(2, 3 and 4) and/or specific interaction between proteins, *e.g.*, an antibody and HER2 (4), motor proteins, and rail filaments such as actin filaments and microtubules (5).

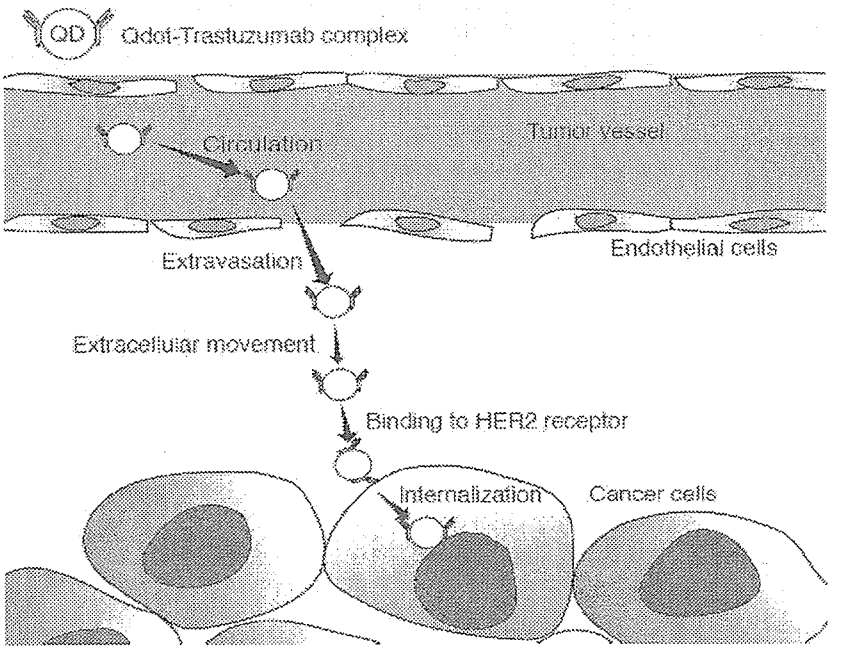

Fig. 6. Delivery of the quantum dots labeled monoclonal antibody. The QT complex in the circulation moved vessel to the interstitial space, then bound to the tumor cells. The QT complex finally reached at the nuclear region through intracellular rail protein.

The molecular mechanism underlying the movement and its cessation during delivery of nano-particles in animal models is the fundamental basis of drug delivery. There have been many different approaches to tumor-targeting "nanocarriers" including anti-cancer drugs, for passive targeting such as Myocet [20], Doxil [21] and for active targeting such as MCC-465 [22], anti-HER2 immunoliposome [23]. There is still very little understanding of the biological behavior of nanocarriers, including such crucial features as their transport in the blood circulation, cellular recognition, translocation into the cytoplasm, and final fate in the target cell. These results suggest that the transport of nanocarriers would be quantitatively analyzable in the tumors of living animals by the present method. This approach thus should afford great potential new insight into particle behavior in complex biological environments. Such new insight in turn will allow rational improvements in particle design to increase the therapeutic index of the tumor targeting nanocarriears.

Nanocrystal semiconductor quantum dots conjugated with antibody may serve fundamentally as new materials controllable for medical purposes including cancer molecular imaging.

Acknowledgments

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Journal of Thrombosis and Haemostasis, 4: 1566–1574

ORIGINAL ARTICLE

Adrenal gland-dependent augmentation of plasminogen activator inhibitor-1 expression in streptozotocin-induced diabetic mice

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Summary. *Background:* Diabetes is associated with an excess risk of cardiac events, and one risk factor for infarction is an elevated level of plasminogen activator inhibitor-1 (PAI-1). *Objectives and methods:* To evaluate whether the glucocorticoid hormones are involved in the diabetes-induced PAI-1 production, we examined expression profiles of *PAI-1* mRNA in adrenalectomized (ADX) mice with streptozotocin (STZ)-induced diabetes. *Results:* The diabetes-induced augmentation of plasma PAI-1 levels and *PAI-1* mRNA expression in the heart and lungs was completely normalized in diabetic ADX mice. The glucocorticoid receptor antagonist RU486 significantly, but only partly suppressed PAI-1 induction in STZ-induced diabetic mice, suggesting that factors other than glucocorticoids are also involved in PAI-1 induction provoked by diabetes. *Conclusion:* Our results suggested that the adrenal gland plays a critical role in the progression of thrombosis in diabetic patients by inducing expression of the *PAI-1* gene.

Keywords: adrenalectomy, diabetes, glucocorticoid, plasminogen activator inhibitor-1, RU486, streptozotocin.

Introduction

Diabetes is associated with several hematologic and rheologic abnormalities that might predispose to thrombosis and lead to an increased risk of cardiac events [1]. Several studies have demonstrated alterations in the plasma proteins involved in blood coagulation and fibrinolysis in diabetic patients [2–5].

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Recent evidence suggests that an increased level of plasminogen activator inhibitor-1 (PAI-1) is an important contributor to the development of acute and chronic cardiovascular disease in diabetics [2–5]. Although PAI-1 concentrations are elevated in the plasma of type 2 (non-insulin-dependent) diabetic patients [6,7], the effects of type 1 (insulin-dependent) diabetes mellitus on plasma PAI-1 levels are controversial. Some reports indicate that plasma PAI-1 levels are increased in type 1 diabetic patients [8–11], whereas others indicate normal levels [6,12–14]. The precise molecular mechanism of diabetes-induced *PAI-1* expression has not yet been fully elucidated. We showed that both total and active PAI-1 levels are increased in the blood of mice with streptozotocin (STZ)-induced diabetes [15]. 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 [16]. The circadian fluctuation of *PAI-1* mRNA expression is augmented in peripheral tissues such as the heart, lungs, and kidneys of diabetic mice [15]. Interestingly, the circadian augmentation of *PAI-1* mRNA expression in these tissues proceeds in parallel with that of plasma corticosterone levels [15].

Transcription of the *PAI-1* gene is induced by large amounts of agents such as glucose [17], transforming growth factor (TGF)-β [18–21], tumor necrosis factor (TNF)-α [18,21–23], insulin [24], angiotensin II [25–27], and glucocorticoids [23,28–33]. Glucocorticoids are stress hormones that exert diabetogenic effects by increasing hepatic glucose production and inducing insulin resistance, resulting in increased plasma insulin levels. Both type 1 [34] and type 2 [35] diabetic patients have elevated circulating cortisol levels resulting from hyperactivation of the hypothalamo-pituitary-adrenocortical (HPA) axis. Diabetes also induces hypercorticosteronemia in rodents injected with STZ [36–38]. Glucocorticoids are critical inducers of *PAI-1* expression in various cells and tissues [28–33], and the *cis*-acting glucocorticoid response element (GRE) is located in the 5′-flanking sequence of the *PAI-1* gene both in humans [39] and in rats [30].

The present study examines whether hypercorticosteronemia is responsible for the diabetes-induced plasma PAI-1 increase by evaluating the expression profiles of *PAI-1* mRNA in tissues of adrenalectomized (ADX) mice with STZ-induced diabetes mellitus. We found that the diabetes-induced plasma PAI-1 increase, and augmentation of *PAI-1* mRNA expression in the heart and lungs (but not in the kidneys and liver) of diabetic mice were completely prevented by ADX. The glucocorticoid receptor antagonist RU486 [40] significantly, but only partly, suppressed PAI-1 induction in the diabetic mice, suggesting that factors other than glucocorticoids are also involved in STZ-induced PAI-1 induction. The present results suggest that the adrenal gland plays a critical role in the diabetes-induced PAI-1 elevation that, in turn, compromises the normal fibrin clearance mechanism and consequently promotes thrombosis in diabetic patients.

Materials and methods

Animals

Male Jcl:ICR mice (Clea Japan Inc., Tokyo, Japan) aged 7–8 weeks underwent bilateral ADX using the dorsal approach under ketamine/xylazine anesthesia [ketamine 91 mg kg^{-1} body weight (BW) and xylazine 3.6 mg kg^{-1} BW, intramuscularly]. The ADX mice were given free access to standard chow (CE-2, Clea Japan Inc.) and to 0.9% NaCl. Sham-operated control mice were given standard chow and water *ad libitum*. After the operation, apparently alert and healthy mice were housed under a 12:12 h light–dark cycle (lights on at 07:00 hours and lights off at 19:00 hours) for at least 2 weeks before the day of the STZ (Sigma, St Louis, MO, USA) injection. Insulin-dependent diabetes was induced by a single intraperitoneal injection of the β-cell toxin STZ (200 mg kg^{-1}) as described [41]. Twenty-one days after the STZ injection, the mice were killed at 21:00 hours and tissues were dissected, quickly frozen and stored in liquid nitrogen.

To study the effects of the RU486 (Sigma), stock RU486 (1 mM in 95% ethanol) diluted to 1 μM in drinking water [42] was administered to the mice starting 18 days from the STZ injection. The mice were then killed 3 days later as described above.

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 (3000 × *g*) in a desktop centrifuge. Humoral factors indicating the development of diabetes were determined in serum samples that were collected and stored at −80 °C. Serum glucose levels were measured using a kit (Wako Pure Chemical Industries, Osaka, Japan). Serum insulin and corticosterone levels were measured using commercially available ELISA (Mercodia AB, Uppsala, Sweden) and EIA (Diagnostic Systems Laboratories, Inc., Webster, TX, USA) kits, respectively.

