Involvement of circadian clock gene Clock in diabetes-induced circadian augmentation of plasminogen activator inhibitor-1 (PAI-1) expression in the mouse heart

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Abstract Diabetes is associated with an excess risk of cardiac events, and one of the risk factors for infarction is the elevated-levels of plasminogen activator inhibitor-1 (PAI-1). To evaluate how the molecular clock mechanism is involved in the diabetes-induced circadian augmentation of PAI-1 gene expression, we examined the expression profiles of PAI-1 mRNA in the hearts of Clock mutant mice with streptozotocin-induced diabetes. Circadian expression of PAI-1 mRNA was blunted to low levels under both normal and diabetic conditions in Clock mutant mice, although the expression rhythm was augmented in diabetic wild-type (WT) mice. Furthermore, plasma PAI-1 levels became significantly higher in WT mice than in Clock mutant mice after STZ administration. Our results suggested that the circadian clock component, CLOCK, is involved in the diabetes-induced circadian augmentation of PAI-1 expression in the mouse heart. © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

Serious adverse cardiovascular events including myocardial infarction, sudden cardiac death and stroke have pronounced circadian rhythm, reaching a peak during the morning hours. The frequency of infarction in this time period is 1.5–3 fold higher than that during other times of the day. Levels of blood plasminogen activator inhibitor-1 (PAI-1), the primary physiological inhibitor of plasminogen activators, peak during the early morning, which might explain the morning onset of myocardial infarctions [1,2]. Maemura et al. [3] described the circadian expression of PAI-1 mRNA in the heart and kidneys of mice, and suggested that the circadian oscillation of PAI-1 gene expression plays an important role in the circadian fluctuation of blood fibrinolytic activity. Assays in vitro have shown that CLOCK:BMAL2 (CLIF) and CLOCK:BMAL1 heterodimers up-regulate human PAI-1 gene expression via E-box (CACGTG) elements located at bp –677 to –672 and at bp –562 to –557 [3].

Clock was the first clock gene identified in vertebrates by forward mutagenesis using N-ethyl-N-nitrosourea in a behavioral screening [4]. When transferred from a light–dark cycle to constant darkness, the behavioral periodicity of homozygous Clock mutants becomes unusually long [4,5]. Clock encodes a basic helix–loop–helix (bHLH)-PAS transcription factor that is a positive regulator of an autoregulatory transcription–translation feedback loop [6]. CLOCK forms heterodimers with BMAL1 (a bHLH-PAS transcription factor) and transactivates other clock genes such as period1 (Per1), Per2, cryptochrome1 (Cry1), and Cry2 via E-box elements in their promoters [6,7]. Circadian output genes such as albumin D-site binding protein (DBP) [8,9], prokineticin 2 [10], Weel [11,12], peroxisome proliferator–activated receptor α (PPARα) [13], and Rev-erbα [14] also have E-box elements in their flanking regions, and the rhythmic expression of these genes is CLOCK-dependent in mammals. Therefore, whether the circadian expression of PAI-1 mRNA is actually reduced in Clock mutant mice in the same manner as that of the mPer genes, DBP, and PPARα, should be of wide interest.

Diabetes is associated with several hematologic and rheologic abnormalities that might predispose to thrombosis and lead to an excessive risk of cardiac events. Numerous studies have demonstrated alterations in the plasma proteins involved in blood coagulation and fibrinolysis in diabetic patients [1,15–17]. Recent evidence suggests that increased PAI-1 production is an important contributor to the development of vascular disease in diabetes [1,15–17]. We found that the levels of both total PAI-1 antigen and active PAI-1 antigen are increased in the blood of mice with streptozotocin (STZ)-induced diabetes [18]. The circadian fluctuation of PAI-1 mRNA expression is extremely augmented in peripheral tissues such as the heart and lungs of diabetic mice. However, the molecular mechanism of such robust circadian PAI-1 expression in the diabetic state has not yet been elucidated.

