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## 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  $24.6 \pm 2.7$  minutes at zeitgeber time (ZT) 2 to  $40.3 \pm 4.3$  minutes at ZT8,  $24.3 \pm 2.3$  minutes at ZT14, and  $31.0 \pm 4.4$  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,<sup>1</sup> and autonomous peripheral oscillators have been defined for most tissues, including the vasculature, heart, and kidney.<sup>2-4</sup> 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).<sup>3,5</sup> The molecular oscillator is composed of interlocking positive and negative transcriptional and translational feedback loops that drive circadian gene expression.<sup>6</sup> Heterodimers of the bHLH-PAS transcription factors CLOCK and/or NPAS2 with BMAL1 constitute the positive limb of this feedback loop,<sup>7</sup> 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.<sup>6,8</sup> A second loop involves the circadian regulation of *Bmal1* transcription by REV-ERB $\alpha$ <sup>9</sup> and ROR $\alpha$ .<sup>10</sup>

### Clinical Perspective p 2095

Aspects of both cardiovascular physiology and the clinical manifestation of cardiovascular disease display diurnal vari-

ation. 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.<sup>11,12</sup> The timing of adverse cardiovascular events corresponds to oscillations in gene and protein expression of known regulators of hemostasis<sup>13,14</sup>; an example is plasminogen activator inhibitor-1 (PAI-1),<sup>15</sup> a time-independent risk factor for cardiovascular disease.<sup>16</sup> Cassettes of genes relevant to vascular injury and integrity have been shown to oscillate in mouse aorta, as does vascular leakage.<sup>2</sup> We have recently implicated the molecular clock in regulating circadian variation in BP and the response to asynchronous stress.<sup>17</sup> 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. *Bmal1*<sup>fl/fl</sup> mice were crossed with Cre<sup>tek</sup> transgenic mice<sup>18</sup> to generate either *Bmal1*<sup>fl/fl</sup>Cre<sup>tek</sup> or *Bmal1*<sup>fl/fl</sup> littermate controls, kindly provided by Dr Chris Bradfield at the University of

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From the Institute for Translational Medicine and Therapeutics, School of Medicine, University of Pennsylvania, Philadelphia (E.J.W., Y.C., D.F.R., T.S.P., G.A.F.); and McArdle Laboratory for Cancer Research, University of Wisconsin School of Medicine and Public Health, Madison (J.A.W., C.A.B.).

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Correspondence to Garret A. FitzGerald, MD, 153 Johnson Pavilion, 3620 Hamilton Walk, Philadelphia, PA 19104. E-mail garret@spirit.gcr.upenn.edu

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Wisconsin; mice were backcrossed to C57BL/6J to the N4 generation. Global Bmal1 knockout mice (Bmal1<sup>-/-</sup>) were compared with Bmal1<sup>+/+</sup> (wild-type [WT]) littermate controls. Fully backcrossed CLOCK<sup>mut</sup> and NPAS2<sup>-/-</sup> 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<sup>fx/fx</sup>Cre<sup>Tek</sup> 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 (CLOCK<sup>mut</sup>, NPAS2<sup>-/-</sup>, and WT, n=10 to 18; Bmal1<sup>-/-</sup> and Bmal1<sup>+/+</sup>, n=4 to 5) or 14 to 17 weeks (Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> and Bmal1<sup>fx/fx</sup>, n=10) with the use of a photochemical injury model described previously.<sup>19</sup> Slight modifications in anesthesia (ketamine, 100 mg/kg; xylazine, 20 mg/kg) and transillumination (543.5-nm laser; 5 mm from vessel) were used. The time to vessel occlusion (TTVO) was measured as the time between rose bengal injection (Acros 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.<sup>20</sup> 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.<sup>21</sup> Circadian rhythm analysis of the individual hourly MAP and HR data was performed as described.<sup>17</sup> Time-series analyses were conducted with the use of generalized estimating equations,<sup>22</sup> as implemented in the package Geepack version 1.0.10 for R-2.4.1 ([www.cran.r-project.org/](http://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 described<sup>17</sup> in male mice aged 10 to 15 weeks (CLOCK<sup>mut</sup>, NPAS2<sup>-/-</sup>, and WT, n=7 to 9) or 18 to 20 weeks (Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> and Bmal1<sup>fx/fx</sup>, n=3 to 6). Total RNA was isolated from tissues with an RNeasy Mini-Kit (Qiagen) and from cells as described.<sup>23</sup> Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR were performed as described,<sup>23</sup> 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 ANOVA 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