Northern blot analysis

Total RNA was extracted from tissues using guanidinium thiocyanate extraction followed by ISOGEN (Nippon Gene Co. Ltd, Tokyo, Japan). Poly(A)$^+$ RNA was purified from total RNA using the GenElute mRNA Miniprep Kit (MRN70; Sigma-Aldrich, St Louis, MO, USA). Total RNA (20 μg) or poly(A)$^+$ RNA (5 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, Wilmington, DE, USA) by passive capillary transfer. The probes generated from cDNA fragments of *PAI-1* (bases: 138–689; GenBank accession number M33960), serum and glucocorticoid-induced kinase (*SGK*) (bases: 41–800; GenBank accession number AF139638) and *GAPDH* (bases: 133–575; GenBank accession number M17701) were hybridized and detected as described [15]. Samples were normalized to the amount of *GAPDH* mRNA.

Measurement of plasma total PAI-1 and active PAI-1 levels

Platelet-poor plasma was obtained from blood that was immediately mixed with 0.2 volumes of 65 mM sodium citrate, pH 7.2 and centrifuged as described above. Plasma total PAI-1 and active PAI-1 levels were measured using Total Murine PAI-1 and Mouse PAI-1 Activity ELISA kits (both from Molecular Innovations, Inc., Southfield, MI, USA), respectively.

Statistics

Data are expressed as mean ± SEM. Results were statistically analyzed by the one-way analysis of variance (ANOVA) with $P < 0.05$ as the criterion for statistical significance.

Results

Figure 1A and B show that food and water intake was significantly increased after STZ administration in sham-operated mice. On the contrary, the STZ injection did not significantly alter food and water consumption in ADX mice, although ADX alone significantly increased water intake (Fig. 1A,B). Both injected STZ and ADX slightly but significantly decreased body weight, although STZ had no additive effect on the changes in the body weight of ADX mice (Fig. 1C).

Serum glucose levels were increased about 5.3- and 6.0-fold by STZ injected into sham-operated and ADX mice, respectively, although ADX alone significantly decreased the glucose levels by 45% (Fig. 2A). Serum insulin levels were obviously decreased not only by STZ but also by ADX, although STZ had no additive effect on serum insulin levels in ADX mice (Fig. 2B). The injection of STZ notably increased the serum corticosterone levels in sham-operated mice (Fig. 2C) and slightly but significantly increased the low level in ADX mice.

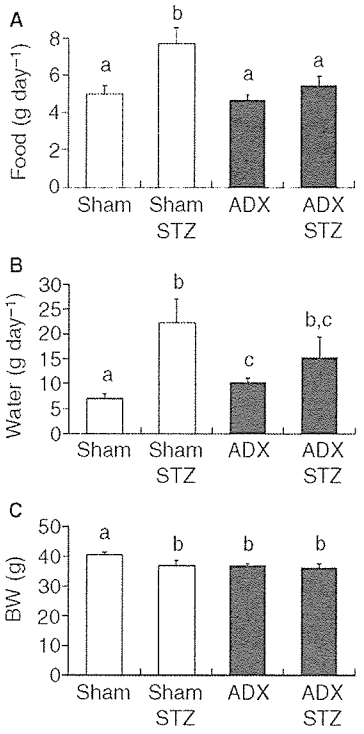

Fig. 1. Effects of streptozotocin (STZ)-induced diabetes on (A) food intake, (B) water intake, and (C) body weight in sham-operated and adrenalectomized (ADX) mice. Bilateral ADX was performed at least 2 weeks before STZ injection. Twenty-one days after STZ injection, food and water intake was evaluated throughout the day. Values are mean ± SEM. Open and filled columns indicate values for sham-operated ($n = 9$ and $n = 10$ for control and diabetic mice, respectively) and ADX ($n = 8$ and $n = 7$ for control and diabetic mice, respectively) mice, respectively. Different characters indicate statistically significant ($P < 0.05$).

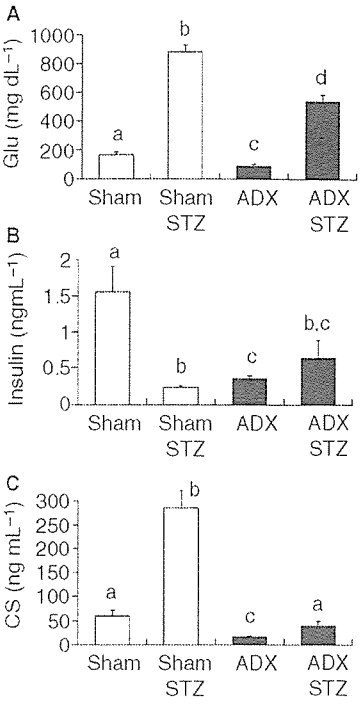

Fig. 2. Effects of streptozotocin (STZ)-induced diabetes on serum levels of (A) glucose, (B) insulin, and (C) corticosterone in sham-operated and adrenalectomized (ADX) mice. Humoral markers of diabetes were examined in serum samples obtained from decapitated mice (see Materials and methods). Values are mean ± SEM. Open and filled columns indicate values for sham-operated ($n = 9$ and $n = 10$ for control and diabetic mice, respectively) and ADX ($n = 8$ and $n = 7$ for control and diabetic mice, respectively) mice, respectively. Different characters indicate statistically significant ($P < 0.05$).

Figure 3 shows that the injected STZ induced *PAI-1* mRNA expression in a tissue-dependent manner. Levels of *PAI-1* mRNA were significantly augmented in the heart and lungs, although the mRNA levels were not changed in the liver and kidneys of mice injected with STZ. Cardiac mRNA levels of the *PAI-1* gene were induced more than twofold by STZ in sham-operated mice. Adrenalectomy decreased the *PAI-1* mRNA levels, and the diabetes-induced augmentation of *PAI-1* mRNA expression was completely prevented by ADX in both the heart and lungs of diabetic mice. Hepatic *PAI-1* mRNA levels were not changed by STZ but significantly increased by ADX. Furthermore, ADX-dependent augmentation of *PAI-1* gene expression was normalized by STZ.

Plasma total and active PAI-1 levels were obviously increased by STZ in sham-operated mice (Fig. 4) whereas the diabetes-induced increase in plasma PAI-1 levels was abolished in the ADX mice. These plasma PAI-1 profiles were closely associated with the *PAI-1* mRNA levels in the heart and lungs (Fig. 3), although unlike the mRNA levels, ADX alone did not significantly affect plasma PAI-1 levels.

Three days of RU486 administered via the drinking water slightly but significantly increased the amount of food and water consumed by STZ-induced diabetic mice and decreased their body weight, but did not affect these parameters in control mice (Fig. 5). RU486 significantly but only slightly suppressed diabetes-induced hyperglycemia in the mice given STZ, but did not affect serum glucose levels in control mice (Fig. 6A). RU486 did not affect serum insulin levels either in control or in STZ-induced diabetic mice (Fig. 6B). RU486 significantly suppressed diabetes-induced *PAI-1* mRNA expression in the hearts of STZ-injected mice but not in control mice (Fig. 7). RU486 also significantly suppressed diabetes-induced *SGK* mRNA expression in the STZ-injected mice, but not in control mice.

Three days of RU486 administration significantly suppressed diabetes-induced plasma increases in total and active PAI-1 levels (Fig. 8) but did not affect plasma PAI-1 levels in control mice.

Discussion

A single injection of STZ induced hyperglycemia in ADX and in sham-operated mice (Fig. 2), although diabetes-induced increases in food and water intake and changes in body weight were significantly reduced and diminished, respectively in the ADX mice (Fig. 1). Appetite in humans

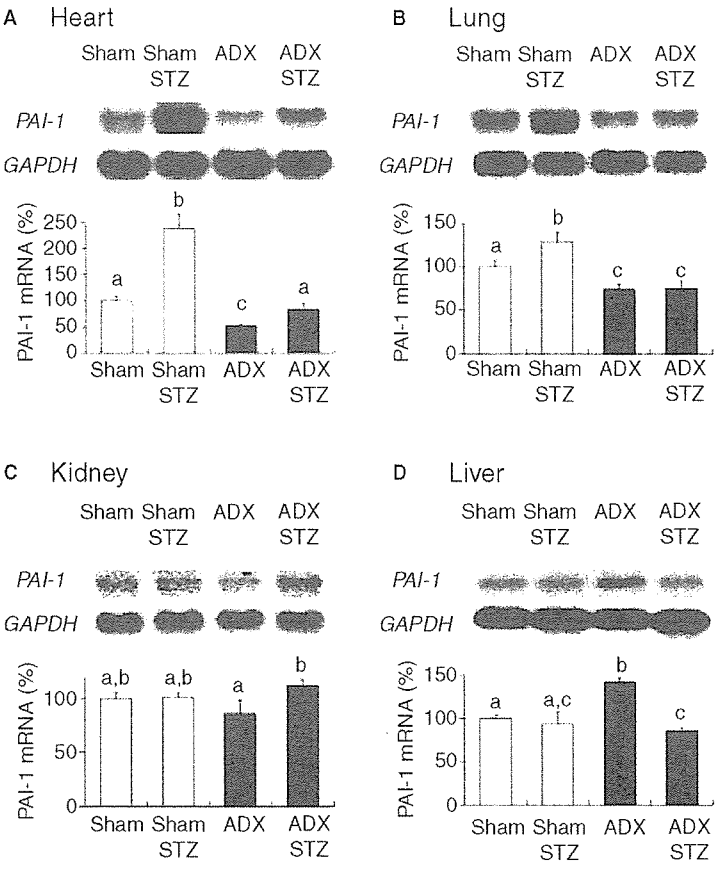

Fig. 3. Expression of *PAI-1* mRNA in (A) heart, (B) lungs, (C) kidneys, and (D) liver of sham-operated and adrenalectomized (ADX) mice with streptozotocin-induced diabetes. Messenger RNA levels of *PAI-1* gene quantified from Northern blots are plotted. Total RNA (20 μg) from the heart, lungs, and kidneys, and poly(A)$^+$ RNA (5 μg) from the liver were analyzed. Maximum value of sham-operated control mice is expressed as 100%. Values are mean ± SEM. Open and filled columns indicate values for sham-operated (n = 9 and n = 10 for control and diabetic mice, respectively) and ADX (n = 8 and n = 7 for control and diabetic mice, respectively) mice, respectively. Different characters indicate statistically significant (P < 0.05).