To evaluate how the molecular clock mechanism is involved in the diabetes-induced circadian augmentation of PAI-1 gene expression, we compared the expression profiles of PAI-1 mRNA in the hearts of wild-type (WT) and circadian rhythm
mutant Clock mice with STZ-induced diabetes mellitus. Streptozotocin reduces the expression of glucose transporter 2 (GLUT2) without affecting proinsulin mRNA expression or the total RNA yield and protein content in pancreatic β-cells [19]. We also compared levels of total PAI-1 antigen in the blood of WT and Clock mutant mice with diabetes mellitus. The present results suggest that CLOCK, the core circadian clock component, is involved in the diabetes-induced circadian augmentation of PAI-1 expression in peripheral tissues, which consequently decreases fibrinolytic activity in a time-dependent manner.

2. Materials and methods

Animals. Clock mutant mice were derived from animals supplied by J.S. Takahashi (Northwestern University, Evanston, IL.) that originally had the Clock allele on BALB/c and C57BL/6J backgrounds. A breeding colony was established by further backcrossing with C57BL/6J mice [5]. Male mice at 8–12 weeks of age were maintained under a 12:12 h light-dark cycle (lights on at 06:00 and lights off at 18:00). Insulin-dependent diabetes was induced by a single intraperitoneal injection of the β-cell toxin streptozotocin (STZ, 200 mg/kg) as described [20]. Twenty-six days later, the mice were sacrificed at 2:00, 8:00, 14:00, and 20:00 h, and tissues were dissected, quickly frozen and stored in liquid nitrogen.

Measurement of serum glucose, insulin, and corticosterone levels. Immediately before tissue isolation, blood was withdrawn from the mice and centrifuged for 10 min at maximum speed in a desktop centrifuge. Serum samples were collected and stored at −80 °C. Humoral factors indicating the development of diabetes were determined in these serum samples. Serum glucose levels were measured using a kit (Wako Pure Chemical Industries, Osaka, Japan). Serum insulin and corticosterone (CS) levels were measured using commercially available ELISA (Mercodia AB, Sweden) and EIA (Diagnostic Systems Laboratories, Inc., TX, USA) kits, respectively.

Northern blot analysis. Total RNA was extracted from tissues using guanidinium thiocyanate followed by ISOGEN (Nippon Gene Co., Ltd.; Japan). Total RNA (20 μg) from tissues at each time point was denatured, separated on 1% agarose/0.7 M formaldehyde gels, and blotted onto nylon membranes (GeneScreen Plus; DuPont, USA) by passive capillary transfer. The probes generated from cDNA fragments of PAI-1 (bases: 1138-1602; GenBank Accession No. J03179) were hybridized of GAPDH (mercaptoethylamine) without affecting proinsulin mRNA expression or the total RNA yield and protein content in pancreatic β-cells [19]. We also compared levels of total PAI-1 antigen in the blood of WT and Clock mutant mice with diabetes mellitus. The present results suggest that CLOCK, the core circadian clock component, is involved in the diabetes-induced circadian augmentation of PAI-1 expression in peripheral tissues, which consequently decreases fibrinolytic activity in a time-dependent manner.

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3. Results

Fig. 1A and B shows that food and water intake was significantly increased after STZ administration in both WT (food, df = 39, P < 0.01; water, df = 39, P < 0.01) and homozygous Clock mutant mice (food, df = 35, P < 0.01; water, df = 35, P < 0.01). The STZ injection did not significantly increase the body weight of the WT and Clock mutant mice (df = 40, P = 0.61 and df = 32, P = 0.54, respectively) (data not shown), although both WT and Clock mutant control mice (vehicle injection) weighed slightly but significantly more during the study (df = 38, P < 0.01 and df = 38, P < 0.01, respectively; data not shown).

Serum glucose levels averaged about 5-fold more in diabetic than in control mice of both genotypes (WT, F[1,32] = 367, P < 0.01; Clock, F[1,33] = 399, P < 0.01) (Fig. 1C). Serum insulin levels varied in a circadian manner in control mice of both genotypes (WT, F [3,16] = 7.89, P < 0.01; Clock, F[3,16] = 3.43, P < 0.05), while the acrophase of Clock mutant mice was delayed for several hours compared with that of WT mice (Fig. 1D). Streptozotocin continuously decreased insulin levels in both WT (F[1,32] = 66.0, P < 0.01) and Clock mutant mice (F[1,33] = 15.0, P < 0.01).