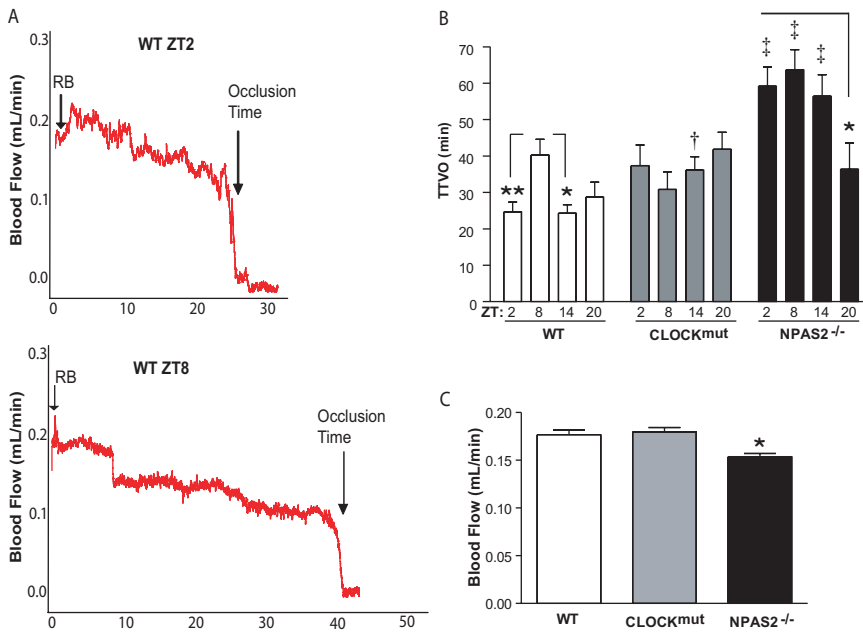
With the use of a photochemical injury model in the mouse femoral artery, mean TTVO was recorded at 4 ZTs: ZT2, ZT8, ZT14, and ZT20 in CLOCK<sup>mut</sup> and NPAS2<sup>-/-</sup> mice and WT controls. A diurnal variation in TTVO was observed in WT mice (Figure 1A and 1B; *P*=0.02), with significantly 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.<sup>13,24,25</sup> 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 CLOCK<sup>mut</sup> 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<sup>-/-</sup> 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: CLOCK<sup>mut</sup> mice had a small but significant increase in TTVO at ZT14 compared with WT (*P*<0.05), whereas NPAS2<sup>-/-</sup> 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. CLOCK<sup>mut</sup> mice, on the other hand, retained normal baseline blood flow, in accordance with their normotensive phenotype<sup>17</sup> and their more subtle effect on absolute TTVO.

### Depression of Endothelial BMAL1 Alters Gene Expression in the Vasculature

Endothelial cells (ECs) express key components of the molecular oscillator (Data Supplement Figure IIA); however,



**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  $18$ ],  $P = 0.273$  [CLOCK<sup>mut</sup>,  $n = 10$  to  $14$ ], and  $P = 0.035$  [NPAS2<sup>-/-</sup>,  $n = 12$  to  $15$ ]). \* $P < 0.05$  and \*\* $P < 0.01$  for comparisons within genotype, † $P < 0.05$  and ‡ $P < 0.01$  for comparisons across genotype. C, Combined baseline Doppler blood flow (BF) for all time points ( $P < 0.0001$ ). NPAS2<sup>-/-</sup> have significantly lower baseline blood flow compared with WT controls ( $0.154 \pm 0.003$  and  $0.177 \pm 0.005$  mL/min,  $n = 83$  and  $n = 64$ , respectively; \* $P < 0.01$ ), whereas there was no difference between WT and CLOCK<sup>mut</sup> ( $0.180 \pm 0.005$  mL/min,  $n = 63$ ). No diurnal variation in blood flow across the 4 time points was evident (data not shown).

these cells, when isolated, did not display rhythmic expression of oscillator components after serum shock (Data Supplement Figure IIB through IIF), which is effective in other cell types such as vascular smooth muscle cells (VSMCs)<sup>3,17</sup> and fibroblasts.<sup>26</sup> We analyzed tissues from Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> and Bmal1<sup>fx/fx</sup> mice for the presence of both the Bmal1<sup>fx</sup>-unexcised and Bmal1<sup>fx</sup>-excised alleles, as described previously.<sup>27</sup> In the absence of Cre<sup>Tek</sup>, the Bmal1<sup>fx/fx</sup> mice revealed only the Bmal1<sup>fx</sup>-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 Tek<sup>Cre</sup> is also expressed in cells of hematopoietic lineage,<sup>18</sup> we found the presence of both the Bmal1<sup>fx</sup>-unexcised and Bmal1<sup>fx</sup>-excised alleles in all organs examined from Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> mice. However, the Bmal1<sup>fx</sup>-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,<sup>28</sup> Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> mice are of normal body weight and have normal complete blood counts (data not shown); they appear to be otherwise healthy.

