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Daily injection of insulin attenuated impairment of liver circadian clock oscillation in the streptozotocin-treated diabetic mouse

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Abstract Recent studies have shown a dampened amplitude of clock gene rhythm in the heart and liver of streptozotocin (STZ)-treated rats and mice, however it is unknown whether impairment is due to dysfunction of the suprachiasmatic nucleus (SCN) or not. Rhythmic expression of mPER2 was dampened in the STZ-treated mouse liver but not SCN and cerebral cortex. Injection of insulin could normalize an impairment of *mPer2* and mPER2 expression rhythm in the liver, when it was injected at nighttime, but not at daytime. In the present study, we demonstrated that insulin-dependent diabetes impaired oscillation of the peripheral clock gene and its product. Insulin injection can recover dampened oscillation of the peripheral clock depending on its injection time.

Keywords: Circadian; *db*/*db*; Clock gene; Restricted feeding; Streptozotocin

1. Introduction

Diabetes mellitus is a syndrome characterized by hyperglycemia resulting from the absolute or relative impairment of insulin secretion and/or insulin action. There are two main types of diabetes mellitus known as type I (insulin-dependent) and type II (insulin-independent) [1]. Recent evidence suggests that the binding of NPAS2 and CLOCK to the E-box depends on the ratio of NAD to NADH [2]. These molecules are essential components of the respiratory enzyme chain and the ratio between them fluctuates according to changes in cellular metabolism. Thus, diabetes may affect molecular mechanism of circadian clock in the liver through impairment of energy metabolism. Interestingly, recent study showed that all clock and clock-controlled genes examined possess circadian advanced and dampened rhythms of gene expression in the streptozotocin (STZ)-induced type I diabetic rat heart [3] and mouse liver [4]. However, these experiments still possess a question whether impairment of clock gene expression refers to impairment of suprachiasmatic nucleus (SCN) clock function or that of peripheral clock function. In this experiment, we examined mPER2 expression in the SCN and *mPer2* and mPER2 in the liver in STZ-treated diabetic mice.

Diabetes mellitus induced by STZ treatment is a syndrome characterized by hyperglycemia resulting from the absolute impairment of insulin secretion, therefore insulin treatment is necessary to attenuate the diabetic syndrome. Therefore, we examined whether daily insulin injection can recover the impairment of circadian expression of *mPer2* and mPER2 in the STZ-treated mouse liver. We injected insulin at nighttime or daytime onset in the light–dark (LD) housing condition, because daily injection of insulin may facilitate the recovery of circadian oscillation, when it was injected during eating time but not during resting time.

2. Materials and methods

2.1. Animals and induction of diabetes

Male ddY mice (6 weeks old) were obtained from Takasugi Experimental Animals (Saitama, Japan). Animals were maintained on a LD cycle (LD 12 h light: 12 h dark, with lights on at 8:30 a.m.) at a room temperature of 23 °C and allowed free access to food and water. All animals were treated in accordance with the Law (No. 105) and Notification (No. 6) of the Japanese Government.

2.2. Experimental design

Diabetes was induced by a single intraperitoneally injection of STZ (200 mg/kg; Wako Chemical Osaka, Japan) at Zeitgeber time (ZT) 11 (day 0) into 15-week-old mice and blood glucose level was checked 5 days after STZ injection. Five days after STZ, animals were sacrificed every 6 h from ZT 0 to 18 on day 5 ($n = 3$–4 for each time point) for measurement of *mPer2* and mPER2 expression.

In order to elucidate the effect of insulin on impairment of *mPer2* and PER2 expression, mice were divided into three groups. The first group animals were injected with vehicle (0.1 M citrate sodium buffer) at ZT 11 (day 0), and 5 days after vehicle injection, animals received a subcutaneous injection of saline at ZT 11.5 or ZT 23.5 for further 6 consecutive days. On the next day of final injection (day 10), mice were sacrificed at ZT 0, 6, 12, and 18 ($n = 3$ for each time point). The second and third group animals were injected with STZ (200 mg/kg) at ZT 11 (day 0), and 5 days after STZ injection, animals received a subcutaneous injection of saline for second group or insulin (5 IU/kg, Sigma) for third group at ZT 11.5 or ZT 23.5 for further 6 consecutive days. On the next day of final injection (day 10), mice were sacrificed at ZT 0, 6, 12, and 18 ($n = 3$–5 for each time point). Next, we examined the acute effect of insulin on the impairment. Five days after STZ injection mice were received saline or insulin (5 IU/kg) at ZT 11.5, and then sacrificed 2 or 4 h after insulin injection.