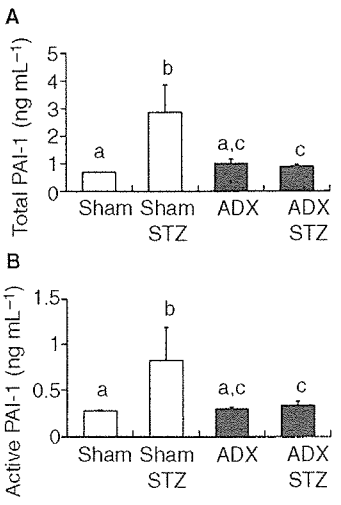

Fig. 4. Plasma (A) total and (B) active plasminogen activator inhibitor-1 levels in sham-operated and adrenalectomized (ADX) mice with streptozotocin-induced diabetes. Values are mean ± SEM. Open and filled columns indicate values for sham-operated (n = 9 and n = 10 for control and diabetic mice, respectively) and ADX (n = 8 and n = 7 for control and diabetic mice, respectively) mice, respectively. Different characters indicate statistically significant (P < 0.05).

and other animals parallels changes in glucocorticoid hormone levels, and ADX induces a depression in feeding although the underlying mechanisms have not been fully elucidated [43]. Plasma corticosterone levels were extremely elevated in STZ-injected sham-operated mice (Fig. 2), as discussed below. Plasma insulin levels were remarkably decreased not only by injected STZ but also by ADX (Fig. 2), as described [44]. The ADX-induced decrease in plasma insulin levels seemed to be caused by the suppression of daily food consumption [45].

The HPA axis of patients with diabetes mellitus is hyperactivated, especially in the presence of poor glycemic control and ketoacidosis [46–48]. Levels of circulating cortisol are elevated in both type 1 [34] and type 2 [35] diabetic patients, and diabetes induces hypercorticosteronemia in STZ-injected mice [15,36,37]. Glucocorticoids are critical inducers of *PAI-1* expression in a variety of cells and tissues [23,28–33]. In fact, the *cis*-acting GRE is located in the 5′-flanking sequence of the *PAI-1* gene both in humans [39] and rats [30]. We therefore postulated that *PAI-1* expression induced by hypercorticosteronemia constitutes a risk factor for cardiovascular diseases in diabetic patients.

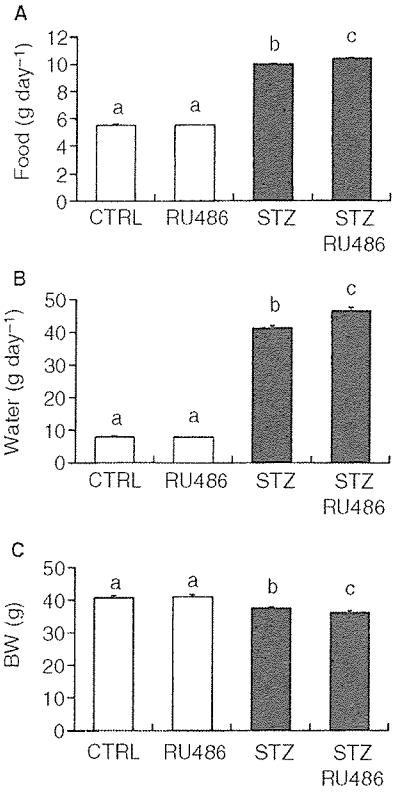

Fig. 5. Effects of RU486 administered via drinking water on (A) food intake, (B) water intake, and (C) body weight in streptozotocin (STZ)-induced diabetic mice. Parameters were evaluated on day 3 after RU486 administration. Values are mean ± SEM. Open and filled columns indicate values for non-diabetic (n = 5 for both control and RU486-administered mice) and STZ-induced diabetic (n = 6 and n = 8 for control and RU486-administered mice, respectively) mice, respectively. Different characters indicate statistically significant ($P < 0.05$).

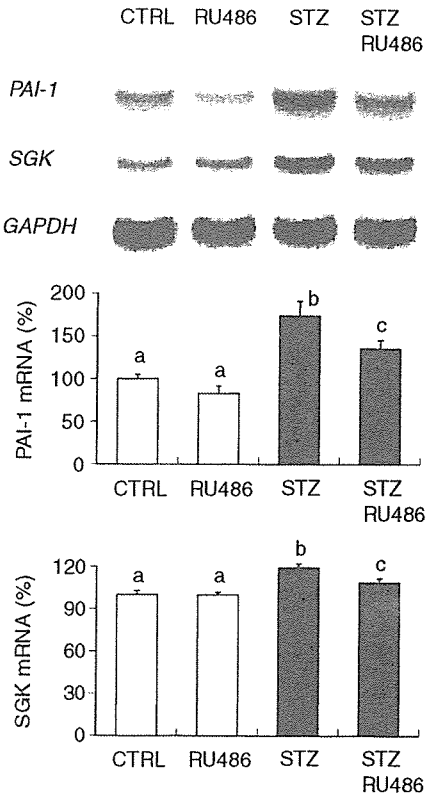

Fig. 7. Expression of *PAI-1* and *SGK* mRNAs in the hearts of mice with streptozotocin (STZ)-induced diabetes administered with RU486. Messenger RNA levels of genes were quantified from Northern blots. Maximum value of non-diabetic control mice is expressed as 100%. Values are mean ± SEM. Open and filled columns indicate values for non-diabetic (n = 5 for both control and RU486- administered mice) and STZ-induced diabetic (n = 6 and n = 8 for control and RU486- administered mice, respectively) mice, respectively. Different characters indicate statistically significant ($P < 0.05$).

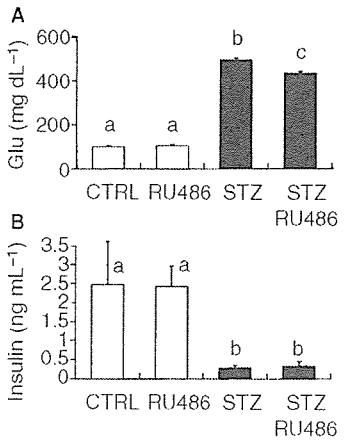

Fig. 6. Effects of RU486 administered via drinking water on serum levels of (A) glucose and (B) insulin in streptozotocin (STZ)-induced diabetic mice. Humoral markers of diabetes were examined in serum samples from decapitated mice (see Materials and methods). Values are mean ± SEM. Open and filled columns indicate values for non-diabetic (n = 5 for both control and RU486-administered mice) and STZ-induced diabetic (n = 6 and n = 8 for control and RU486-administered mice, respectively) mice, respectively. Different characters indicate statistically significant ($P < 0.05$).