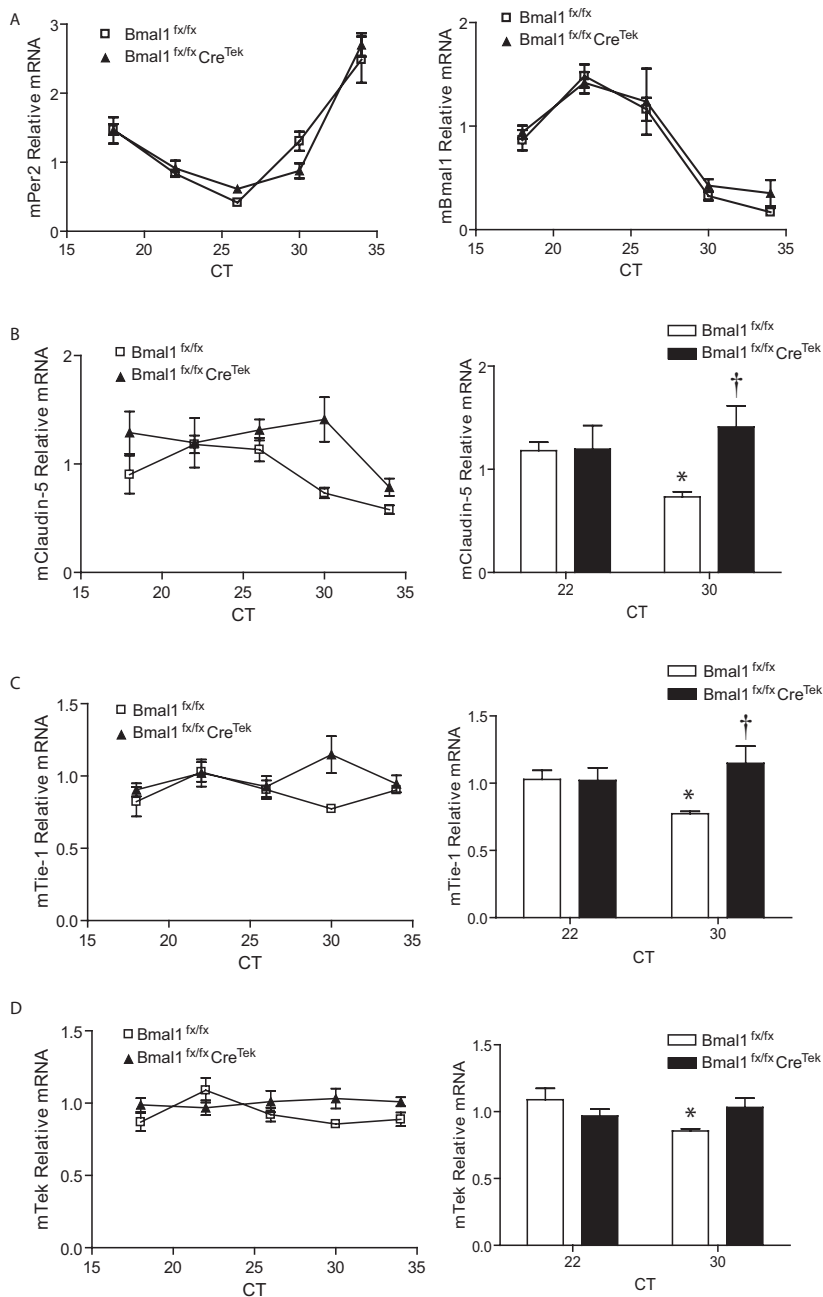
Gene expression in mouse aortas harvested from Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> and Bmal1<sup>fx/fx</sup> control mice every 4 hours for 16 hours in DD was analyzed by real-time PCR for transcripts previously determined to be transcribed rhythmically in this tissue.<sup>2</sup> Clock genes *Per2* and *Bmal1* were both rhythmically expressed in Bmal1<sup>fx/fx</sup> and Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> 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 rhythmic expression in Bmal1<sup>fx/fx</sup> ( $P = 0.02$  and  $P = 0.03$ , respectively) but not Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> 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 Bmal1<sup>fx/fx</sup> ( $P = 0.09$ ) or Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> mice ( $P = 0.9$ ; Figure 2D). Some

genes associated with both ECs and VSMCs (*Pai-1*, *Icam2*) were altered in Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> mice, whereas the 2 VSMC-specific transcripts analyzed (*Titin-Cap* and *Calponin-3*) were not rhythmically or differentially expressed in either Bmal1<sup>fx/fx</sup> or Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> animals (Data Supplement Figure IIID and data not shown).