2.3. RT-PCR

Mice were deeply anesthetized with ether and intracardially perfused with ice-cold saline to remove excess blood. A block-shaped sample of liver (approximately 100 mg wet weight) was taken for measurement of liver clock gene expression by RT-PCR. Total RNA from each sample

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Abbreviations: LD, light–dark; SCN, suprachiasmatic nucleus; STZ, streptozotocin; ZT, Zeitgeber time

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was extracted separately by RNA-Solv Reagent (Omega BioTeK, GA). A One-Step RT-PCR System Platinum Version (Invitrogen, CA) was used for a RT-PCR containing 100 ng of RNA. *mPer2* and *β-actin* cDNA was amplified using GeneAmp9900 (Applied Biosystems, CA). Procedure of RT-PCR was the same as that reported in our previous paper [5,6].

2.4. mPER2 immunohistochemistry

Mice ($n = 3$ mice for each time point) were deeply anesthetized with ether and intracardially perfused with 0.1 M phosphate buffer (PB) (pH 7.4) containing 4% paraformaldehyde (PFA). Brains and livers were removed, post-fixed in 0.1 M PB containing 4% PFA for 24 h at 4 °C, and transferred into 20% sucrose in 0.1 M PB for 72 h at 4 °C. Immunoreactivity process was exactly the same as that reported in our previous paper [7].

2.5. Measurement of the number of mPER2 immunoreactive cells in the SCN and liver

The number of mPER2 immunoreactive (mPER2-ir) cells of SCN was counted by Scion Image Beta 4.02. The threshold value was set to 120, and the nuclear size was set as a minimum of 10 and a maximum of 40 pixels. mPER2-ir cell numbers from five slices, that contain mPER2-ir cells from rostral to caudal SCN, were averaged and used for cell number of each mouse. In the liver, three photographs around central vein of hepatic lobe were taken per individual mouse. These photographs were printed (65×85 mm) and the number of mPER2-ir cells of liver contained in the division defined arbitrarily (40×50 mm) was counted by un-notified person. Then, averaged cell number was calculated from each animal, and averaged cell number and standard error was obtained from three animals for each time point.

2.6. Measurement of blood glucose concentration

Just prior to liver isolation, 0.5 ml of blood was withdrawn from the mouse heart and serum glucose level was measured by Novo Assist Plus (Novo Nordic Pharma, Tokyo, Japan) in the manner recommended by The manufacturer.

2.7. Statistical analysis

The values were expressed as means ± S.E.M. The significance of differences between groups at each time point was determined by the Student's t test. Rhythmicity of each group was tested by one-way ANOVA. Two-way ANOVA was used for comparing the daily expression rhythm of the two groups.

3. Results

3.1. Effect of STZ on mPer2 gene expression and mPER2-ir cells in the SCN and liver

The success of diabetes induction was assessed by measuring both body weight and serum glucose level 5 days after STZ injection. Body weight was significantly decreased in STZ-treated mice (28 ± 0.10, $n = 12$) compared to vehicle mice (34 ± 0.69, $n = 12$) 5 days after the treatment. Serum glucose level was 3.8-fold higher in STZ-treated mice (570 ± 13 mg/100 ml $P < 0.01$, Student's t test) compared with vehicle mice (151 ± 5 mg/100 ml). Fig. 1A and B shows daily *mPer2* gene expression of vehicle- or STZ-treated group in the liver. In STZ group, *mPer2* gene expression was dampened at ZT 18. Two-way ANOVA analysis (treatment × time course) demonstrated a significantly different expression in *mPer2* ($F(3, 20) = 17.4$, $P < 0.01$) between vehicle- and STZ-treated group.