The present study showed that plasma PAI-1 levels are elevated in an adrenal gland-dependent manner in mice with STZ-induced diabetes. The half-life of PAI-1 in the circulating blood is relatively short (approximately 6 min) [49], suggesting that *de novo* synthesis of PAI-1 is important. The present study showed that ADX prevented diabetes-induced increases in plasma PAI-1 levels and in *PAI-1* mRNA expression in the heart and lungs. Chronic administration of the glucocorticoid receptor antagonist RU486 significantly, but only partially, suppressed *PAI-1* gene expression in mice with STZ-induced diabetes (Fig. 7), although ADX completely suppressed the diabetes-induced augmentation of *PAI-1* expression in these mice (Fig. 3). These results suggest that factors other than glucocorticoids are also involved in STZ-induced *PAI-1* expression. Adrenalectomy exerts several effects on metabolic parameters such as food consumption, body weight gain, body fat content, thermogenesis, insulin resistance, plasma levels of insulin, glucose, and triglycerides [50]. However, some of these effects cannot be mimicked by RU486 [50]. The present study showed that ADX obviously attenuated STZ-induced increases in food intake and blood glucose levels in contrast to RU486,

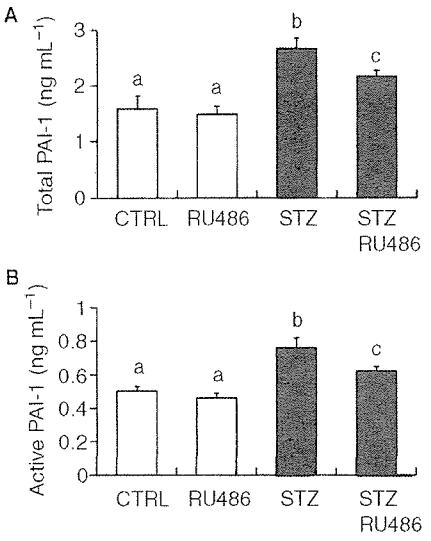

Fig. 8. Plasma (A) total and (B) active plasminogen activator inhibitor-1 levels in the RU486-treated diabetic mice. Values are mean ± SEM. Open and filled columns indicate values for non-diabetic (n = 5 for both control and RU486- administered mice) and streptozotocin-induced diabetic (n = 6 and n = 8 for control and RU486- administered mice, respectively) mice, respectively. Different characters indicate statistically significant ($P < 0.05$).

which enhanced STZ-induced food intake and only slightly suppressed the increase in blood glucose. The effects on cardiac *PAI-1* mRNA expression in STZ-injected diabetic mice, ADX mice, diabetic ADX mice, RU486-treated mice and RU486-treated diabetic mice correlated with blood glucose levels. Hyperglycemia is a potent inducer of *PAI-1* expression [51–53]. Therefore, changes in blood glucose levels might be involved in the ability of both ADX and RU486 to attenuate diabetes-induced *PAI-1* expression. A defect in catecholamines caused by adrenodemedullation diminishes the diabetogenic effect of STZ [54], suggesting that the diabetogenic effect in STZ-injected ADX mice and in RU486-treated mice is not totally comparable. Another possible explanation for the different effect of ADX and RU486 on diabetic-induced *PAI-1* expression is that mineralocorticoid receptor (MR)-mediated transactivation by glucocorticoids is involved in diabetes-induced *PAI-1* expression, because although glucocorticoids bind to both glucocorticoid receptor (GR) and MR, their affinity for MR is higher (K_d 1 nM compared with 10 nM for GR) [55]. Whether glucocorticoids are directly or indirectly involved in diabetes-induced *PAI-1* gene expression remains to be determined. Regardless, we showed that the adrenal gland is involved in the plasma PAI-1 increase in mice with STZ-induced diabetes. To our knowledge, this is the first report to show the role of the adrenal gland in PAI-1 regulation *in vivo*.

Proinflammatory cytokines and growth factors, such as interleukin (IL)-1, IL-6, TNF-α epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and TGF-β, also regulate *PAI-1* expression [18–23,27,56–59]. Among these humoral factors, TNF-α and TGF-β induce *PAI-1* gene expression in a variety of cell types. We therefore examined the effect of ADX on circulating levels of TNF-α and TGF-β1 as representative humoral factors in STZ-induced diabetic mice (Fig. S1). We found that ADX did not significantly affect the levels of these cytokines.

The present study showed that the effect of the STZ-induced diabetes on *PAI-1* mRNA expression levels was tissue-specific as reported [37]. Streptozotocin significantly increased *PAI-1* mRNA levels in the heart and lungs, but not in the liver and kidneys (Fig. 3). Interestingly, the effect of the ADX alone on *PAI-1* gene expression was also tissue-specific. The expression of *PAI-1* was suppressed in the heart and lungs of ADX mice, while levels were significantly increased in the liver. Moreover, ADX blocked the STZ-induced augmentation of *PAI-1* mRNA levels in the heart and lungs. The expression profiles of *PAI-1* mRNA in the heart and lungs were closely associated with the plasma total PAI-1 levels, suggesting that these tissues are involved in the plasma PAI-1 increase in mice with STZ-induced diabetes. Although the present study could not determine the underlying mechanism of ADX-induced *PAI-1* mRNA expression in the liver, STZ notably normalized this process. Adrenalectomy decreases plasma levels of insulin, glucose, and triglycerides, body fat content, and the liver glycogen content [50,60]. We recently reported that expression levels of several hepatic genes involved in glucose and lipid metabolism are critically affected in ADX mice [44]. Adrenalectomy-induced metabolic changes that are reversed by STZ-induced diabetes might be involved in the liver-specific augmentation of *PAI-1* gene expression in the ADX mice.

The effects of STZ, RU486, and of RU486 administered to STZ-induced diabetic mice on cardiac *PAI-1* mRNA expression were very similar to those on *SGK* mRNA expression (Fig. 7). The immediate early gene *SGK* contains a GRE in its promoter region [61], and it is transcriptionally induced by serum and glucocorticoids [62]. Glucocorticoids can induce *SGK* gene transcription via GR in isolated cardiac cells [63], although the role of SGK in cardiac physiology is unknown [55]. Recent studies have shown that SGK is involved in the glucocorticoid-induced inhibition of pancreatic insulin secretion [64] and that its mRNA expression is up-regulated in the kidneys of STZ-induced diabetic mice [65]. These observations considered together with the present findings suggest that hypercorticosteronemia is responsible for the diabetes-induced up-regulation of *PAI-1* gene expression as well as that of *SGK* gene expression in mice injected with STZ.

The ADX mice expressed detectable levels of plasma corticosterone (Fig. 2) and STZ slightly but significantly increased this level. The enzyme 11β-hydroxysteroid dehydrogenase 1 (11βHSD1) acts predominantly as a reductase *in vivo*, facilitating glucocorticoid action by converting circulating receptor-inactive 11-ketoglucocorticoids to active glucocorticoids, and it is abundantly expressed in many tissues [55]. Therefore, 11βHSD1 expressed outside the adrenal gland might be responsible for the diabetes-induced plasma increase in corticosterone levels in ADX mice.

Blood PAI-1 levels change in a circadian manner and peak during the early morning, which might explain the morning onset of myocardial infarctions [5,66,67]. Maemura *et al.* [68] 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, the basic helix-loop-helix (bHLH)-PAS transcription factors are involved in an autoregulatory transcription-translation feedback loop [69] and up-regulate human *PAI-1* gene expression via E-box (CACGTG) elements located at bp −677 to −672 and at bp −562 to −557 [68]. We showed that the circadian expression of *PAI-1* mRNA is augmented in the hearts of STZ-induced diabetic mice [37]. Furthermore, the diabetes-induced circadian augmentation of *PAI-1* mRNA is prevented in homozygous *Clock* mutant mice [36], suggesting that a core component of the circadian clock, CLOCK, is involved in diabetes-induced *PAI-1* mRNA expression *in vivo*. However, serum corticosterone levels were increased both in wild-type and in *Clock* mutant mice in that study, although diabetes-induced *PAI-1* gene expression was obviously suppressed in the *Clock* mutant mice [36]. These results together with the present finding that the adrenal gland contributes to diabetes-induced *PAI-1* expression, indicate that CLOCK- and adrenal gland-dependent transactivation mechanisms are both independently essential for diabetes-induced *PAI-1* gene expression in mice. The results of the present study suggest that metabolic as well as genetic determinants [36] are very important for the regulation of plasma PAI-1 levels in diabetic patients.

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Supplementary material

The following supplementary material can be found at http://www.blackwell-synergy.com/loi/jth:

Fig S1. Serum (A) Tumor necrosis factor (TNF)-α and (B) transforming growth factor (TGF)-β1 levels in sham-operated and adrenalectomized (ADX) mice with streptozotocin-induced diabetes. Serum levels of TNF-α and TGF-β1 were measured using BioSource Mouse TNF-α ELISA (BioSource, Camarillo, CA, USA) and Quantikine Mouse/Porcine/Rat TGF-β1 ELISA (R&D Systems, Minneapolis, MN, USA) kits, respectively. Values are mean ± SEM. Open and filled columns indicate values for sham-operated ($n = 9$ and $n = 10$ for control and diabetic mice, respectively) and ADX ($n = 8$ and $n = 7$ for control and diabetic mice, respectively) mice, respectively. Different characters indicate statistically significant ($P < 0.05$).

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ORIGINAL ARTICLE

CLOCK is involved in obesity-induced disordered fibrinolysis in *ob/ob* mice by regulating *PAI-1* gene expression

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Summary. *Background*: An increased level of obesity-induced plasma plasminogen activator inhibitor-1 (PAI-1) is considered a risk factor for cardiovascular disease. *Aim*: The present study investigates whether the circadian clock component CLOCK is involved in obesity-induced PAI-1 elevation. *Methods*: We examined plasma PAI-1 and mRNA expression levels in tissues from leptin-deficient obese and diabetic *ob/ob* mice lacking functional CLOCK protein. *Results*: Our results demonstrated that plasma PAI-1 levels were augmented in a circadian manner in accordance with the mRNA expression levels in *ob/ob* mice. Surprisingly, a *Clock* mutation normalized the plasma PAI-1 concentrations in accordance with the mRNA levels in the heart, lung and liver of *ob/ob* mice, but significantly increased *PAI-1* mRNA levels in adipose tissue by inducing adipocyte hypertrophy in *ob/ob* mice. The *Clock* mutation also normalized tissue PAI-1 antigen levels in the liver but not in the adipose tissue of *ob/ob* mice. *Conclusion*: These observations suggest that CLOCK is involved in obesity-induced disordered fibrinolysis by regulating *PAI-1* gene expression in a tissue-dependent manner. Furthermore, it appears that obesity-induced PAI-1 production in adipose tissue is not closely related to systemic PAI-1 increases *in vivo*.