Gene expression in lung ECs and VSMCs harvested from Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> and Bmal1<sup>fx/fx</sup> controls was analyzed by real-time PCR. *Bmal1* expression was lower by  $\approx 40\%$  in ECs from Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> versus Bmal1<sup>fx/fx</sup> ( $P < 0.05$ ), whereas expression was unaltered in VSMCs (Data Supplement Figure IIIB). Importantly, depression of *Bmal1* did not alter expression of *Clif* (*Bmal2*) in ECs or VSMCs or in whole aortas harvested from Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> mice (Data Supplement Figure IIIB and IIID). Interestingly, depression of *Bmal1* expression specifically in ECs coincided with a marked increase in *Npas2* expression ( $P < 0.05$ ), as we have previously reported in Bmal1-null aortas.<sup>17</sup> Increased *Per2* expression was also observed in isolated ECs ( $P < 0.05$ ) but not in VSMCs or whole aortas (Data Supplement Figure IIIB and Figure 2A). Changes in EC circadian gene expression did not correspond with alterations in expression of output genes *Pai-1* or *Thrombomodulin* (*Thbd*)<sup>29</sup> (Data Supplement Figure IIIC), although subtle changes in *Pai-1* expression were observed in whole aorta (Data Supplement Figure IIID). Circadian oscillations of *Thbd* have been reported in the lung and heart,<sup>29</sup> but no significant oscillation was observed in whole aortas from either Bmal1<sup>fx/fx</sup> ( $P = 0.150$ ) or Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup> mice ( $P = 0.413$ ; Data Supplement Figure IIID).

### Depression of Endothelial BMAL1 Affects Thrombogenesis

An augmented thrombotic response to vascular injury at ZT14 compared with ZT8 was confirmed in the Bmal1<sup>fx/fx</sup> control mice used for this study ( $36.4 \pm 2.6$  versus  $66.8 \pm 7.5$  minutes, respectively;  $P < 0.05$ ; Figure 3A and 3B). Interest-

