Night-time restricted feeding normalises clock genes and *Pai-1* gene expression in the *db/db* mouse liver

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Abstract

Aims/hypothesis. An increase in PAI-1 activity is thought to be a key factor underlying myocardial infarction. Mouse *Pai-1* (*mPai-1*) activity shows a daily rhythm in vivo, and its transcription seems to be controlled not only by clock genes but also by humoral factors such as insulin and triglycerides. Thus, we investigated daily clock genes and *mPai-1* mRNA expression in the liver of *db/db* mice exhibiting high levels of glucose, insulin and triglycerides.

Methods. Locomotor activity was measured using an infrared detection system. RT-PCR or in situ hybridisation methods were applied to measure gene expression. Humoral factors were measured using measurement kits.

Results. The *db/db* mice showed attenuated locomotor activity rhythms. The rhythmic expression of *mPer2* mRNA was severely diminished and the phase of *mBmal1* oscillation was advanced in the *db/db* mouse

liver, whereas *mPai-1* mRNA was highly and constitutively expressed. Night-time restricted feeding led to a recovery not only from the diminished locomotor activity, but also from the diminished *Per2* and advanced *mBmal1* mRNA rhythms. Expression of *mPai-1* mRNA in *db/db* mice was reduced to levels far below normal. Pioglitazone treatment slightly normalised glucose and insulin levels, with a slight reduction in *mPai-1* gene expression.

Conclusions/interpretation. We demonstrated that Type 2 diabetes impairs the oscillation of the peripheral oscillator. Night-time restricted feeding rather than pioglitazone injection led to a recovery from the diminished locomotor activity, and altered oscillation of the peripheral clock and *mPai-1* mRNA rhythm. Thus, we conclude that scheduled restricted food intake may be a useful form of treatment for diabetes.

Keywords Clock gene · *db/db* · Diabetes · Insulin · Liver · Peripheral oscillator · Restricted feeding

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Abbreviations: PB, phosphate buffer · RF, restricted feeding · SCN, suprachiasmatic nucleus · STZ, streptozotocin · ZT, Zeitgeber time

Introduction

Various physiological and behavioural phenomena are under the control of an endogenous circadian rhythm [1]. The molecular circadian clock system is thought to be based on transcriptional/translational feedback loops consisting of several ‘clock genes’ and their products [2]. For example, in mammals, CLOCK, a member of the basic helix-loop-helix-PAS domain that contains transcription factors, heterodimerises with BMAL1 and activates transcription of the *Per1/2* and *Cry1/2* genes. On the other hand, CRY1/2 proteins negatively regulate the E-box binding ability of CLOCK: BMAL1 [2]. Clock genes are expressed not only in the suprachiasmatic nucleus (SCN) of the hy-

pothalamus where the central clock exists, but also in other brain regions and various peripheral tissues. Recent study shows that mammalian peripheral tissues contain an oscillator that couples to a central oscillator under normal conditions [3], and the clock genes in these peripheral tissues show clear circadian rhythms [4]. Plasminogen activator inhibitor type 1 (PAI-1), a primary regulator of the fibrinolytic cascade, has two functional E-box motifs in the promoter [5], and its gene expression is regulated by the circadian clock [6].

In vitro investigations have found that both glucose and insulin stimulate the production and subsequent release of PAI-1 in human vascular endothelial and smooth muscle cells [7, 8]. In addition, it was suggested that hyperglycaemia is responsible for stimulating transcription of the *Pai-1* gene in vascular smooth muscle cells [9]. Hyperglycaemia, which results from the absolute or relative impairment of insulin secretion and/or insulin action is a characteristic of diabetes mellitus, of which there is Type 1 and Type 2 [10].

The *db/db* mouse is a recognised animal model of Type 2 diabetes patients. These mice, which lack functional leptin receptors [11, 12], become identifiably obese around 3 to 4 weeks of age and show symptoms of insulin resistance. Diabetes in *db/db* mice can be divided into two stages [13, 14]: the hyperinsulinaemic phase (phase I) and the hypoinsulinaemic phase (phase II) after the age of 2 to 3 months. Increased plasma insulin levels seen at the age of 10 days continue to increase until the age of 2 to 3 months (phase I), at which time there is an abrupt decrease followed by a return to normal levels. Blood glucose content is relatively normal or slightly elevated during phase I. However, after the age of 2 to 3 months (phase II), the blood glucose level markedly increases and remains at a high level until death.

Application of glucose to rat-1 cell culture reduces *Per1* and *Per2* gene expression and resets the circadian rhythm of clock gene expression [15]. In addition, acute administration of insulin increases *Per1* gene expression in rat-1 cell culture [16]. These findings provided the impetus for our present investigation of clock gene and *Pai-1* gene expression in the liver of Type 1 diabetic *db/db* mice, since these mice exhibit hyperglycaemia and hyperinsulinaemia.

