours later, at the start of the light cycle in the present studies.

BP displays a well-defined circadian rhythmicity that might be expected to interact with thrombogenesis in conditioning the temporal variability in cardiovascular events. We have shown previously that BMAL1, CLOCK, and NPAS2 all influence BP and/or its circadian rhythmicity in mice.\textsuperscript{17} In those studies, global deletion of BMAL1 completely abolished the rhythm in BP while also resulting in a hypotensive phenotype independent of time. Here we found that restricted depression of BMAL1 did not disrupt the temporal oscillation in BP. However, it depressed pressure during the active phase. Although HR was elevated, suggesting activation of the baroreflex, we did not detect an alteration in plasma catecholamines. Similarly, the diurnal variation in plasma nitrates/nitrites was unaltered. It is possible that the changes in BP are secondary to the unexpected impact of endothelial BMAL1 deletion on activity. Further studies will be necessary to elucidate the mechanism that underlies this observation.

In conclusion, we provide the first evidence that thrombogenicity in vivo is subject to diurnal variation. Disruptions of genes that are intrinsic to the core oscillator—CLOCK, NPAS2, and BMAL1—impinge on this phenomenon. Recently, Gauguier and colleagues\textsuperscript{46} associated gene variants in BMAL1 with susceptibility to hypertension and diabetes, consistent with previous observations involving this and other clock components in mice.\textsuperscript{17,47} The present observations suggest that the molecular clock may interact with environmental variables, such as stress,\textsuperscript{17} to regulate cardiovascular risk by influencing not only hypertension but also thrombosis and perhaps other aspects of cardiovascular dysfunction.

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Disclosures
None.

References
Clinical cardiovascular events such as myocardial infarction and stroke undergo diurnal variation, but it has been unclear whether this reflects a role for the molecular clock or temporal variation in exposure to environmental triggers. Studies of platelet aggregation ex vivo may be confounded by selection of subpopulations because of platelet activation in vivo. Here we describe diurnal variation in the time to thrombotic vascular occlusion in vivo, subsequent to a photochemical vascular injury. This pattern was disrupted or altered when circadian clock genes (Bmal1, Clock, or Npas2) were either mutated or deleted, manipulations that also affect blood pressure. Selective deletion of a single circadian clock gene, Bmal1, from the endothelium alone was sufficient to alter thrombogenesis in vivo. The impact of molecular clock components on thrombogenesis and blood pressure may interact with environmental variables to contribute to the diurnal variation in cardiovascular events observed in humans.
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