Keywords: adipose tissue, circadian clock, *Clock* mutant mouse, *ob/ob* mouse, obesity, plasminogen activator inhibitor-1.

Introduction

Obesity is an independent risk factor for the development of atherosclerosis and cardiovascular disease [1,2]. The inhibition of fibrinolysis and obesity are closely connected, and elevated levels of plasma plasminogen activator inhibitor-1 (PAI-1), the primary physiological inhibitor of plasminogen activators, is regarded as a main cause of decreased fibrinolytic activity [1–3]. Various cells *in vitro* produce PAI-1, which is widely distributed in tissues such as vessel walls (endothelial and smooth muscle cells), macrophages, the liver, and adipose tissue, and its expression is regulated by several cytokines, hormones, and metabolic factors such as tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), insulin, glucocorticoids, angiotensin II, some fatty acids, and glucose [4,5]. Pharmacological agents such as thiazolidinediones, metformin, and AT_1-receptor antagonists reduce adipose PAI-1 expression [5]. In addition, energic weight loss leads to a decrease in plasma PAI-1 levels, which increase again if weight is regained [4]. The same effect has been achieved by surgically removing fat [4]. Studies of animal models support these clinical findings. Plasma PAI-1 levels increase 4- to 6-fold in an age-dependent manner in leptin-deficient obese and diabetic *ob/ob* mice compared with lean littermates in accordance with the increased expression of *PAI-1* mRNA in adipose tissue [6]. Therefore, several recent studies that aimed to determine the source of PAI-1 have focused on adipose tissue [4,5,7].

Serious adverse cardiovascular events, including myocardial infarction, sudden cardiac death, pulmonary embolism, critical limb ischemia and aortic aneurysm rupture, all have pronounced circadian rhythmicity, reaching a peak during the morning [8–10]. The frequency of infarction during this period is 1.5- to 3-fold higher than that at other times of the day. Levels of blood PAI-1 peak during the early morning, which might explain the morning onset of myocardial infarctions [11,12]. Maemura *et al.* [13] 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 upregulate human *PAI-1* gene expression via E-box (CACGTG) elements located at base pairs −677 to −672 and −562 to −557 [13,14].

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Clock was the first clock gene to be identified in vertebrates by forward mutagenesis using *N*-ethyl-*N*-nitrosourea in a behavioral screening [15]. When transferred from a light–dark cycle to constant darkness, the behavioral periodicity of homozygous *Clock* mutants becomes unusually long [15,16]. *Clock* encodes a basic helix-loop-helix (bHLH)– Per-Arnt-Sim (PAS) transcription factor that is a positive regulator of an autoregulatory transcription–translation feedback loop [17]. CLOCK forms heterodimers with BMAL1 (a bHLH-PAS transcription factor) and transactivates other clock genes such as period 1 (*Per1*), *Per2*, cryptochrome 1 (*Cry1*) and *Cry2* via E-box elements in their promoters [17,18]. Circadian output genes such as albumin D-site binding protein (*DBP*) [19,20], prokineticin 2 [21], Wee1 [22,23], peroxisome proliferator-activated receptor α (*PPARα*) [24] and Rev-erbα [25] also have E-box elements in their flanking regions and the rhythmic expression of these genes is CLOCK-dependent in mammals. The circadian expression of *PAI-1* mRNA is reduced along with that of other CLOCK-regulated circadian genes in *Clock* mutant mice [23,26–28].

We recently showed that plasma PAI-1 concentrations are elevated in a circadian manner in streptozotocin (STZ)-induced diabetic mice, which are considered to be an animal model of insulin-dependent type 1 diabetes [29]. The diabetes-induced plasma PAI-1 increase in these mice seemed to result from transcriptional activation of the PAI-1 gene in several tissues induced by the STZ [29]. Moreover, CLOCK appears to be involved in the diabetes-induced increase in PAI-1, because plasma PAI-1 increases induced by STZ are obviously suppressed in *Clock* mutant mice [26].

In the present work we initially determined that plasma PAI-1 levels are increased in a circadian manner in leptin-deficient *ob/ob* mice. Circadian expression of *PAI-1* mRNA was also augmented in tissues from these mice. Thereafter, to determine the contribution of CLOCK to obesity-induced PAI-1 production, we crossed genetically obese *ob/ob* mice with *Clock* mutant mice expressing dominant negative CLOCK protein. We found that mutation of the circadian clock gene *Clock* tissue dependently affected obesity-induced *PAI-1* mRNA expression in the mice. Surprisingly, the obesity-induced elevation of plasma PAI-1 levels was suppressed in *Clock*-mutated *ob/ob* mice (*Clk/Clk;ob/ob* mice), although *PAI-1* mRNA expression in adipose tissue was additively increased. Our findings suggest that the circadian clock component CLOCK plays a critical role in obesity-induced fibrinolytic disorders. Furthermore, obesity-induced PAI-1 production in adipose tissue appears not to be important for systemic PAI-1 increases *in vivo*, in contrast to previous speculation that adipose tissue is a major contributor to plasma PAI-1 elevation under conditions of obesity.

Materials and methods

Animals

Heterozygous *ob/+* mice were supplied by G. S. Hotamisligil (Harvard University, Boston, MA, USA) and maintained on a C57BL/6J genetic background. *Clk/Clk;ob/ob* mice were derived by crossing with homozygous *Clock* mutant mice harboring one mutated leptin allele (*ob/+*). *Clock* mutants were derived from mice supplied by J. S. Takahashi (Northwestern University, Evanston, IL, USA) that originally had the *Clock* allele on BALB/c and C57BL/6J backgrounds. A breeding colony was established by further backcrossing with Jcl:ICR mice (Clea Japan Inc., Tokyo, Japan) and mating with *ob/+* mice. The resulting F1 pups were heterozygous for the deletion of both *Clock* and *leptin* (*ob*) and were further crossed to generate mice with all of the genotypes. At the age of 12–15 weeks, male mice were maintained under a 12:12 h light–dark cycle (lights on at 0:00 h and lights off at 12:00 h). After at least 2 weeks of light entrainment, the mice were killed at 14:00 h (except for those described in Fig. 1) and tissues were dissected, quickly frozen and stored in liquid nitrogen.

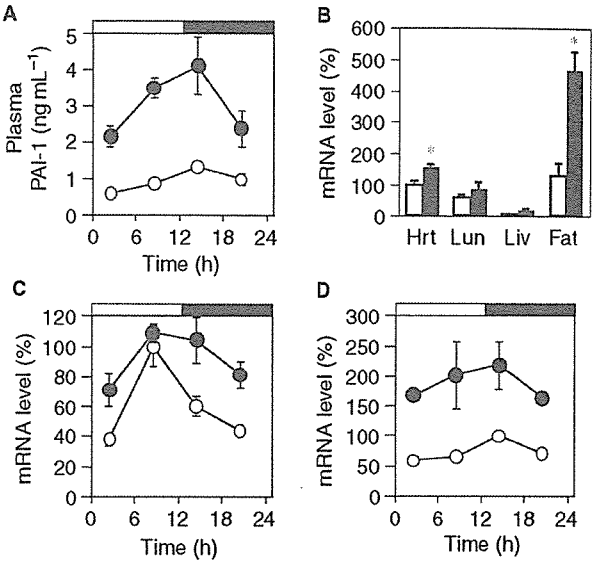

Fig. 1. Circadian augmentation of plasma plasminogen activator inhibitor-1 (PAI-1) levels in leptin-deficient *ob/ob* mice. (A) Circadian profile of plasma total PAI-1 levels in WT and *ob/ob* mice. (B) Tissue distribution of *PAI-1* mRNA in WT and *ob/ob* mice. Mice were killed at 14:00 h, and then total RNA was extracted from dissected tissues. Levels of *PAI-1* mRNA were determined from Northern blots. The value of the WT heart is expressed as 100%. In each tissue, genotypes were compared using Student's *t*-test (*$P < 0.05$). Hrt, heart; Fat, epididymal adipose tissue; Liv, liver; Lun, lung. (C–D) Circadian expression profile of *PAI-1* mRNA in the heart (C) and epididymal fat (D) of *ob/ob* mice. Maximum value of WT mice is expressed as 100%. Values are means ± SEM ($n = 3$–6). Open and filled circles/bars indicate values for WT and *ob/ob* mice, respectively. Horizontal open and solid bars indicate lights on and lights off, respectively.

Measurement of serum metabolic parameters

Mouse blood was centrifuged for 10 min at maximum speed in a desktop centrifuge. Serum samples were collected and stored at −80 °C. Serum glucose, triglyceride (TG), free fatty acids (FFA), and total cholesterol (T-Cho) levels were measured using kits (Wako Pure Chemical Industries Ltd, Osaka, Japan).

Serum insulin, TNF-α, and TGF-β levels were measured using Mercodia Mouse Insulin ELISA (Mercodia AB, Uppsala, Sweden), BioSource Mouse TNF-α ELISA (BioSource, Camarillo, CA, USA), and Quantikine Mouse/Porcine/Rat TGF-β1 ELISA (R&D Systems, Minneapolis, MN, USA) kits, respectively.