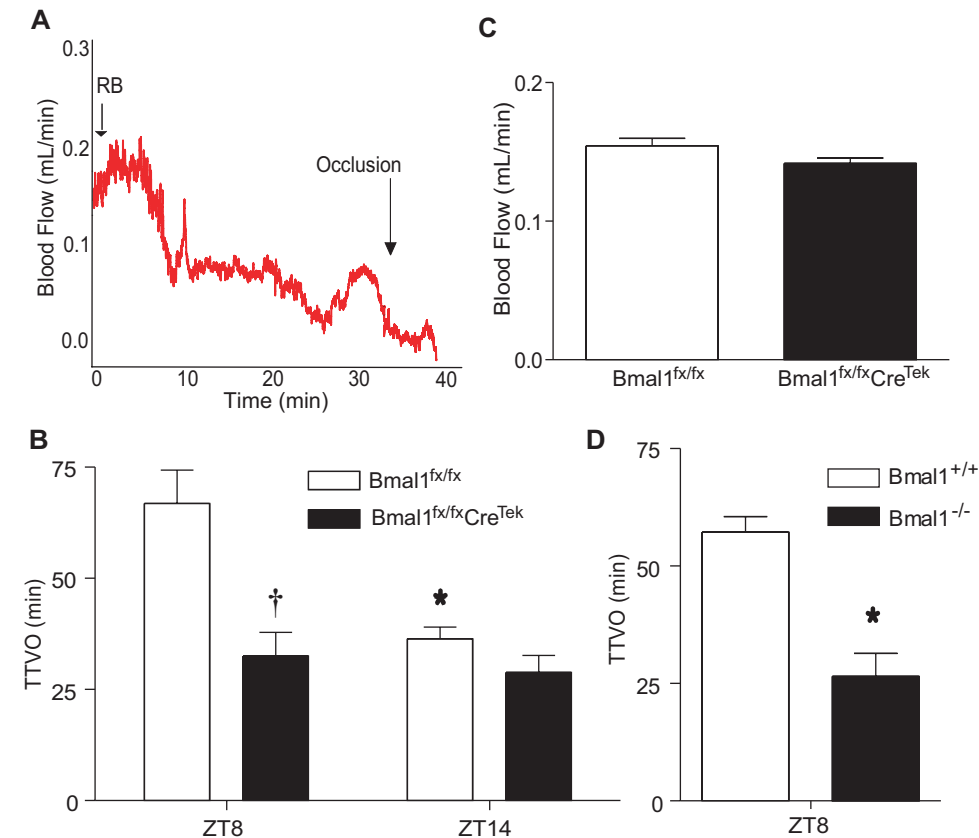


**Figure 2.** Endothelial BMAL1 regulates the expression of endothelial-associated genes. Real-time PCR analysis of aortas harvested from Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> mice and their Bmal1<sup>fl/fl</sup> littermate controls at 4-hour intervals from CT18-CT34. A, *mPer2* (left panel) and *mBmal1* (right panel) displayed similar rhythmic profiles in Bmal1<sup>fl/fl</sup> ( $P < 0.0001$  both genes) and Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> mice ( $P = 0.007$ , *mPer2* and  $P = 0.009$ , *mBmal1*). B, *mClaudin-5* displayed rhythmic expression (left panel) in Bmal1<sup>fl/fl</sup> ( $P = 0.022$ ) but not Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> ( $P = 0.215$ ). Analysis of CT22 vs CT30 (right panel);  $*P < 0.05$  for Bmal1<sup>fl/fl</sup> CT22 vs CT30,  $\dagger P < 0.01$  for Bmal1<sup>fl/fl</sup> vs Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> at CT30. C, *mTie-1* displayed rhythmic expression (left panel) in Bmal1<sup>fl/fl</sup> ( $P = 0.032$ ) but not Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> ( $P = 0.323$ ). Analysis of CT22 vs CT30 (right panel);  $*P < 0.01$  for Bmal1<sup>fl/fl</sup> CT22 vs CT30,  $\dagger P < 0.001$  for Bmal1<sup>fl/fl</sup> vs Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> at CT30. D, *mTek* did not display rhythmic expression (left panel) in Bmal1<sup>fl/fl</sup> ( $P = 0.086$ ) or Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> ( $P = 0.924$ ). Analysis of CT22 vs CT30 (right panel);  $P = 0.044$ ,  $*P < 0.05$  for Bmal1<sup>fl/fl</sup> CT22 vs CT30. Relative mRNA was calculated with the use of  $\beta$ -actin expression ( $n = 3$  to 6, all genes).

ingly, this phenomenon was lost in the Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> animals: Similar TTVO was recorded at ZT8 and ZT14 ( $32.5 \pm 5.3$  and  $28.9 \pm 3.8$  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<sup>-/-</sup>) in comparison to Bmal1<sup>+/+</sup> littermate controls ( $26.6 \pm 4.8$  versus  $57.2 \pm 3.3$ , 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<sup>fl/fl</sup> and Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> (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<sup>fl/fl</sup>Cre<sup>Tek</sup> and Bmal1<sup>fl/fl</sup> littermate controls. A robust diurnal variation in MAP, HR, and activity was apparent in Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> and Bmal1<sup>fl/fl</sup> 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.<sup>30</sup> Bmal1<sup>fl/fl</sup> and Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> mice had similar 24-hour period harmonics for MAP ( $30.1 \pm 3.5\%$  versus  $28.9 \pm 2.3\%$ ), HR ( $26.1 \pm 2.9\%$  versus  $29.0 \pm 3.3\%$ ), and activity ( $25.3 \pm 3.4\%$  versus  $25.1 \pm 1.4\%$ ).



**Figure 3.** Depression of endothelial BMAL1 affects diurnal variation of and absolute TTVO. A, Representative Doppler flow tracing after photochemical injury of a  $Bmal1^{fx/fx}Cre^{Tek}$  femoral artery at ZT8. Rose bengal injection and vessel occlusion are marked with arrows. B, Mean TTVO ( $n=10$ ;  $P=0.007$ ). \* $P<0.05$  for  $Bmal1^{fx/fx}$  ZT8 vs ZT14. † $P<0.01$  for  $Bmal1^{fx/fx}Cre^{Tek}$  vs  $Bmal1^{fx/fx}$  at ZT8. C, Combined baseline Doppler blood flow for  $Bmal1^{fx/fx}$  and  $Bmal1^{fx/fx}Cre^{Tek}$  mice ( $n=23$  to 25). D, Mean TTVO for  $Bmal1^{+/+}$  and  $Bmal1^{-/-}$  at ZT8 ( $n=4$  to 5; \* $P<0.05$ ).