Indeed, strategies that have been shown to prevent diabetes, such as diet and exercise or administration of thiazolidinediones, also decrease plasma PAI-1 concentrations [17, 18, 19]. Thus, in this study, we allowed diabetic *db/db* mice daily access to food for only a limited period of time and examined whether this restricted feeding or pioglitazone administration led to a recovery from abnormal clock gene and *mPai-1* gene expression in the liver.

Materials and methods

Animals. Male C57BL/KsJ-*db/db* Jcl (*db/db*) and C57BL/KsJ-*db/+m* Jcl (*db/+*) mice were purchased from Clear Japan (Tokyo, Japan). Male *ddY* mice were obtained from Takasugi Experimental Animals (Saitama, Japan). Animals were maintained on a light–dark cycle (12 h light, 12 h dark, with lights on at 08.30 hours) at room temperature (23 °C), and were given free access to food and water except for during daily restricted feeding (RF) experiments. All animals were treated in accordance with the Principles of Laboratory Animal Care, and with the Law (no. 105) and Notification (no. 6) of the Japanese Government.

RNA isolation and RT-PCR. Mice ($n=3-6$ mice per time point) were deeply anaesthetised with ether and intracardially perfused with ice-cold saline. After perfusion, the liver was isolated, frozen in liquid nitrogen and stored at -80 °C until RNA isolation. Total RNA was extracted using Isogen reagent (Nippon Gene, Tokyo, Japan). After RNase-free DNase treatment, 100 ng of total RNA was reverse transcribed and amplified using the Superscript One-Step RT-PCR System (Invitrogen, Calif., USA) in a GeneAmp PCR System 9700 (Applied Biosystems, Calif., USA). A more detailed description of the specific primer pairs of clock (*mPer1*, *mPer2* and *mBmal1*), *mPai-1* and β -actin genes, and the semi-quantitative RT-PCR method used for measuring the expression level of mRNA can be found in Minami et al. [20]. Briefly, each target clock gene cDNA was co-amplified with β -actin cDNA in a single PCR tube. The PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide and analysed by an EDAS-290 system (Kodak, N.Y., USA). The intensity of PCR product of the target gene was normalised to the intensity of β -actin. The amplitude (ratio of peak : trough) and phase determined by this method were reproducible and found to be comparable to those determined by Northern blot [21]. Thus, the present experimental conditions are considered capable of detecting a daily change of clock gene expression in the mouse liver.

In situ hybridisation. The quantity of *mPer1* and *mPer2* mRNA expression in the SCN was assessed by in situ hybridisation as previously described [22]. On the day of the experiment, mice were deeply anaesthetised by ether and were intracardially perfused with 0.1 mol/l phosphate buffer (PB; pH 7.4) containing 4% paraformaldehyde at the following Zeitgeber times (ZT; ZT 0 is defined as lights on, and ZT 12 as lights off): 0, 6, 12 and 18. Six and four mice were used for each time point in *db/+* mice and *db/db* mice respectively. After animals were killed, brains were removed, post-fixed in 0.1 mol/l PB containing 4% paraformaldehyde for 24 h at 4 °C, and transferred into 20% sucrose in 0.1 mol/l PB for 72 h at 4 °C. Brain slices (40 μ m thick) containing the temporal cortex and the SCN were made using cryostat (Microm, HM505E; Heidelberg, Germany) then placed in 2 $\times$ standard saline citrate until processing for hybridisation. Slices were first treated with 1 μ g/ml proteinase K in 10 mmol/l Tris-HCl buffer (pH 7.5) containing 10 mmol/l EDTA for 10 min at 37 °C, followed by 0.25% sodium dodecyl sulphate) containing 33 P-labelled cRNA probes for 16 h at 60 °C. Radioisotope (α -[33 P] UTP; PerkinElmer Life Sciences, Boston, Mass., USA)-labelled antisense cRNA probes were made from restriction-enzyme-linearised cDNA templates. After high-stringency post-hybridisation wash in 2 $\times$ standard saline citrate/50% formamide, slices were treated with RNaseA (10 μ g/ml) for 30 min at 37 °C. Slices were washed in 2 $\times$ standard saline citrate/50% formamide and air-dried. Images were visualised by autoradiogram using BioMax MR film (Eastman Kodak, Rochester, N.Y.,

USA) and analysed with an image analysing system (MCID; Imaging Research, St. Catharines, Ont, Canada) after conversion into optical density by ^{14}C -autoradiographic microscans (Amersham Pharmacia Biotech, Little Chalfont, UK).

We subtracted the intensity of the optical density in the corpus callosum from that in the SCN and regarded this value as the net intensity in the SCN. The intensity values of sections taken from rostral to caudal areas of the SCN (four sections per mouse) were then summed; the sum was considered to be a measure of the amount of clock gene data analysis. Four to six animals made up each ZT data point, and the values were expressed as means $\pm$ SEM.

RF experimentation. RF experimentation was performed as previously described [23] with the exception that food could only be accessed for 4 h during the night. In brief, after 1 day of fasting (termed day 0), mice were allowed access to food for 4 h from ZT 18 to ZT 22 for 6 consecutive days (day 1 to day 6) or 12 consecutive days (day 1 to day 12). In RF groups, animals were killed at ZT 0, 6, 12 and 18 on day 6 or day 12 under RF conditions. In groups with free access to food and water, animals were killed under these conditions.