Northern blot analysis

Total RNA was extracted from tissues using guanidinium thiocyanate followed by ISOGEN (Nippon Gene Co. Ltd, Tokyo, 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, Boston, MA, USA) by passive capillary transfer. The probes generated from cDNA fragments of *PAI-1* (bases 138–689; GenBank accession number M33960) and *GAPDH* (bases 133–575; GenBank accession number M17701) were hybridized and detected as described [30]. Samples were normalized against the corresponding *GAPDH* RNA levels.

PAI-1 antigen extraction

Frozen tissue fragments of about 0.2 g were homogenized in 1 mL of Tris-buffered saline (5 mM Tris–HCl, pH 7.4) supplemented with 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate, and incubated overnight at 4 °C on a tilting table. After centrifugation at 3000 × *g*. for 60 min, the protein concentration of the supernatants was determined using Micro BCA Protein Assay kit (Pierce, Rockford, IL, USA). Levels of PAI-1 antigen were evaluated using commercially available ELISA kit as described below.

Measurement of plasma total PAI-1 and active PAI-1 levels

We obtained platelet-poor plasma by immediately mixing blood with 0.2 volumes of 65 mM sodium citrate, pH 7.2, followed by centrifugation at maximum speed in a desktop centrifuge. Plasma total PAI-1 and active PAI-1 levels were measured using total murine PAI-1 and Mouse PAI-1 Activity ELISA kits (both from Molecular Innovations Inc., Southfield, MI, USA), respectively.

Histological analysis of adipose tissue

Adipose tissues were embedded immediately in 4% carboxymethyl cellulose sodium and frozen in liquid nitrogen. Sections of 10 μm for epididymal fat examination were cut, mounted on silanized slides, fixed in 63% methanol, 27% chloroform, 6% formaldehyde and 4% acetic acid (fixing solution) and stained with hematoxylin and eosin.

Statistics

Data are expressed as means ± SEM. Group variations were statistically analyzed using one-way analysis of variance (ANOVA) and further tested by Student's *t*-test with $P < 0.05$ as the criterion for statistical significance.

Results

An initial examination of the plasma PAI-1 levels in leptin-deficient *ob/ob* mice revealed extremely augmented circadian fluctuation of the plasma PAI-1 concentrations (Fig. 1A). To determine the source of these elevated PAI-1 levels, various tissues from lean and obese mice were analyzed by Northern blotting (Fig. 1B). While both the heart and adipose tissues from *ob/ob* mice contained more *PAI-1* mRNA than those from lean compartments of these organs, the increase was most obvious in the adipose tissue. Lung and liver *PAI-1* mRNA levels were slightly, but not significantly, increased in *ob/ob* mice. A subsequent examination of the daily expression profile of *PAI-1* mRNA in the heart and adipose tissues of *ob/ob* mice (Fig. 1C,D) showed that the mRNA levels were augmented in a circadian manner in these tissues.

The *Clock* mutation enhanced the increase in the body weight (BW) of *ob/ob* mice, but did not affect the weight of the lean compartments (Fig. 2A). Obesity-induced hyperglycemia and hyperinsulinemia were developed in *ob/ob* mice (Fig. 2A–C) as reported [31]. However, the*Clock* mutation surprisingly suppressed both hyperglycemia and hyperinsulinemia in *ob/ob* mice without reducing the BW, but did not affect these parameters in the lean compartments (Fig. 2A–C) as we recently reported [32]. Serum T-Cho and TG levels were increased in *ob/ob* mice, and additively increased by the *Clock* mutation (Fig. 2D,E). The *Clock* mutation also induced an increase in serum T-Cho and TG levels in the lean compartments (Fig. 2D,E) as recently described [33]. Serum FFA levels were increased in *ob/ob* mice, and were not affected by the *Clock* mutation both in *ob/ob* mice and in lean compartments (Fig. 2F). Serum TNF-α and TGF-β1 levels did not differ among the genotypes (Fig. 2G,H).

The effects of the *Clock* mutation and a leptin deficiency on *PAI-1* mRNA expression levels considerably differed among tissues. Leptin-deficient obesity did not affect *PAI-1* mRNA expression in the heart, but the *Clock* mutation significantly reduced mRNA levels in both *ob/ob* mice and in lean compartments (Fig. 3A). Obesity also did not induce *PAI-1* mRNA expression in the lungs, but the *Clock* mutation significantly reduced the mRNA levels in *ob/ob* mice (Fig. 3B). The *Clock* mutation suppressed the significant amount of *PAI-1* mRNA expression induced by obesity in the liver of *ob/ob* mice (Fig. 3C). Obesity-induced *PAI-1* mRNA expression was further enhanced by the *Clock* mutation in the adipose tissue of*ob/ob* mice (Fig. 3D). Notably, the *Clock* mutation obviously enhanced the obesity-induced *PAI-1* mRNA expression in the adipose tissue of *ob/ob* mice, but significantly decreased that in the heart, lungs and liver of *ob/ob* mice (Fig. 3A–D). We also evaluated PAI-1 protein levels in the liver (Fig. 3E) and adipose tissue (Fig. 3F). We showed that hepatic PAI-1 antigen levels were significantly increased in *ob/ob* mice, and completely normalized to the wild-type (WT) levels in *Clk/Clk;ob/ob* mice

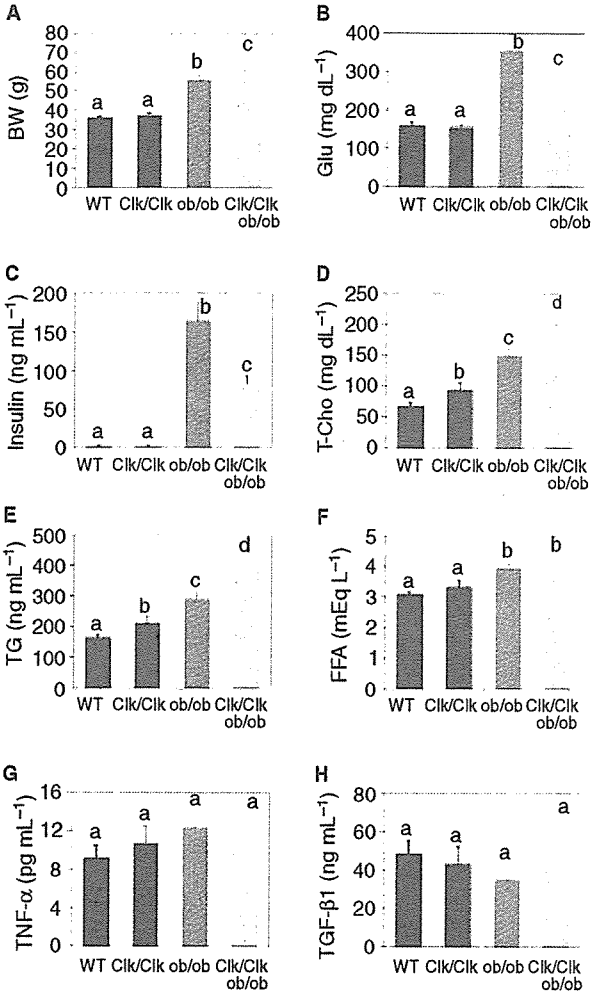

Fig. 2. Metabolic parameters of *Clock* mutated leptin-deficient *ob/ob* mice. Serum was collected from wildtype (WT), homozygous *Clock* mutant (*Clk/Clk*), leptin-deficient (*ob/ob*), and *Clk/Clk;ob/ob* mice (see *Materials and methods*). Values are means ± SEM (n = 12–15). Different characters indicate statistical significance ($P < 0.05$). BW, body weight; Glu, glucose; T-Cho, total cholesterol; TG, triglyceride; FFA, free fatty acids.

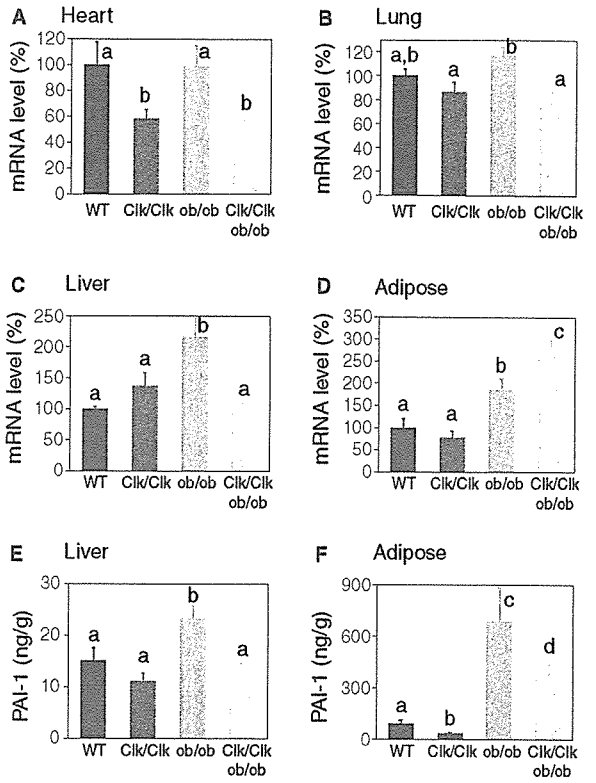

Fig. 3. Expression profiles of PAI-1 mRNA (A–D) and PAI-1 antigen (E–F) of *Clock* mutated leptin-deficient *ob/ob* mice. Total RNA was extracted from tissues of 12–15-week-old male WT, homozygous *Clock* mutant (*Clk/Clk*), leptin-deficient (*ob/ob*), and *Clk/Clk;ob/ob* mice (see *Materials and methods*) and *PAI-1* mRNA levels were determined from Northern blots. Maximum value of WT mice is expressed as 100%. Tissue PAI-1 antigen levels are expressed as antigen (ng) per protein (g). Values are means ± SEM (n = 7–11). Different characters indicate statistical significance ($P < 0.05$).

along with the *PAI-1* mRNA levels. In adipose tissue, PAI-1 antigen levels were increased more than 7-fold in *ob/ob* mice, although the mRNA levels were increased only 2-fold compared with those in WT mice. Adipose PAI-1 antigen levels in *Clk/Clk;ob/ob* mice were 4-fold higher than those in WT mice. Adipose PAI-1 antigen levels were significantly higher in *ob/ob* mice than in *Clk/Clk;ob/ob* mice (Fig. 3F), although the mRNA levels in *ob/ob* mice were significantly lower than those in *Clk/Clk;ob/ob* mice (Fig. 3D).