Next, we observed daily change of cells expressing mPER2 immunoreactivity at four time points in the liver of vehicle- or STZ-treated mice. mPER2-ir cells in the liver were located across almost entire hepatic tissue at ZT 18. Along with central vein, mPER2-ir positive nuclei were well observed as the dark spots (Fig 1C). Although vehicle-treated animal showed a clear

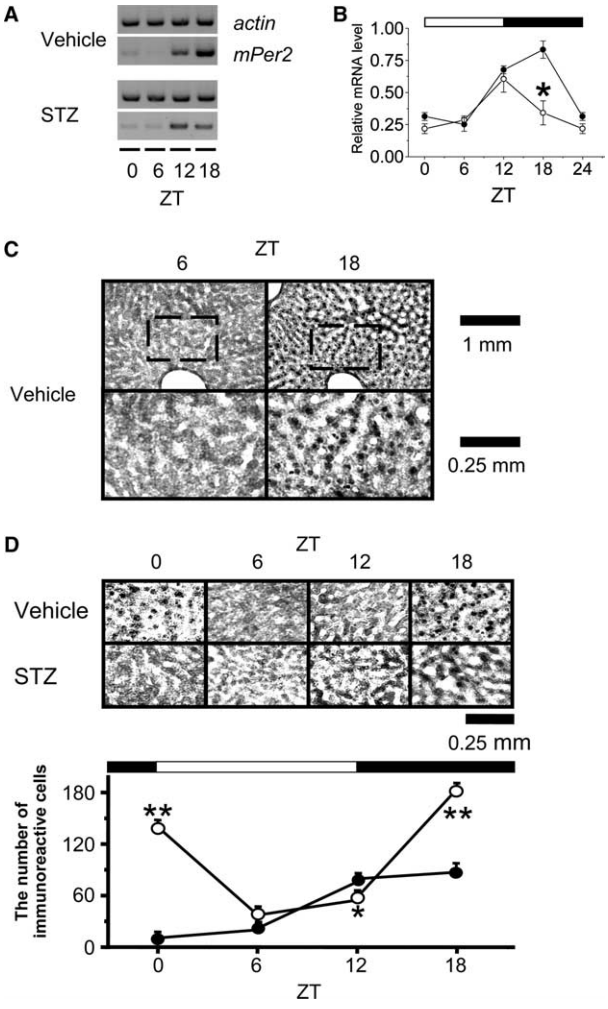

Fig. 1. Effect of STZ on *mPer2* and mPER2-ir cells expression in the liver on day 5. Mice were injected with STZ (200 mg/kg) or vehicle and housed for next 5 days (day 0–5). (A) The *mPer2* mRNA expression demonstrated by RT-PCR for analysis. (B) Time course of *mPer2* mRNA expression in the vehicle- (closed circle) ($n = 3$ for each time point) and STZ-treated (open circle) ($n = 4$ for each time point) group mice liver. Relative mRNA levels of *mPer2* are shown as ratios to *β-actin*. *, $P < 0.05$ *vs. vehicle (Student's t* test). (C) Representative sections of hepatic slice. Square area (40×50 mm) along with central vein shows the tissue area in which the number of mPER2-ir cells (upper panel) was counted, and the enlargement was shown in the lower panel. (D) Representative sections containing mPER2-ir cells in the liver of vehicle- or STZ-treated mice. Daily fluctuation of mPER2-ir cell numbers in the liver of vehicle- ($n = 3$ for each time point) or STZ-treated mice ($n = 3$ for each time point). *, $P < 0.05$; **, $P < 0.01$ vs. vehicle (Student's t test).

daily rhythm of mPER2-ir cell numbers, at ZT 6 there were relatively few mPER2-ir cells (Fig. 1C). The positive cell numbers were dramatically reduced at ZT 0 and 18 in the STZ-treated mice (Fig. 1D).

Although mPER2-ir SCN cells were located across almost entire SCN at ZT 12 and 18 (Fig. 2), mPER2 immunoreactivity was preferentially observed in the nucleus of SCN cells. In both vehicle- or STZ-treated mice, a clear daily rhythm of mPER2-ir cell was observed in the SCN with a peak at ZT 12. Thus, daily rhythm of mPER2-ir cells in the SCN did not differ between both groups (Fig. 2), Similar to the SCN, daily

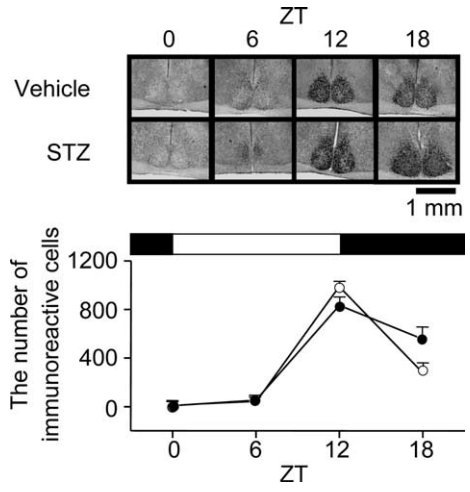

Fig. 2. Effect of STZ on the number of mPER2-ir cells in the SCN. Upper panel shows representative sections containing mPER2-ir cells in the SCN of mice. Lower panel shows a daily fluctuation of mPER2-ir cell numbers in the SCN of vehicle- ($n = 3$ for each time point) or STZ-treated mice ($n = 3$ for each time point).

rhythm of mPER2 positive cell numbers in the cerebral cortex is same between both groups (data not shown).