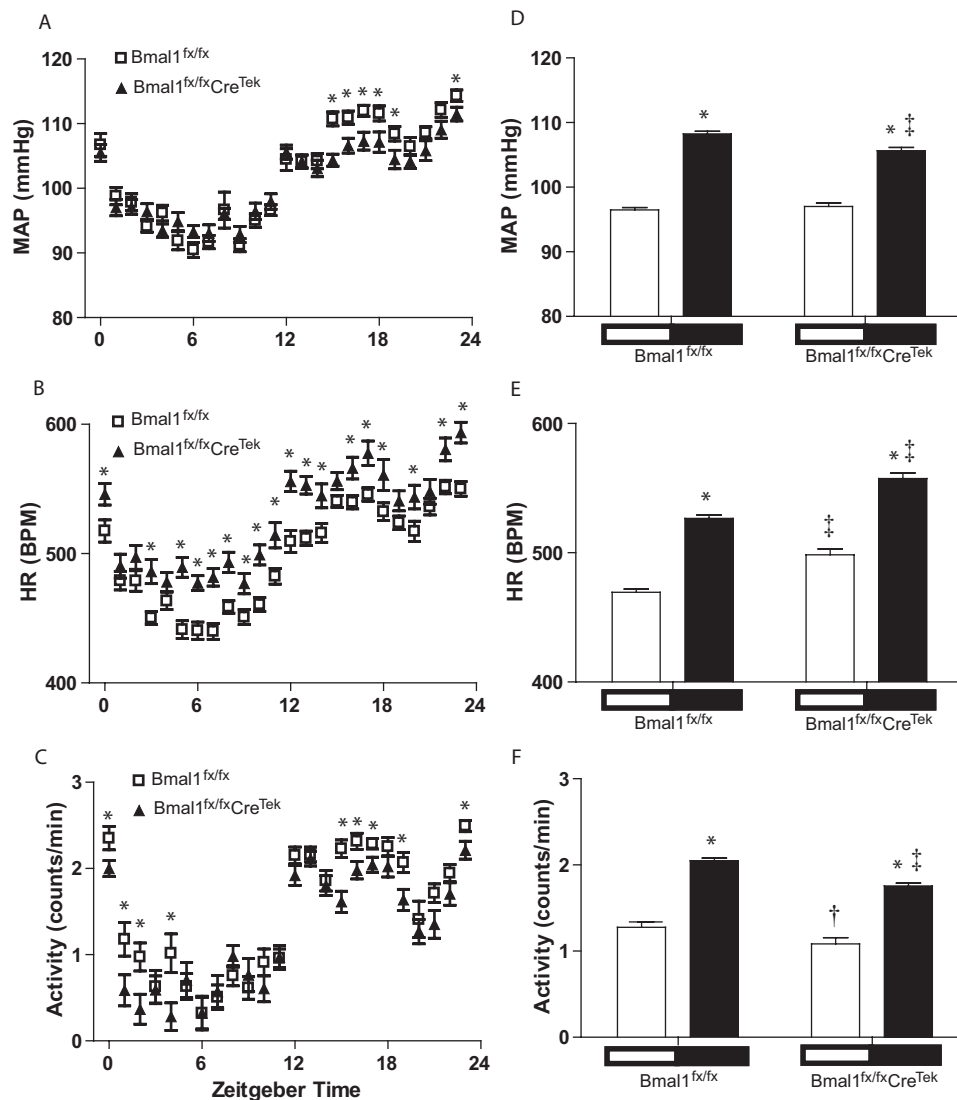
MAP, HR, and activity were also increased during the dark cycle relative to the light cycle<sup>20</sup> in both  $Bmal1^{fx/fx}$  and  $Bmal1^{fx/fx}Cre^{Tek}$  mice (Figure 4D to 4F;  $P<0.0001$ ).  $Bmal1^{fx/fx}Cre^{Tek}$  mice had significantly lower MAP at distinct time points within the active phase versus  $Bmal1^{fx/fx}$  mice (Figure 4A;  $P<0.05$ ). MAP was also lower in  $Bmal1^{fx/fx}Cre^{Tek}$  mice during the active phase with a 12-hour average (Figure 4D;  $P<0.0001$ ). HR was significantly higher in  $Bmal1^{fx/fx}Cre^{Tek}$  mice during both the rest and active phases versus  $Bmal1^{fx/fx}$  controls (Figure 4B and 4E;  $P<0.0001$ ).