Locomotor activity rhythm. Mice were housed individually in transparent plastic cages (31 $\times$ 20 $\times$ 13 cm) under light–dark (12 h, 12 h) conditions and their locomotor activity was measured using an area sensor (F5B; Omron, Tokyo, Japan) with an infrared detector. Locomotor activity was continuously recorded in 6-min epochs by personal computer. Chi square periodogram analysis [24] was applied to examine the periodicity ranging from 20- to 30-h periods. In general, Qp value calculated by chi square periodogram is related to the robustness of circadian rhythm [25]; a high Qp value indicates strong robustness of rhythm. Daily locomotor activity counts were also calculated.

Measurement of blood glucose, insulin and triglycerides. Immediately prior to liver isolation, 0.5 ml of blood was withdrawn from the mouse heart, and blood glucose was measured by Novo Assist Plus (Novo Nordic Pharma, Tokyo, Japan) according to manufacturer's instructions. Insulin and triglycerides were measured in the plasma using the Insulin Measurement Kit (Morinaga, Tokyo, Japan) and Triglyceride E-test (Wako, Tokyo, Japan) according to manufacturer's instructions.

Drugs. Streptozotocin (STZ) was purchased from Wako Chemical (Osaka, Japan). STZ was dissolved in 0.1 mol/l sodium citrate buffer (pH 5.2) and intraperitoneally injected (200 mg/kg) into 13-week-old *ddY* mice.

Pioglitazone (10 mg/kg, p.o.; Wako Chemical, Osaka, Japan) suspended in 0.5% carboxymethylcellulose was administered to *db/db* mice (10 weeks old) at ZT 12 for 12 days. On day 13, mice were killed at ZT 6 or ZT 18, with no drug administration. Control *db/db* and *db/+* mice were administered with 0.5% carboxymethylcellulose at a dose of 10 ml/kg.

Statistical analysis. The values are expressed as means $\pm$ SEM. Rhythmicity of each group was tested by one-way ANOVA. The significance of differences between groups at each time point was determined by the Student's *t* test. Differences were considered significant when the associated probability value was less than 0.05.

Results

Locomotor activity and daily expression of clock genes in the *db/db* mouse liver. Using a semi-quantitative RT-PCR method, we examined clock (*mPer1*, *mPer2*, *mBmal1*) and *mPai-1* gene expression at four time points in the liver of *db/+* and *db/db* mice placed in light–dark conditions. We used both phase I (6–8 weeks old) and phase II (13–14 weeks old) *db/db* mice to study the effect of diabetic progress on clock gene mRNA expression. The *db/+* mice showed clear daily locomotor activity rhythms under light–dark conditions (Fig. 1a). The mRNA levels of all clock genes examined showed a clear daily rhythm for both age groups of *db/+* mice (Fig. 1c). One-way ANOVA revealed a significant rhythmicity in all clock gene expression in *db/+* mice (phase I: *mPer1*, F [3, 20]=8.3, $p<0.01$; *mPer2*, F [3, 20]=12.9, $p<0.01$; *mBmal1*, F [3, 19]=8.4, $p<0.01$; phase II: *mPer1*, F [3, 19]=11.9, $p<0.01$; *mPer2*, F [3, 19]=12.218, $p<0.01$; *mBmal1*, F [3, 19]=22.9, $p<0.01$). Levels of *mPer1* and *mPer2* mRNA peaked during the night (ZT 12 and ZT 18 respectively), while *mBmal1* mRNA levels peaked in the morning (ZT 0).

The daily locomotor activity rhythm of phase I *db/db* mice was moderately attenuated (Fig. 1a). All phase I *db/db* mice ($n=8$) showed significant rhythmicity as evaluated by chi square periodogram analysis. All clock gene mRNA was expressed in a daily fashion under these experimental conditions (*mPer1*, F [3, 8]=7.7, $p<0.01$; *mPer2*, F [3, 8]=4.3, $p<0.01$; *mBmal1*, F [3, 8]=37.4, $p<0.01$, one-way ANOVA) in phase I *db/db* mice, but the peak time of *mPer2* mRNA was advanced from ZT 18 to ZT 12. While *mPer1* and *mPer2* mRNA expression were low at ZT 6 in *db/+* mice, moderately expressed *mPer1* and *mPer2* could be observed at ZT 6 in the phase I *db/db* mouse liver (Fig. 1b, c; *mPer1*: $df=9$, $p<0.05$; *mPer2*: $df=11$, $p<0.01$, Student's *t* test vs *db/+*). On the other hand, *mBmal1* expression in phase I *db/db* mice was similar to that in *db/+* mice.