3.2. Effect of daily insulin injection on the expression of *mPer2* gene and mPER2-ir cells in the SCN and liver of STZ-treated mice

We examined the effect of insulin injection for 6 consecutive days on *mPer2* gene expression and mPER2-ir cells in the SCN and liver of STZ-treated mice. Serum glucose level was decreased 30 min after insulin injection at both injection times (225 ± 61 mg/100 ml for ZT 11.5 injection; 253 ± 10 mg/100 ml for ZT 23.5 injection), but it returned to high level 6.5 h after insulin injection (464 ± 17 mg/100 ml for ZT 11.5 injection; 484 ± 42 mg/100 ml for ZT 23.5 injection). In the liver, STZ-saline group again showed a dampened *mPer2* gene expression (Fig. 3A–D) and weak expression rhythm of mPER2-ir cell number (Fig. 3E). Two-way ANOVA analysis (treatment $\times$ time course) demonstrated a significant difference of *mPer2* expression between STZ-saline and STZ-insulin groups ($F(3, 23) = 34.0$, $P < 0.01$), when insulin was administrated at ZT 11.5 (Fig. 3A and B).

Two-way ANOVA analysis (treatment $\times$ time course) demonstrated no significant difference of *mPer2* expression between STZ-saline and STZ-insulin groups ($F(3, 23) = 1.5$, $P > 0.05$), when insulin was injected at ZT 23.5 (Fig. 3C and D). mPER2-ir positive cells were clearly increased at ZT 18 in comparison with the saline injection in STZ-treated mice (Fig. 3E), when insulin was injected at ZT 11.5.

In the SCN, a significant daily rhythm in the number of mPER2-ir positive cells was found with peak at ZT 12 in either saline or insulin injection group. Thus, insulin injection at ZT 11.5 or 23.5 did not affect mPER2-ir expression in the SCN (Fig. 4).

3.3. Acute effect of insulin on the expression of *mPer2* and mPER2-ir cells in the liver of STZ-treated mice

In order to reveal that acute injection of insulin is sufficient for the recovery of the impairment, we examined the acute effect of insulin injection on *mPer2* expression and number of