Norepinephrine ( $P<0.05$ ) and epinephrine ( $P<0.05$ ) were unaltered in  $Bmal1^{fx/fx}Cre^{Tek}$  mice (Table). Although a diurnal variation in total plasma nitrates was evident, no difference was detected between  $Bmal1^{fx/fx}$  and  $Bmal1^{fx/fx}Cre^{Tek}$  mice (Table). Activity was significantly reduced in  $Bmal1^{fx/fx}Cre^{Tek}$  mice versus  $Bmal1^{fx/fx}$  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 CLOCK<sup>31–33</sup> and CRY<sup>34</sup> on the circadian oscillation of plasma PAI-1 and its mRNA.<sup>15,35,36</sup> 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.<sup>14</sup> Total PAI-1 was significantly increased at ZT8 ( $0.59\pm 0.10$  ng/mL) and ZT14 ( $1.00\pm 0.14$  ng/mL) in comparison to ZT2 ( $0.17\pm 0.04$  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 ( $1.81\pm 0.05$  versus  $2.28\pm 0.16$  ng/mL;  $P<0.05$ ). Oscillations in plasma levels of these enzymes were also assayed in  $Bmal1^{fx/fx}$  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 CLOCK<sup>mut</sup>, NPAS2<sup>-/-</sup> 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<sup>-/-</sup> 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 *Bmal1*-deficient animals had significantly lower plasma PAI-1 levels versus control at ZT14 (data not shown).



**Figure 4.** Depression of endothelial BMAL1 alters BP, HR, and activity. Telemetric recordings for MAP (A), HR (B), and activity (C) averaged each hour from 3 consecutive 24-hour periods in *Bmal1<sup>fx/fx</sup>* ( $n=10$ ) and *Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup>* ( $n=8$ ). Values are plotted at ZT, where ZT0–12 is lights on (rest phase) and ZT12–24 is lights off (active phase). A, MAP was significantly lower in *Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup>* at distinct time points during the active phase ( $*P<0.05$ ). B, HR was significantly higher in *Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup>* during rest and active phases ( $*P<0.05$ ). C, Activity was significantly lower in *Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup>* at distinct time points within rest and active phases ( $*P<0.05$ ). Telemetric recordings taken separately during rest and active phases for MAP (D), HR (E), and activity (F) in *Bmal1<sup>fx/fx</sup>* vs *Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup>*. MAP, HR, and activity were significantly higher during active vs rest phases for both genotypes (D, E, F,  $*P<0.0001$ ). D, MAP was significantly lower in *Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup>* vs *Bmal1<sup>fx/fx</sup>* during the active phase ( $\ddagger P<0.0001$ ). E, HR was significantly higher in *Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup>* vs *Bmal1<sup>fx/fx</sup>* during rest and active phases ( $\ddagger P<0.0001$ ). F, Activity was significantly lower in *Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup>* vs *Bmal1<sup>fx/fx</sup>* during rest ( $\dagger P<0.05$ ) and active ( $\ddagger P<0.0001$ ) phases. White boxes indicate lights on (rest phase); dark boxes, lights off (active phase).

## 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  $\approx 12$  hours later.<sup>13</sup> 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 (CLOCK<sup>mut</sup>) or endothelium alone (*Bmal1<sup>fx/fx</sup>Cre<sup>Tek</sup>*) 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 CLOCK<sup>mut</sup> at a single time point and at almost all time points in NPAS2<sup>-/-</sup> mice, whereas it was shortened when BMAL1 was

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

	ZT	Bmal1 <sup>fl/fl</sup>	Bmal1 <sup>fl/fl</sup> Cre <sup>Tek</sup>
Norepinephrine	3	1.5±0.5	1.7±0.4
Norepinephrine	15	1.7±0.6	3.0±0.8
Epinephrine	3	4.8±0.7	5.6±0.9
Epinephrine	15	9.6±2.2	7.3±1.0
Nitrate+nitrite	3	1.9±0.2	1.6±0.2
Nitrate+nitrite	15	5.0±0.7*	3.9±0.3*

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.