The daily locomotor activity rhythm of phase II *db/db* mice was severely diminished (Fig. 1a). About 75% of the phase II *db/db* mice (6/8) did not show significant rhythmicity as evaluated by chi square periodogram analysis. Daily counts of locomotor activity were significantly lower in phase II *db/db* mice (1824 $\pm$ 659) than in *db/+* mice (6078 $\pm$ 1190) ($df=8$, $p<0.01$, Student's *t* test). The rhythmicity of *mPer2* mRNA was attenuated (F [3, 8]=2.2, $p=0.17$, one-way ANOVA). There was elevated *mPer1* mRNA expression (Fig. 1, right column; ZT 0: $df=7$, $p<0.05$; ZT 6: $df=8$, $p<0.01$; ZT 12: $df=8$, $p<0.01$, Student's *t* test vs *db/+*). However, the rhythmicity of *mPer1* and *mBmal1* mRNA expression was still significant (*mPer1*: F [3, 8]=12.8, $p<0.01$; *mBmal1*, F [3, 8]=20.7, $p<0.01$, one-way ANOVA). The *mBmal1* mRNA rhythm was diminished and advanced in phase II *db/db* mice but not in *db/+* mice (Fig. 1b, c).

Fig. 1. Daily locomotor activity rhythm and fluctuation of clock genes in the liver of *db/+* and *db/db* mice. Left panels: phase I (6- to 8-week-old) mice; right panels: phase II (13- to 14-week-old) mice. **a.** Representative double-plotted actograms showing locomotor activity records of *db/+* (upper) and *db/db* (lower) mice under light-dark (12 h, 12 h) conditions. The uppermost light and dark bars indicate light and dark periods respectively. Right panel shows result of chi square periodogram analysis. Horizontal axis shows the period and vertical axis shows the amplitude (Qp). The ascending

straight line in the periodograms represents $p < 0.01$. **b.** Representative electrophoresis photographs of PCR products from the *db/+* and *db/db* mouse liver at ZT 0, 6, 12 and 18. **c.** Daily mRNA abundance of each clock (*mPer1*, *mPer2*, *mBmal1*) and *mPai-1* gene was plotted as a relative mRNA level that was normalised to *β-actin* mRNA. The lowest value was set to 1. Each value represents the mean $\pm$ SEM ($n = 3-6$ mice at each time point). ** $p < 0.01$, * $p < 0.05$ vs *db/+* at each time point (Student's *t* test). Open circles, *db/+*; closed circles, *db/db*

Fig. 2. Daily *mPer1* and *mPer2* mRNA expression in the suprachiasmatic nucleus (SCN) of *db/+* and phase II *db/db* mice. Upper: representative autoradiograms showing *mPer1* (a) and *mPer2* (b) mRNA of *db/+* (upper) and *db/db* (lower) mice at each ZT. Lower: expression of *mPer1* (a) and *mPer2* (b) mRNA levels of each genotype (13–14 weeks of age) at ZT 0, 6, 12 and 18. Each value represents the mean $\pm$ SEM ($n=4-6$ mice per time point). Open circles, *db/+*; closed circles, *db/db*.

Daily expression of *mPai-1* gene in the *db/db* mouse liver. In the liver of phase I *db/+* mice, *mPai-1* expression was very weak at all sampling times (Fig. 1c), and not significant ($F [3, 8]=2.3$, $p=0.14$, one-way ANOVA), whereas phase I *db/db* mice exhibited a clear daily rhythm of *mPai-1* mRNA with a peak at ZT 18 (Fig. 1c; $F [3, 8]=15.0$, $p<0.01$, one-way ANOVA). Phase II *db/+* mice exhibited weakly expressed *mPai-1* during the day ($F [3, 8]=17.9$, $p<0.01$, one-way ANOVA), whereas *db/db* mice exhibited strongly expressed *mPai-1* throughout the entire day with a peak at ZT 12 (Fig. 1c; $F [3, 8]=3.7$, $p=0.06$, one-way ANOVA).

Daily expression of *mPer* genes in the SCN of *db/db* mice. We examined *mPer1* and *mPer2* expression in the SCN of phase II *db/db* mice at four time points. In contrast to *mPer* mRNA expression in the liver, *mPer1* mRNA expression in the SCN was not affected (Fig. 2; $F [1, 26]=1.5$, $p=0.22$, two-way ANOVA) and *mPer2* mRNA expression in the SCN was slightly affected (Fig. 2; $F [1, 30]=4.9$, $p<0.05$, two-way ANOVA).

Fig. 3. Effect of streptozotocin (STZ) on locomotor activity, and clock and *mPai-1* gene expression. **a.** Representative double-plotted actograms showing daily locomotor activity before and after vehicle (upper) or STZ (lower) injection (arrows). The uppermost light and dark bars indicate light and dark periods respectively. Right panel shows result of chi square periodogram analysis. Horizontal axis shows the period and vertical axis shows the amplitude (Qp). The ascending straight line in the periodograms represents $p<0.01$. **b.** Representative elec-

trophoresis photographs of PCR products from 5 days after vehicle injection (ctrl) and STZ injection (STZ) at ZT 0, 6, 12 and 18. **c.** Daily mRNA abundance of each clock and *mPai-1* gene from control (ctrl) and STZ-treated mice was plotted as a relative mRNA level that was normalised to β -actin mRNA. The lowest value was set to 1. Each value represents the mean $\pm$ SEM ($n=3$ mice per time point). ** $p<0.01$, * $p<0.05$ vs control (Student's *t* test). Open circles, control; closed circles, STZ.