The circadian expression of PAI-1 mRNA peaked in the hearts of normal WT mice at 14:00 (F[3,15] = 15.2, P < 0.01) (Fig. 2A). The oscillation was augmented (F[3,29] = 51.6, P < 0.01) and the peak levels were increased about 1.8-fold after STZ administration in WT mice (Fig. 2A). However, rhythmic PAI-1 mRNA expression was damped in the hearts of control Clock mutant mice (F[3,16] = 1.84, P = 0.18). The STZ-injection increased cardiac PAI-1 mRNA levels by about 1.3-fold (F[1,32] = 13.8, P < 0.01) but the expression did not become significantly rhythmic (F[3,16] = 0.362, P = 0.78) in Clock mutant mice.

On the other hand, robust circadian expression of DBP mRNA peaked at 08:00 in the hearts of both normal and diabetic WT mice (F[3,15] = 105, P < 0.01 and F[3,14] = 26.2, P < 0.01, respectively) (Fig. 2B). The expression profiles did not differ between normal and diabetic WT mice (F[1,29] = 0.429, P = 0.52). However, the rhythmic expression of DBP mRNA was completely abolished in the hearts of both normal (F[3,16] = 0.709, P = 0.56) and diabetic (F[3,16] = 0.529, P = 0.67) Clock mutant mice. Levels of DBP mRNA expression did not significantly differ between control and diabetic Clock mutant mice (F[3,32] = 0.059, P = 0.81).

Plasma levels of total PAI-1 antigen were obviously increased by STZ in both WT and Clock mutant mice (F[1,32] = 45.1, P < 0.01 and F[1,33] = 23.8, P < 0.01, respectively) (Fig. 3). Peak to peak comparisons revealed that STZ increased the plasma PAI-1 levels from 0.85 ± 0.10 to 2.28 ± 0.35 and from 0.72 ± 0.11 to 1.31 ± 0.12 in WT and Clock mutant mice, respectively. However, it should be noted that the plasma PAI-1 levels were significantly higher in the diabetic WT mice than in the diabetic Clock mutant mice (F[1,32] = 7.34, P < 0.05), although the levels did not statistically differ between the genotypes under normal conditions (F[1,32] = 2.30, P = 0.139). Thus, the Clock mutation diminished the diabetes-induced increase in plasma PAI-1 levels.

Serum CS levels varied in a circadian manner in control mice of both genotypes (WT, F[3,16] = 15.4, P < 0.01; Clock, F[3,16] = 3.50, P < 0.05), while the acrophase of Clock mutant mice was delayed compared with that of WT mice (Fig. 4). Streptozotocin increased CS levels in a circadian manner in both WT (F[1,31] = 8.16, P < 0.01) and Clock mutant mice (F[1,33] = 26.1, P < 0.01).

4. Discussion

The fibrinolytic system undergoes obvious changes in both Type 1 (insulin-dependent) and Type 2 (non-insulin-depen-
dent) diabetes mellitus. Although PAI-1 concentrations are elevated in the plasma of Type 2 diabetic patients [22,23], the effects of Type 1 diabetes mellitus on plasma PAI-1 levels are controversial. Some reports indicate that plasma PAI-1 levels are increased in Type 1 diabetic patients [24–27], whereas others indicate normal levels [22,28–30]. However, the molecular mechanism of diabetes-induced PAI-1 expression has not been elucidated.

To evaluate how the molecular clock mechanism is involved in the diabetes-induced circadian augmentation of PAI-1 gene expression, we examined the expression profiles of PAI-1 mRNA in the hearts of Clock mutant mice with STZ-induced diabetes. The cardiac levels of PAI-1 mRNA expression and the plasma PAI-1 levels were significantly increased in the diabetic WT mice. Surprisingly, the circadian augmentation of cardiac PAI-1 expression was suppressed in STZ-injected Clock mutant mice, suggesting that the transcription factor CLOCK is involved in the diabetes-induced circadian increase in plasma PAI-1 levels. We also found that the circadian expression of DBP mRNA was not affected by STZ-induced diabetes in mice as found in diabetic rats [31], although the circadian expression of DBP mRNA [8,9] as well as that of PAI-1 [3,32,33] is positively regulated by CLOCK via the E-box element. These results indicated that the circadian transcription mechanisms of the PAI-1 and DBP genes differ at least under diabetic conditions, although CLOCK is involved in the circadian transactivation of both genes. Recent studies have revealed that class I (β) bHLH-PAS proteins such as BMAL1 (MOP3/ARNT3/TIC) and BMAL2 (CLIF/MOP9), as well as class II (α) bHLH-PAS proteins such as CLOCK and MOP4 (NPAS2) are involved in the E-box-dependent circadian transactivation of mammalian clock and clock-regulated output.
genes. The diversity of bHLH-PAS protein complexes seems to contribute to the gene-specific transcriptional regulation via the E-box elements under various physiological conditions such as diabetes.