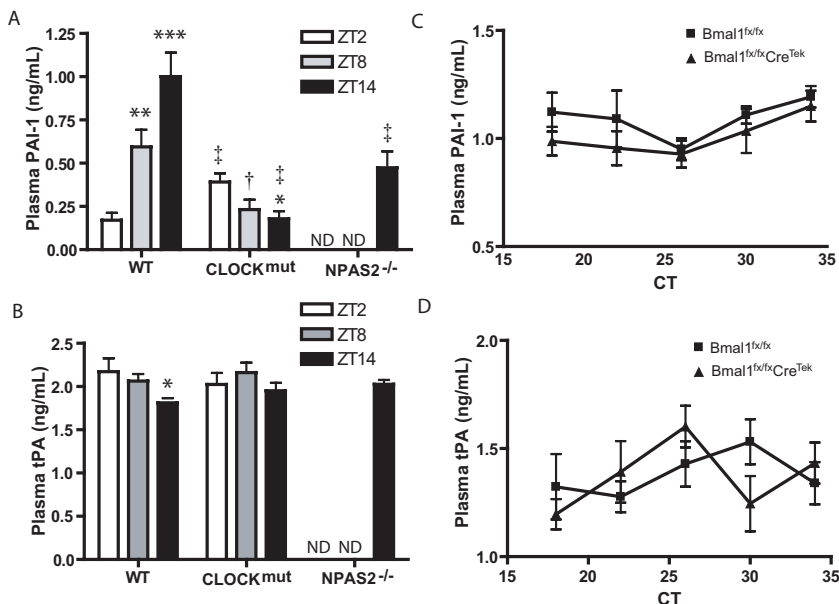
deleted either selectively in ECs or globally. Interestingly, shortened TTVO in Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> or Bmal1-null mice corresponded with enhanced *Npas2* expression in vascular cells.<sup>17</sup> Previously, we have observed distinct time-dependent and -independent influences of clock components on BP.<sup>17</sup> 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 shock, in contrast to other cell types, such as fibroblasts<sup>26</sup> or VSMCs,<sup>3,17</sup> failed to induce oscillatory gene expression in our hands. Recently, Takeda et al<sup>29</sup> reported low-amplitude rhythms in gene expression in cultured human umbilical vein ECs by Northern blot analysis. Depression of BMAL1 in ECs disrupts oscillatory expression of EC-specific genes such as *Claudin-5*, *Tie-1*, and *Tek* ex vivo, whereas oscillation of VSMC-specific genes or core clock components shared among many cell types in aortic tissue, such as *Per2* and *Bmal1*, is retained. Alterations in the core clock components *Bmal1*, *Npas2*, and *Per2* became evident in ECs when

analyzed in isolation. The depression of Bmal1 expression by ≈40% in Bmal1<sup>fl/fl</sup>Cre<sup>Tek</sup> mice is likely an underestimation of EC gene deletion because the isolation procedure from mouse lung did not produce a completely pure population of ECs. Although these observations are suggestive, they do not define an autonomous oscillator in the vascular endothelium. Perhaps more frequent sampling would have permitted detection more definitively of endothelial gene oscillations. On the one hand, rather than retaining an intrinsic rhythm ex vivo, ECs may be supported in vivo by rhythmic systemic physical and/or hormonal signals.<sup>37</sup> Alternatively, they may be subject to input from other cycling cell types, such as VSMCs, and they may not themselves be capable of driving rhythmic gene expression (as our in vitro data might suggest).

Multiple mechanisms are likely to impinge on diurnal variation in thrombogenesis. Indices of human platelet function and platelet count have been reported to oscillate in some but not all studies.<sup>38–42</sup> Given that platelets are anucleate fragments of megakaryocytes,<sup>43</sup> 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.<sup>44</sup> Parenthetically, excretion of the major 11-dehydro metabolite of thromboxane B<sub>2</sub>, which derives predominantly from platelets under physiological conditions in vivo,<sup>45</sup> 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<sup>14</sup>; 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,<sup>33</sup> because we found that depression of endothelial BMAL1 failed to affect



**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 ( $P<0.0001$ ,  $n=8$  to 16). \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  for comparisons vs ZT2 within genotypes. † $P<0.05$ , ‡ $P<0.01$  for comparisons across genotypes. B, Mean plasma tPA ( $P<0.05$ ,  $n=8$  to 16). \* $P<0.05$  for WT ZT2 vs ZT14. C, Mean plasma PAI-1 ( $P=0.004$  for CT,  $n=3$  to 6). D, Mean plasma tPA ( $P=0.254$ ,  $n=3$  to 6). Data shown are mean±SEM. ND indicates not detected.

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.<sup>11,12</sup> 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.<sup>17</sup> 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<sup>46</sup> associated gene variants in BMAL1 with susceptibility to hypertension and diabetes, consistent with previous observations involving this and other clock components in mice.<sup>17,47</sup> The present observations suggest that the molecular clock may interact with environmental variables, such as stress,<sup>17</sup> 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.

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### CLINICAL PERSPECTIVE

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.