The *Clock* mutation induced adipocyte hypertrophy in epididymal adipose tissue from lean mice (Fig. 4A,B). Furthermore, the adipocyte hypertrophy induced by the leptin deficiency was obviously enhanced by the *Clock* gene mutation in *ob/ob* mice (Fig. 4C,D).

Plasma antigen levels of total (Fig. 5A) and active (Fig. 5B) PAI-1, which were remarkably increased by the leptin-deficient obesity in *ob/ob* mice, were normalized by the *Clock* mutation (Fig. 5).

Discussion

Obesity induces PAI-1 production both in humans and in experimental animals [6]. However, few investigators have described the circadian profile of plasma PAI-1 levels under conditions of obesity. In the current study, we discovered that plasma PAI-1 levels are increased in a circadian manner in leptin-deficient obese and diabetic *ob/ob* mice. This circadian augmentation of the plasma PAI-1 concentrations appeared to be regulated at the level of gene expression, because tissue *PAI-1* mRNA levels were augmented in a circadian manner in accordance with the plasma antigen levels in *ob/ob* mice. Expression levels of *PAI-1* mRNA were extremely augmented in a circadian manner in adipose tissue from *ob/ob* mice, although levels in other tissues, such as the heart, lungs and liver, of these mice were only slightly increased compared with those of their lean compartments. These results suggested that obesity-induced *PAI-1* mRNA expression is regulated in a tissue-specific manner as reported [6]. The present observations indicated that circadian transactivation of the *PAI-1* gene might be involved in the obesity-induced plasma PAI-1 elevation.

As noted above, CLOCK is the essential component for circadian *PAI-1* gene expression *in vivo*, because tissue *PAI-1*

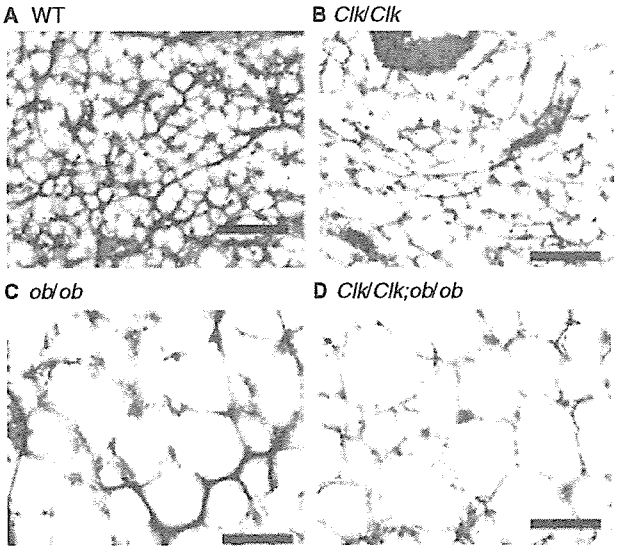

Fig. 4. Histological analysis of adipose tissue in *Clock* mutated leptin-deficient *ob/ob* mice. Epididymal adipose tissues from 12–15-week-old male WT, homozygous *Clock* mutant (*Clk/Clk*), leptin-deficient (*ob/ob*), and *Clk/Clk;ob/ob* mice (see *Materials and methods*) were stained with hematoxylin and eosin. Bars indicate 100 μm.

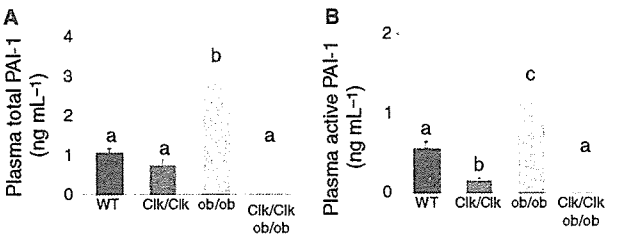

Fig. 5. Plasma total (A) and active (B) PAI-1 levels in *Clock* mutated leptin-deficient *ob/ob* mice. Plasma was collected from WT, homozygous *Clock* mutant (*Clk/Clk*), leptin-deficient (*ob/ob*), and *Clk/Clk;ob/ob* mice (see *Materials and methods*). Values are means ± SEM (n = 12–15). Different characters indicate statistical significance ($P < 0.05$).

mRNA levels are circadian [13] and the mRNA rhythm is abolished in homozygous *Clock* mutant mice [26,27]. We examined whether the CLOCK molecule is involved in the obesity-induced plasma PAI-1 increase in *Clk/Clk;ob/ob* mice (see Materials and methods). The results showed that the *Clock* gene mutation normalized the plasma PAI-1 concentrations augmented by obesity in*ob/ob* mice by affecting the mRNA expression levels in a tissue-dependent manner. The *Clock* mutation significantly suppressed obesity-induced *PAI-1* mRNA expression in the heart, lungs and liver, but obviously enhanced the expression in adipose tissue from *ob/ob* mice. These findings suggested that CLOCK-dependent transactivation via the E-box element(s) located in the flanking region of *PAI-1* gene is impaired in the heart, lungs and liver of *Clk/Clk;ob/ob* mice. On the other hand, the increase in *PAI-1* mRNA levels in the adipose tissue of *Clk/Clk;ob/ob* mice seemed to indirectly result from altered fat-cell features as discussed below. Furthermore, the amount of mRNA expression of the *PAI-1* gene in adipose tissue appeared to contribute only slightly to the plasma PAI-1 levels in *ob/ob* mice as suggested [34]. In the present study, plasma PAI-1 as well as *PAI-1* mRNA and antigen levels in tissues were examined only at the peak time (14:00 h) of plasma PAI-1 levels [29]. In homozygous *Clock* mutant mice, circadian fluctuation of both *PAI-1* mRNA expression and plasma PAI-1 levels is completely flattened at the bottom levels of those of WT mice (N. Ohkura *et al.*, unpublished data). Therefore, *PAI-1* mRNA expression and plasma PAI-1 levels in *Clk/Clk;ob/ob* mice seemed to be lower than those in *ob/ob* mice throughout the day, although both *PAI-1* mRNA and plasma PAI-1 levels are circadian in *ob/ob* mice, as shown in Fig. 1.

The *Clock* mutation induced adipocyte hypertrophy in epididymal adipose tissues from lean mice (Fig. 4A,B). Furthermore, the *Clock* mutation obviously enhanced the adipocyte hypertrophy induced by the leptin deficiency in *ob/ob* mice (Fig. 4C,D). Significantly more PAI-1 is secreted per adipocyte in obese donors than in individuals within the normal weight range [4,5]. More PAI-1 is produced by large than by small fat cells, independently of the fat depot [4,5]. The present study found that BW was additively increased by the *Clock* gene mutation in leptin-deficient *ob/ob* mice. Serum TG levels were also additively elevated by the *Clock* gene mutation in*ob/ob* mice. These observations appear to be closely associated with enlarged fat cells in addition to an increased adipose tissue mass in *Clk/Clk;ob/ob* mice. The adipose tissue mass is generally determined by two distinct processes: the formation of new adipocytes from precursor cells (adipocyte differentiation), and an increase in adipocyte size because of fat storage (adipocyte hypertrophy) [35]. Shimba *et al.* [36] recently demonstrated that BMAL1 is involved in adipocyte differentiation *in vitro*. Using fibroblasts derived from homozygous *Clock* mutant embryos, we found that the CLOCK molecule is also involved in adipocyte differentiation *in vitro* (G. I. Atsumi *et al.*, unpublished data). These findings and our present results suggest that the bHLH transcription factors of circadian clock components such as CLOCK and BMAL1 play a critical role in the regulation of both differentiation and fat storage of adipocytes *in vivo*. Mutations of these molecules might suppress adipocyte differentiation and induce hypertrophy. Furthermore, *Clock* mutation-induced hypertrophy of adipocytes seemed to result from a leptin-independent mechanism, as the *Clock* mutation additively induced adipocyte hypertrophy in leptin-deficient *ob/ob* mice. Such hypertrophy might be key to explaining the *PAI-1* mRNA accumulation in adipose tissue of *Clk/Clk;ob/ob* mice.

