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The molecular clock regulates circadian transcription of tissue factor gene

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ABSTRACT

Tissue factor (TF) is involved in endotoxin-induced inflammation and mortality. We found that the circadian expression of *TF* mRNA, which peaked at the day to night transition (activity onset), was damped in the liver of *Clock* mutant mice. Luciferase reporter and chromatin immunoprecipitation analyses using embryonic fibroblasts derived from wild-type or *Clock* mutant mice showed that CLOCK is involved in transcription of the *TF* gene. Furthermore, the results of real-time luciferase reporter experiments revealed that the circadian expression of *TF* mRNA is regulated by clock molecules through a cell-autonomous mechanism via an E-box element located in the promoter region.

1. Introduction

The frequency of thromboembolic events such as pulmonary embolism, cerebral infarction and acute myocardial infarction peaks between morning and noon in an apparently circadian fashion [1,2]. Fluctuations in the levels of coagulation factors that influence the hemostatic balance might contribute to these adverse outcomes. Levels of factor VII (FVII), FVIII, proteins C and S, antithrombin and plasminogen activator inhibitor 1 (PAI-1) fluctuate in humans and rodents with circadian rhythms [3].

The basic helix–loop–helix (bHLH)-PAS transcription factors CLOCK and BMAL1 are positive regulators of the autoregulatory transcription-translation feedback loops of molecular circadian clocks that are located in most tissues [4]. CLOCK and BMAL1 heterodimerize and transactivate other clock genes such as *Per1*, *Per2*, *Cry1* and *Cry2*, and downstream clock-controlled genes via E-box (CACGTG) elements in their promoters [4]. CLOCK (or its ortholog NPAS2) and BMAL1 (or its ortholog BMAL2) are involved in the circadian transcription of FVII [5], PAI-1 [6], and TM [7] genes.

Tissue factor (TF) initiates the primary step of the coagulation cascade and also impacts non-hemostatic processes, such as atherosclerosis, primary tumor growth and metastasis [8]. We recently demonstrated that mRNA expression of the *TF* gene in mouse tissues is circadian [9] and the present study examines the molecular mechanisms involved in this process.

2. Materials and methods

2.1. Animals

Male Jcl:ICR (Clea Japan Inc., Tokyo, Japan) and homozygous *Clock* mutant mice on a Jcl:ICR background [10] at 7–8 weeks of age were maintained under a 12:12 h light–dark cycle (lights on at Zeitgeber time 0 (ZT 0) and lights off at ZT 12) for at least 2 weeks before the day of experimentation. Animal care, handling and experimentation proceeded under the approval of our institutional Animal Care and Use Committee (Permission #2009-020).

2.2. Quantitative reverse transcription (RT)-PCR

Total RNA was extracted using RNAiso (Takara Bio Inc., Otsu, Japan). Single-stranded cDNA was synthesized using PrimeScript™ RT reagent kits with gDNA Eraser (Takara Bio Inc., Otsu, Japan). Real-time RT-PCR proceeded using SYBR® Premix Ex Taq™ II (Takara Bio Inc., Otsu, Japan) and a LightCycler™ (Roche Diagnostics, Mannheim, Germany). The reaction conditions were 95 °C for 10 s followed by 45 cycles of 95 °C for 5 s, 57 °C for 10 s and 72 °C for 10 s. The primer sequences were as described [9]. The amount of target mRNA was normalized relative to that of 18S rRNA.

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2.3. Cell cultures

We maintained embryonic fibroblasts prepared from wild-type and homozygous *Clock* mutant mice in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich St. Louis, MO, USA) supplemented with 10% FBS (SAFC Biosciences, Lenexa, KN, USA) at 37 °C under a humidified 5% CO_2 atmosphere.

2.4. Construction of reporter and expression vectors

The mouse *TF* promoter region spanning −1020 to +80 (numbers are distances in base pairs from the putative transcription start site, +1) was amplified by PCR and the product was ligated into the pGL4.12-Basic luciferase reporter vector (Promega). Expression vectors for mouse CLOCK, dominant negative CLOCK protein (CLOCKΔ19), BMAL1, PER2, and CRY1 were constructed using a cDNA generated from the mouse liver RNA by RT-PCR. All coding regions were ligated into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA, USA).

2.5. Luciferase reporter assays

We seeded fibroblasts prepared from wild-type or *Clock* mutant mice at a density of 1×10^5/well in 24-well culture plates. The cells were transfected 18 h later with 100 ng/well of reporter and 0.5–1.0 μg/well (total) of expression vectors. The pRL-TK vector (0.5 ng/well; Promega) was also co-transfected as an internal control reporter. Luciferase activity was assessed at 48 h after transfection. The cells were then harvested, lysed and then analyzed using a dual luciferase reporter assay system (Promega). The ratio of firefly (expressed from reporter construct) to *Renilla* (expressed from pRL-TK) luciferase activities in each sample served as a measure of normalized luciferase activity.