Locomotor activity and daily expression of clock genes in the STZ-induced diabetic mouse liver. The phases of clock and clock-controlled gene mRNA rhythms were previously shown to have advanced for approximately 3 hours in the STZ-induced diabetic rat heart [26]. We examined locomotor activity rhythm and clock gene mRNA expression in the liver of STZ-injected mice. The blood glucose level of STZ-injected mice at ZT 12, 5 days after injection (29.4 ± 2.4 mmol/l), was significantly higher than that of control mice (7.6 ± 0.69 mmol/l; $p < 0.01$, Student's *t* test) and comparable to that of phase II *db/db* mice (24.2 ± 1.3 mmol/l). The averaged daily activity counts were slightly reduced from 9488 ± 891 to 7309 ± 2062 by STZ injection ($df=9$, $p=0.36$, Student's *t* test; Fig. 3a). The vehicle injection had no effect on locomotor activity counts ($11\,469 \pm 649$ for before injection, $10\,956 \pm 1964$ for after injection). Although the robustness indicated by Qp values was also slightly reduced by STZ injection, 24-h rhythmicity was maintained in all STZ-treated mice.

In STZ-treated mice, the daily rhythm of *mPer2* mRNA expression was diminished and the peak of the *mBmal1* mRNA rhythm advanced from ZT 0 to ZT 18 (Fig. 3). The peak of the *mPer1* mRNA rhythm was not affected by STZ treatment. However, as seen in *db/db* mice, moderate *mPer1* and *mPer2* mRNA expression was observed at ZT 6 in the STZ-injected mouse liver (Fig. 3b, c; *mPer1*: $df=8$, $p < 0.01$; *mPer2*: $df=8$, $p < 0.01$, Student's *t* test vs control). The expression of *mPai-1* in both control and STZ-treated mice was very low at all ZT points, and there was no significant difference between the two groups ($F [1, 16]=0.07$, $p=0.78$, two-way ANOVA).

RF-induced recovery from abnormal expression of clock and *mPai-1* genes in the *db/db* mouse liver. Phase II *db/+* and *db/db* mice were placed on a night-time RF (ZT 18–22) schedule for 6 or 12 days under light–dark conditions. As shown in Figure 4a, *db/+* mice placed under RF for 12 days maintained clear daily locomotor activity rhythms similar to those seen before RF. The *db/db* mice, which normally have no or a severely diminished locomotor activity rhythm when given free access to food and water, showed an RF-dependent, clear daily locomotor activity rhythm under RF conditions (Fig. 4a). Chi square periodogram analysis demonstrated the recovery of significant rhythmicity. Daily counts of locomotor activity were also increased by RF treatment (1.7 times in *db/+* mice and 4.5 times in *db/db* mice). Thus, there was no significant difference in locomotor activity count between the two groups during the RF period ($df=8$, $p=0.36$, Student's *t* test).

When given free access to food and water, *db/+* mice showed a clear daily expression of clock gene mRNA (Fig. 4). Although 6 days of RF did not affect clock gene expression in *db/+* mice, 12 days of RF re-

duced the amplitude of the rhythm of *mPer2* in these mice. When *db/db* mice were shifted from free access feeding conditions to RF for 6 or 12 days, *mPer2* mRNA rhythmicity changed from severely diminished to robust rhythmic expression in the liver (Fig. 4). In particular, the *mPer2* expression at ZT 6 was reduced to the level seen in *db/+* mice. Furthermore, the peak expression of *mBmal1* mRNA returned to ZT 0 from ZT 18, and the amplitude of the rhythm also returned to the amplitude seen in *db/+* mice. Although *mPai-1* mRNA expression was low in *db/+* mice with free access to food and water, RF treatment lowered the levels of *mPai-1* mRNA in a manner dependent on the RF treatment period (6 or 12 days). Under free access feeding conditions, *db/db* mice exhibited arrhythmic ($F [3, 8]=1.06$, $p=0.41$, one-way ANOVA) high expression of *mPai-1* in the liver. RF treatment for 6 days lowered the *mPai-1* expression in *db/db* mice, and RF for 12 days eliminated the *mPai-1* mRNA expression (Fig. 4c). During RF, the phase of the mRNA expression rhythm of all clock genes in *db/db* mice started to resemble that of control (*db/+*) mice (Fig. 4).