Various specific hormones, cytokines and metabolic factors are implicated in obesity-induced *PAI-1* expression in adipocytes [4,5]. Both TNF-α and TGF-β are representative cytokines that induce *PAI-1* gene expression in adipocytes [4,5]. Interestingly, these cytokines and their receptors are produced in adipose tissue in an obesity-induced manner in both rodents and humans [5] [4]. However, we could not identify any significant association between serum TNF-α or TGF-β1 levels and those of *PAI-1* mRNA in adipose tissue. Several types of

lipoproteins and FFA induce *PAI-1* gene expression [5]. An increase in serum TG levels might also be responsible for the PAI-1 induction in adipose tissue of *Clk/Clk;ob/ob* mice. Furthermore, *PAI-1* mRNA levels are determined not only by the rate of transcription but also by the rate at which mRNA transcripts are degraded [37]. Thus, post-transcriptional regulation of *PAI-1* gene expression might be involved in generating the increased *PAI-1* mRNA levels in the adipose tissue of *Clk/Clk;ob/ob* mice.

The key finding in this study is that the *Clock* mutation normalized plasma PAI-1 levels in obese and diabetic *ob/ob* mice, while the mRNA and antigen levels of PAI-1 were not normalized in the adipose tissue of *Clk/Clk;ob/ob* mice. As noted above, adipose tissue has been generally considered the most likely contributor to elevated plasma PAI-1 concentrations in obese humans [2,4,5]. However, adipose tissue simply as a source of PAI-1 cannot satisfactorily explain the increase in plasma PAI-1 levels in obese people [34]. In fact, PAI-1 levels and PAI-1 antigen contents are not significantly associated in either intra-abdominal or s.c. adipose tissues in *ob/ob* mice, whereas plasma PAI-1 levels are closely correlated with *PAI-1* mRNA and antigen levels in the liver of these mice [34]. We also evaluated PAI-1 protein levels in the liver and adipose tissue (Fig. 3E,F). We found that hepatic PAI-1 antigen levels were significantly increased in *ob/ob* mice, but completely normalized to the WT levels in *Clk/Clk;ob/ob* mice along with the *PAI-1* mRNA levels. These hepatic PAI-1 antigen and plasma PAI-1 levels were closely associated (Fig. 5). In adipose tissue, PAI-1 antigen levels were increased more than 7-fold in *ob/ob* mice, although the mRNA levels were increased only 2-fold compared with those in WT mice. Adipose PAI-1 antigen levels in *Clk/Clk;ob/ob* mice were 4-fold higher than those in WT mice. Adipose PAI-1 antigen levels were significantly higher in *ob/ob* mice than in *Clk/Clk;ob/ob* mice, although the mRNA levels in *Clk/Clk;ob/ob* mice were significantly higher than those in *ob/ob* mice. These observations suggest that post-transcriptional regulation is involved in adipose PAI-1 expression. More importantly, plasma PAI-1 levels appeared to be closely related to the hepatic *PAI-1* mRNA and antigen levels rather than to those in the adipose tissue.

The present study found that the *Clock* mutation rather surprisingly suppressed both hyperglycemia and hyperinsulinemia in *ob/ob* mice, although it enhanced the BW increase and adipocyte hypertrophy in these mice (Figs 2 and 4). These findings suggest that the *Clock* gene mutation improved insulin sensitivity but enhanced obesity in *ob/ob* mice. Sterol regulatory element binding protein 1 (SREBP-1) regulates the transcription of several factors required for lipogenesis, such as fatty acid synthase (*FAS*), acetyl-CoA carboxylase (*ACC*), and ATP-citrate lyase (*ACL*), and the circadian mRNA expression of *SREBP-1* is diminished in *Clock* mutant mice [32]. SREBP-1 is involved in the pathogenesis of hepatic insulin resistance [38]. We also found that the circadian mRNA expression of *ACC* and *ACL* is damped in the liver of *Clock* mutant mice [32]. Taken together, these observations suggest that the *Clock* mutation improves obesity-induced insulin resistance by reducing *de novo* fatty acid synthesis in the liver. In humans, plasma PAI-1 levels are increased under insulin-resistant states and normalized by thiazolidinedione [39–42]. Therefore, the *Clock* mutation might affect hepatic *PAI-1* gene expression by regulating insulin sensitivity in obese *Clk/Clk;ob/ob* mice.

The mechanisms involved in the increased PAI-1 production in obese and diabetic patients have been only partially explained and the origin of PAI-1 remains uncertain [2]. The present study showed that the obesity-induced *PAI-1* mRNA expression in *ob/ob* mice is obviously suppressed in the heart, lungs and liver of *Clk/Clk;ob/ob* mice in accordance with the plasma PAI-1 levels, although the *Clock* mutation significantly increased the mRNA expression in adipose tissue from *ob/ob* mice. However, we could not define whether the *Clock* mutation directly or indirectly reduced the *PAI-1* mRNA levels in tissues by altering metabolic conditions such as lipogenesis and insulin sensitivity. Importantly, our results suggest that the circadian clock component CLCOK is critical for the obesity-induced impairment of fibrinolytic activity, and therefore increases the risk for cardiovascular disease. The current findings further suggested that PAI-1 production in adipose tissue is not a major cause for the systemic increase of plasma PAI-1 levels in obese individuals. Understanding the molecular mechanism of the obesity-induced plasma PAI-1 increase should lead to the identification of novel pharmaceutical targets with which to treat obesity or diabetes-induced hemostatic disorders.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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A novel E4BP4 element drives circadian expression of *mPeriod2*

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ABSTRACT

***Period2* (*Per2*) is an essential component of the mammalian clock mechanism and robust circadian expression of *Per2* is essential for the maintenance of circadian rhythms. Although recent studies have shown that the circadian E2 enhancer (a non-canonical E-box) accounts for most of the circadian transcriptional drive of *mPer2*, little is known about the other *cis*-elements of *mPer2* oscillatory transcription. Here, we examined the contribution of E4BP4 to *Per2* mRNA oscillation in the cell-autonomous clock. Knockdown experiments of *E4BP4* in both Northern blots and real-time luciferase assays suggested that endogenous E4BP4 negatively regulates *Per2* mRNA oscillation. Sequence analysis revealed two putative E4BP4-binding sites (termed A-site and B-site) on mammalian *Per2* promoter regions. Luciferase assays with mutant constructs showed that a novel E4BP4-binding site (B-site) is responsible for E4BP4-mediated transcriptional repression of *Per2*. Furthermore, chromatin immunoprecipitation assays *in vivo* showed that the peak of E4BP4 binding to the B-site on the *Per2* promoter almost matched the trough of *Per2* mRNA expression. Importantly, real-time luciferase assays showed that the B-site in addition to the E2 enhancer is required for robust circadian expression of *Per2* in the cell-autonomous clock. These findings indicated that E4BP4 is required for the negative regulation of mammalian circadian clocks.**

INTRODUCTION

Physiological and behavioral circadian rhythms are features of organisms ranging from bacteria to humans and are driven by an endogenous clock that consists of transcriptional/translational feedback loops of clock genes (1–4). The first clock mutants were isolated by a forward genetics approach using eclosion rhythms as a phenotype to clock components in *Drosophila* (5). These mutant flies exhibited similar defects in locomotor activity rhythms and the corresponding molecular defects were later identified in the *period* (*per*) gene (6,7). Since then, several additional clock genes, including *timeless*, *clock*, *cycle*, *doubletime* and recently *vrille*, have been identified in *Drosophila* (8–12). Orthologs of most *Drosophila* circadian clock genes have been identified in mammals, highlighting general conservation of the clock mechanism.

Three mammalian homologues (*Per1*, *Per2* and *Per3*) of the *Drosophila circadian clock gene per* have been identified (13–19). Gene targeting studies have demonstrated that $mPer2^{Brdm1}$ mutant mice display a short-circadian period followed by a loss of circadian rhythmicity in constant darkness (20). In contrast, a deletion of *mPer1* only shortens the period length and *mPer3* knockout mice have an essentially normal clock (21,22), indicating that *Per2* plays a prominent role among the three mammalian *Per* genes. Moreover, constitutively overexpressed *mPer2* mRNA rapidly damps cellular rhythm (23), indicating that robust circadian expression of *Per2* is essential for the maintenance of circadian rhythms.

Recent studies have shown that the circadian E2 enhancer (a non-canonical E-box) accounts for most of the circadian transcriptional drive of the *mPer2* gene by CLOCK:BMAL1 (24,25), but little is known about the other *cis*-elements of *mPer2* oscillatory transcription.

The bZIP transcription factor *E4BP4* (also called *NFIL3*) is a mammalian homologue of *vrille* (*vri*) that functions as a key negative component of the *Drosophila* circadian clock (12,26,27). E4BP4 probably plays an important role in the phase-delaying process of chickens as a light-dependent suppressor of *cPer2* (28). Although E4BP4 is believed to be involved in the mammalian circadian clock (25,29,30), direct evidence has yet to support a requirement for E4BP4-mediated regulation of these clocks.

Here, we show that E4BP4 functions as a repressor of *Per2* transcription through a novel E4BP4-binding site in the promoter. We also show that E4BP4 binding is required for

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