We could not exclude the possibility that the diabetes-induced circadian augmentation of PAI-1 mRNA expression was caused by a CLOCK-regulated indirect mechanism, because the transcription of PAI-1 mRNA is regulated by multiple signals such as those from insulin precursors (proinsulin and split proinsulin) [34–36], glucose [37], lipids [38], reactive oxygen species [39], glucocorticoids [40], and inflammatory mediators such as transforming growth factor β (TGFβ), tumor necrosis factor α (TNFα), and interleukin-1α (IL-1α) [41–43].

Glucocorticoid is a major inducer of PAI-1 expression in obvious cells and tissues [40,44,45]. In fact, the cis-acting glucocorticoid response element is located in the 5′-flanking sequence of the PAI-1 gene [40]. We previously suggested that hypercortisolemia in STZ-induced diabetic mice causes a tissue- and time-dependent increase in PAI-1 expression [18]. However, serum CS levels in the present study were increased in a circadian manner both in WT and in Clock mutant mice. These observations suggest that CLOCK-regulated circadian augmentation of PAI-1 mRNA expression in diabetic mice is independent of the increase in serum CS levels.

PAI-1 mRNA expression is induced by various humoral factors such as IL-1α, TNFα, and TGFβ not only in cardiovascular cells but also in cardiac myocytes [43,46,47]. This acute induction of PAI-1 mRNA expression was thought to locally elevate PAI-activity in the heart [47]. However, our present results are not sufficiently complete to indicate whether or not STZ-induced PAI-1 mRNA expression is specific for some cardiac regions. An immunohistochemical approach should evaluate the region-specific PAI-1 induction in the heart of diabetic mice.

It should be noted that in the diabetic mice, plasma PAI-1 levels peaked during the early night (Fig. 3), whereas these levels are increased during the early morning in humans [1,2]. Circadian expression of PAI-1 mRNA is directly regulated by circadian clock molecules in peripheral tissues [3]. Recent studies of circadian gene expressions in circulating blood mononuclear cells have revealed that peripheral circadian clocks oscillate in an antiphasic manner between nocturnal rodents [48] and diurnal humans [49]. Antiphasic circadian expression of PAI-1 mRNA between nocturnal rodents and humans might result from the antiphasic circadian activation of CLOCK-dependent transcription as well as that of Per2 mRNA in peripheral mononuclear leukocytes [48,49]. Considering that the phase of peripheral clocks is quite different between nocturnal rodents and humans, the increase in plasma PAI-1 levels during the early evening in mice might explain the high incidence of myocardial infarction in diabetic humans.

Recent molecular dissection of the circadian clock has revealed that the CLOCK-regulated circadian transactivation of clock or clock-controlled output genes plays an important role in the various physiological rhythms that are related to some diseases. The core circadian components PER2 and CRY1 are involved in tumor progression by inhibiting the expression of hypoxia-induced vascular endothelial growth factor (VEGF) mRNA in a circadian fashion [50]. The present study is the first to suggest that a positive component of the circadian autoregulatory feedback loop, CLOCK, is involved in the cardiovascular disorders associated with diabetes mellitus by affecting the circadian expression of PAI-1 mRNA. The half-life of PAI-1 in circulating blood is relatively short [51], suggesting that de novo synthesis of PAI-1 is important. The molecular elucidation of circadian PAI-1 expression in diabetestes should bring about new insight into the mechanism of fibrinolytic disorders in diabetic patients.

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