We examined the blood insulin, glucose and triglyceride levels before and after RF treatment in *db/db* mice. RF for 12 days lowered the insulin level at ZT 6 ($df=5$, $p < 0.05$, Student's *t* test), blood glucose level at all sampling points (ZT 0: $df=5$, $p < 0.05$; ZT 6: $df=5$, $p < 0.05$; ZT 12: $df=5$, $p < 0.01$; ZT 18: $df=5$, $p < 0.01$, Student's *t* test), and triglyceride level (ZT 0: $df=8$, $p < 0.05$; ZT 6: $df=8$, $p < 0.01$; ZT 12: $df=8$, $p < 0.01$; ZT 18: $df=8$, $p < 0.01$, Student's *t* test) of *db/db* mice, and RF for 12 days, lowered the insulin level at ZT 18 ($df=5$, $p < 0.01$, Student's *t* test), blood glucose level (ZT 0: $df=5$, $p < 0.01$; ZT 6: $df=5$, $p < 0.01$; ZT 18: $df=5$, $p < 0.05$, Student's *t* test), and triglyceride level (ZT 0: $df=5$, $p < 0.05$; ZT 12: $df=5$, $p < 0.01$, Student's *t* test) in *db/+* mice (Fig. 5).

Fig. 4. Effect of night-time restricted feeding (RF) on daily clock and *mPai-1* gene expression in the liver of *db/+* (left) and phase II *db/db* (right) mice. **a.** Representative double-plotted actograms showing daily locomotor activity before and after RF (dotted box). The uppermost light and dark bars indicate light and dark periods respectively. Lower panel shows result of chi square periodogram analysis. Horizontal axis shows the period and vertical axis shows the amplitude (Qp). The ascending straight line in the periodograms represents $p < 0.01$. **b.** Representative electrophoresis photographs of PCR products from free access (ad lib) and RF groups of *db/+* and *db/db* mice at ZT 0, 6, 12 and 18 on day 6 (RF 6d) or day 12 (RF 12d). **c.** Expression of clock and *Pai-1* genes from the *db/+* and *db/db* mouse liver at each ZT was plotted as a relative mRNA level that was normalised to β -actin expression. The lowest value was set to 1. Each value represents the mean $\pm$ SEM ($n=3$ –4 mice per time point). ** $p < 0.01$, * $p < 0.05$ (RF 6d vs ad lib), ### $p < 0.01$, # $p < 0.05$ (RF 12d vs ad lib) (Student's *t* test). Open circles, ad lib; closed circles, RF 6d; closed triangles, RF 12d

a

b

c

Fig. 5. Effect of night-time restricted feeding (RF) for 12 days on daily pattern of blood glucose level (a), insulin level (b), and triglycerides level (c) in phase II *db/+* (upper) and *db/db* (lower) mice. Each value represents the mean $\pm$ SEM ($n=3-4$). ** $p<0.01$, * $p<0.05$ (ad lib vs RF 12d) (Student's t test). Open circles, ad lib; closed circles, RF 12d

were examined under RF conditions for 10 days. Interestingly, RF treatment recovered a night (ZT 18)–day (ZT 6) difference of *mPer2* gene expression in STZ-treated mice (15.6 times higher expression for RF, 1.3 times higher with free access feeding, 11 times higher for non-STZ-treated group).

Fig. 6. Effect of night-time restricted feeding (RF) for 12 days on food intake (a) and body weight (b) of *db/db* mice. Each value represents the mean $\pm$ SEM ($n=12-24$). ** $p<0.01$, * $p<0.05$ (ad lib vs RF 6 or RF 12) (Student's t test). Open circles, *db/db* ad lib; closed circles, *db/db* RF; open triangles, *db/+* ad lib; closed triangles, *db/+* RF

RF for 12 days in *db/+* mice led to reduced food intake (Fig. 6; $df=8$, $p<0.01$, Student's t test). However, RF caused no significant effect on body weight in *db/+* mice (day 6: $df=8$, $p=0.12$; day 12: $df=8$, $p=0.18$, Student's t test). RF treatment significantly reduced food intake (day 6: $df=11$, $p<0.05$; day 12: $df=11$, $p<0.01$, Student's t test) and body weight (day 6: $df=11$, $p<0.05$; day 12: $df=11$, $p<0.01$, Student's t test) in *db/db* mice depending on the duration of RF (6 or 12 days).

Alterations in daily clock genes expressed in the livers isolated from *db/db* mice were normalised by RF (Fig. 4). Therefore, alterations of daily clock genes observed in livers isolated from STZ-treated mice

Pioglitazone-induced recovery from abnormal expression of clock and mPai-1 genes in the db/db mouse liver. Treatment of *db/db* mice with pioglitazone may normalise *mPai-1* gene expression through normalisation of humoral influences such as glucose and insulin in the liver without affecting peripheral clock gene expression, as pioglitazone treatment has been reported to decrease the plasma glucose and insulin levels in *db/db* mice [27]. Daily administration of pioglitazone slightly lowered the *mPai-1* gene expression at both ZT 6 and ZT 18 (Fig. 7), and increased *mPer2* gene expression, especially at ZT 6 (Fig. 7). Pioglitazone slightly lowered the plasma glucose and insulin level (Fig. 7) and significantly increased the body weight, especially at ZT 18 ($df=7$, $p<0.01$, Student's t test).