2.6. Chromatin immunoprecipitation analysis

Cross-linked chromatin derived from the liver was sonicated on ice, and nuclear fractions were obtained by centrifugation at 10,000×*g* for 5 min. Supernatants were incubated with antibodies against CLOCK (Alpha Diagnostic International, San Antonio, TX, USA), histone H3 (AcH; Upstate, Billerica, MA, USA), or rabbit-IgG (Santa Cruz Biotechnology). DNA isolated using GeneElute Mammalian Genomic DNA kits (Sigma–Aldrich) was amplified by PCR using the primer pairs, 5′-GTGCCAAGTGGCCTTTAGAC-3′ and antisense 5′-CGCAGTGGCTAGCAGATCAT-3′ for the surrounding E-box in the *TF* promoter, and 5′-CGCGAAAGCGAGTGAC-3′ and 5′-TGCGTTATAAAGGGCCG-3′ for the region surrounding the transcription start site of the *TF* gene. The quantitative reliability of PCR was evaluated by kinetic analysis of the amplified products to ensure that signals were derived only from the exponential phase of amplification. Ethidium bromide staining did not detect any PCR products in these samples.

2.7. Real-time monitoring of circadian bioluminescence

Embryonic fibroblasts prepared from wild-type mice were transfected with *TF* luciferase *(TF-Luc*) vectors containing a native (CACGTG) or a mutated (CTCGAG) E-box sequence. Thereafter, the cells were stimulated with 10 nM dexamethasone for 2 h to synchronize their circadian clocks. Bioluminescence emitted by *TF-Luc*-infected cells was recorded using a real-time monitoring system (Lumicycle, Actimetrics, Wilmette, IL, USA) and its amplitude was calculated using Lumicycle analysis software (Actimetrics).

2.8. Statistical analysis

The significance of differences between two groups was analyzed using Student's *t*-test. The statistical significance of differences among more than two groups was analyzed by ANOVA and Tukey's post hoc comparisons. A 5% level of probability was considered significant.

3. Results

Hepatic mRNA expression levels of *TF* and of *Per2* and *PAI-1* significantly fluctuated in a circadian manner with a peak at the light (rest phase) to dark (active phase) transition in wild-type mice. Circadian expression of *TF* mRNA was abolished in *Clock* mutant mice expressing dominant negative CLOCK protein (Fig. 1).

We investigated the molecular mechanisms underlying our observations in *Clock* mutant mice using reporter gene assays. We created and transfected a luciferase reporter construct containing the regulatory region of the mouse *TF* gene into mouse embryonic fibroblasts (MEFs) derived from wild-type and *Clock* mutant mice (Fig. 2A). Luciferase activity was significantly higher in wild-type than in *Clock* mutated MEFs, suggesting that CLOCK is involved in transcriptional regulation of the *TF* gene. We then over-expressed CLOCK and BMAL1 with or without negative clock components such as PER2 and CRY1 in wild-type MEFs. The results showed that these clock molecules transcriptionally regulated *TF* gene expression in a dose-dependent manner (Fig. 2B). Luciferase activity was not enhanced in the presence of dominant negative CLOCK protein (CLOCKΔ19). Chromatin immunoprecipitation demonstrated that CLOCK directly bound to the region around the E-box sequence (Fig. 2C). Finally, the reporter plasmid containing a native or a mutated E-box sequence was transfected into wild-type MEFs, and luciferase activity was continuously monitored to determine whether the functional E-box element is essential for rhythmic transcription of the *TF* gene (Fig. 2D). The circadian fluctuation of luciferase activity was obviously abolished by the E-box mutation, indicating that rhythmic transcription of the *TF* gene was regulated through the E-box element via a cell-autonomous mechanism.

4. Discussion

Tissue factor is involved in endotoxin-induced coagulation, inflammation and mortality [11]. The mortality of patients with sepsis is dependent on circadian time and peaks between 2:00 am and 6:00 am (just before the active phase) [12]. Notably, LPS-induced mortality fluctuates in a circadian manner and peaks at ZT11 (just before the active phase) in nocturnal mice [13]. We showed here that CLOCK is involved in circadian expression of the *TF* gene in the mouse liver that peaked at the day to night transition (activity onset). These findings suggest that the circadian response to endotoxin stimuli is governed by the molecular clock through regulating circadian inducibility of the *TF* gene.

The amplitude of circadian gene expression was obviously lower in *TF* than in *PAI-1* as we recently reported [9]. CLOCK-dependent circadian transactivation of the *PAI-1* gene seems to be exerted via not only the E-box but also other enhancer elements by directly interacting with non-circadian transcription factors [14]. This seemed to be one cause of the high-amplitude rhythm of circadian *PAI-1* gene expression.

Our previous investigation of circadian variations in mouse coagulation and fibrinolytic parameters revealed that expression levels of *PAI-1* mRNA and plasma fibrinolytic activity (euglobulin clot lysis time) robustly fluctuated in a circadian manner [3,6]. On the other hand, plasma levels of protein C, prothrombin, FX,