Discussion

The initial hypothesis of the present study appears to be that altered humoral factors in Type 2 diabetes will alter peripheral circadian clocks, which in turn will result in abnormal *mPai-1* expression. In the present experiment, we used young (phase I) and middle-aged (phase II) *db/db* mice as an animal model to study the effects of Type 2 diabetes on clock genes and the clock-controlled gene expression of *mPai-1*. We found that the daily oscillation of *mPer2* gene expression was severely attenuated (or blunted in some cases) and that the peak time of *mPer2* mRNA was advanced from ZT 18 to ZT 12 in the liver of phase I and phase II *db/db* mice. In addition, the peak time of *mBmal1* gene

Fig. 7. Effect of pioglitazone (10 mg/kg) administration for 12 days on body weight (a), blood glucose level (b), insulin level (c) and *mPai-1* and *mPer2* gene expression (d–f). On day 13, mice were killed at ZT 6 or ZT 18 under withdrawal of drug. **f.** Representative electrophoresis photographs of PCR products from the *db/+* and *db/db* mouse liver at ZT 6 and 18. **d, e.** mRNA abundance of *mPer2* and *mPai-1* gene was plotted as a relative mRNA level that was normalised to β -actin mRNA. The lowest value was set to 1. Each value represents the mean $\pm$ SEM ($n=3-4$ mice at each time point). ** $p<0.01$, * $p<0.05$ (Student's *t* test)

mRNA oscillation had also advanced from ZT 0 to ZT 18. While the peak time of *mPer1* was not altered, *mPer1* mRNA was highly expressed at ZT 6 in both phase I and phase II *db/db* mice. These results suggest that oscillation of the peripheral oscillator is advanced and attenuated specifically in phase II *db/db* mice.

The phase of the clock gene mRNA oscillation rhythm advances by about 3 h in the STZ-treated rat heart [26]. Similar results were obtained from our present study of the STZ-treated liver of mice. STZ disrupts pancreatic beta cells and induces Type 1 diabetes [28]. Our results suggest that Type 2 diabetes also induces phase advances of clock gene mRNA in the peripheral tissues. Although the amplitudes of

clock gene oscillation were reportedly not altered in the STZ-induced diabetic rat heart [26], we observed a diminished oscillation of *mPer2* mRNA in both the STZ-injected and *db/db* mouse liver. The different species and/or tissues used in these studies may explain this discrepancy. Furthermore, we injected higher doses of STZ than Young et al. [26] (200 mg/kg vs 65 mg/kg respectively), which may have caused more severe diabetes in the mice used in this study, resulting in diminished *mPer2* mRNA oscillation.

Expression of *mPai-1* mRNA in the liver of young wild (data not shown) and *db/+* mice was very low. The *db/+* mice at 13 to 14 weeks old exhibited weak *mPai-1* expression that peaked at night, and older mice (6 months to 1 year old) also exhibited weak *mPai-1* expression in the liver (data not shown). Comparatively, *mPai-1* mRNA in the 13- to 14-week-old *db/+* mice was higher than that in the 6- to 8-week-old *db/+* mice, possibly due to an ageing effect. Interestingly, 6- to 8-week-old *db/db* mice strongly exhibited expression of *mPai-1* in the liver at ZT 18. This may be explained by the hypothesis that some factors [29] possessing promoter activity on *mPai-1* gene expression are increased at this age in *db/db* mice under the control of clock regulation. The constant high level of *mPai-1* mRNA expression observed in 13- to 14-

week-old *db/db* mice may be related to the increased factors [29] for elevation of *mPai-1* expression, and also to the reduced rhythmicity of clock genes such as *mPer1*, *mPer2*, *mBmal1* and *Bmal2* for arrhythmicity of *mPai-1* expression. An increase in blood glucose, insulin and triglyceride levels produces *Pai-1* mRNA expression in the liver [30], and insulin and triglycerides synergistically induce *Pai-1* biosynthesis in hepatocytes [30]. Previously published data suggest that the CLOCK:BMAL2 (or BMAL1) heterodimer may be responsible for regulating *mPai-1* circadian expression [6]. All of these factors together may cause a constant high level of *mPai-1* gene expression in the liver of *db/db* mice.

Thus, an elevation of blood insulin and triglycerides may strongly induce *mPai-1* mRNA expression in the liver of *db/db* mice, but not in STZ-treated mice. Surprisingly, we found that *mPai-1* mRNA was not elevated in STZ-treated mice. In fact, STZ-treated mice exhibited an elevation of glucose, but not insulin or triglycerides. Such an observation might suggest that alterations in the circadian clock in the *db/db* and STZ-treated mice are independent of the changes in *mPai-1* expression.

Clock gene mRNA oscillation in the SCN did not differ between *db/db* and control (*db/+*) mice. This result leads us to believe that the central oscillator in the SCN is not altered in Type 1 diabetic *db/db* mice and that the diabetes-induced advanced and attenuated oscillation of the peripheral clock does not result from change in the central SCN clock oscillation. Phase II *db/db* mice showed an extremely attenuated locomotor activity rhythm under free access conditions. As clock gene oscillation in the SCN was normal in phase II *db/db* mice, attenuation and arrhythmicity of the locomotor activity and clock gene mRNA rhythm in the liver did not result from a diminishing of the core SCN oscillator but rather from abnormalities stemming from the output mechanism mediating locomotor activity and peripheral clock resetting. Similar results were reported using old animals, in which clock gene expression in the SCN was maintained but locomotor activity was severely diminished [31]. Thus, obesity may be responsible for the reduction of locomotor activity, and hyperphagia may be responsible for the arrhythmicity of locomotor activity in *db/db* mice.

A circadian rhythm in obese Zucker (*falfa*) rats [32, 33] that have a functionally defective leptin receptor [12, 34] showed significant deficits in both the daily mean and circadian amplitude of body temperature and activity, but both rhythms persisted irrespectively of lighting conditions with a period similar to that of lean control mice [32]. The *db/db* mice, on the other hand, had an extremely attenuated locomotor activity rhythm, and about 75% of phase II *db/db* mice lost rhythmicity. Although both *falfa* rats and *db/db* mice have functionally deficient leptin receptors [11, 12],

unlike *db/db* mice, *falfa* rats become severely hyperglycaemic because of their different genetic background [33]. Obese *falfa* rats are relatively normoglycaemic or only slightly hyperglycaemic [35, 36, 37]. Thus, the severe circadian deficits seen in *db/db* mice may be related to their more severe diabetic symptoms in comparison with *falfa* rats.

Our next aim was to elucidate the recovery effect of RF on the abnormal expression of clock and *mPai-1* genes in *db/db* and STZ-treated mice. To do this, we conducted RF at night when animals normally eat chow. Night-time RF not only led to the reappearance of locomotor activity, but also to the recovery of the robust clock gene mRNA oscillation in *db/db* mice. We demonstrated that the advanced expression of *mBmal1* mRNA under free access conditions was normalised under night-time RF conditions in *db/db* mice. A diminished oscillation of *mPer2* and *mBmal1* was also recovered with night-time RF. Interestingly, RF treatment reduced the *mPai-1* mRNA expression in the liver of *db/db* as well as *db/+* mice. Indeed, RF treatment lowered the plasma glucose, insulin and triglyceride levels in *db/db* mice, and also glucose and triglycerides in *db/+* mice. It has been revealed that RF is one of the strong entraining factors of peripheral circadian clocks not only in normal but also in *clock/clock* [20, 38] and STZ-treated mice (present result), as well as a modulator of circulating factors such as glucose, insulin and triglycerides. RF treatment reduced the glucose, insulin and triglyceride levels in *db/db* mice. However, these levels are still high in comparison with those in *db/+* mice. Thus, changing the level of circulation factors may not be directly responsible for expression of *mPai-1*, because RF reduced *mPai-1* expression in the *db/db* mice to levels far below normal. We should consider the role of other humoral factors such as angiotensin II, NEFA and glucocorticoids [29] in the regulation of *Pai-1* gene expression.

RF increased the activity counts and reproduced 24-h rhythmicity in the locomotor activity of *db/db* mice. Therefore, we could not rule out the possibility that the RF produced recovery of gene expression through "exercise" or through food-related signals in *db/db* mice. Further experimentation is required for dissociation of these two factors.

As pioglitazone was reported to reduce plasma glucose and insulin levels under fasting by increasing the body weights [27], this drug was expected to normalise the *mPai-1* expression in *db/db* mice without affecting clock gene expression. Similarly to in a previous paper [27], pioglitazone slightly reduced glucose and insulin levels as well as *mPai-1* expression. When pioglitazone was co-applied with insulin, pioglitazone strongly produced the expression of *Pai-1* gene in HepG2 cells [39] and 3T3-L1 cells [40]. Such a facilitatory effect of pioglitazone might cancel out the reduction of *mPai-1* gene caused by lowering glu-

cose and insulin in pioglitazone-treated *db/db* mice. Abnormal expression of *mPer2* in the liver of *db/db* mice was facilitated by the treatment with pioglitazone. Although at present we do not know the mechanism of the augmentation of *mPer2*, the increase in body weight caused by pioglitazone at ZT 6 may facilitate the *mPer2* gene expression. Indeed, food intake increased the *mPer2* gene expression in the liver of normal fasted mice (unpublished observations).

Thus, RF treatment rather than pioglitazone administration leads to not only the successful recovery of peripheral clock gene oscillation, but also to the normalisation of *Pai-1* gene expression, which is evidence for the usefulness of RF treatment for diabetes mellitus patients.

In summary, we demonstrated impaired clock gene mRNA rhythm in the *db/db* mouse liver. A restricted feeding schedule was sufficient to recover peripheral clock gene expression and also reduce *mPai-1* gene expression in diabetic mice. Thus, scheduled restricted food intake may be a useful form of treatment for diabetes mellitus patients.

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