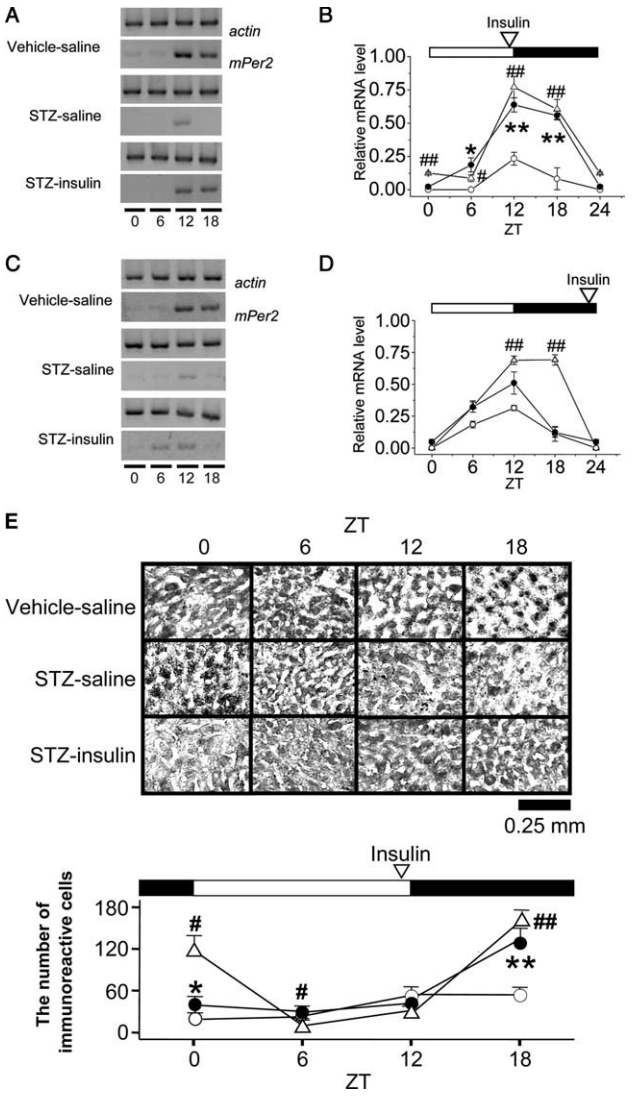

Fig. 3. Effect of insulin on the expression of *mPer2* and mPER2-ir cells in the liver of STZ-treated mice. Mice were injected with STZ (200 mg/kg) or vehicle, and housed for next 5 days (day 0–5). Then, STZ-treated mice were injected with insulin (closed circle) (5 IU/kg) or saline (open circle). Vehicle-treated mice were injected with saline (open triangle) at ZT 11.5 (A) and (B) or 23.5 (C) and (D), respectively, for 6 consecutive days (day 5–10). (A) and (C) The *mPer2* mRNA expression demonstration by RT-PCR. (B) and (D) Expression of *mPer2* mRNA in the vehicle-saline ($n = 3$), STZ-saline ($n = 3$) or STZ-insulin injected groups at ZT 11.5 or ZT 23.5 ($n = 4$–5 for each time point). Relative mRNA levels of *mPer2* are shown as ratios to *β-actin*. *, $P < 0.05$; **, $P < 0.01$ STZ-insulin vs. STZ-saline; $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$ vehicle-saline vs. STZ-saline (Student's t test). (E) Representative sections containing mPER2-ir cells in the liver of each group (vehicle-saline, STZ-saline, or STZ-insulin at ZT 1 1.5). Lower panel shows daily fluctuation of mPER2-ir cell numbers in the liver of vehicle-saline ($n = 3$ for each time point) or STZ-saline or STZ-insulin-treated mice ($n = 3$ for each time point). *, $P < 0.05$; **, $P < 0.01$ STZ-insulin vs. STZ-saline; $^{\#}$, $P < 0.01$; $^{\#\#}$, $P < 0.05$; vehicle-saline vs. STZ-saline (Student's t test).

mPER2-ir cells in the STZ-treated mouse liver. Serum glucose level was decreased 2 h (258 ± 74 mg/100 ml for insulin injection; 604 ± 29 mg/100 ml for saline injection, $P < 0.01$, Student's t test) and 4 h (417 ± 28 mg/100 ml for insulin injection; 559 ± 7 mg/100 ml for saline injection, $P < 0.01$, Student's t test) after insulin injection, but it returned to high level 6 h

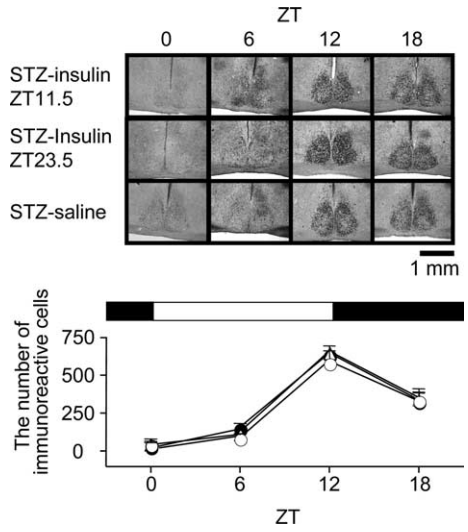

Fig. 4. Effect of insulin on number of mPER2-ir cells in the SCN of STZ-treated mice. STZ-treated mice were injected with insulin (5 IU/kg) at ZT 11.5 (closed circle) or 23.5 (open circle), or injected with saline at ZT 11.5 (open triangle) for 6 consecutive days (day 5–10). Upper panel shows representative mPER2 immunostained sections containing the SCN of each group. Lower panel shows daily fluctuation of mPER2-ir cell numbers in the SCN of each group ($n = 3$).

after (501 ± 6 mg/100 ml for insulin injection; 581 ± 40 mg/100 ml for saline injection). Insulin injection increased mPER2-ir liver cell in STZ-treated mouse 2 and 4 h after injection (Fig. 5B). Similar to the data of mPER2-ir expression, acute injection of insulin elevated *mPer2* gene expression in STZ-treated mouse liver 2 h after injection (Fig. 5A).

4. Discussion

In the present experiment, we found that the daily oscillation of *mPer2* and mPER2-ir cell was severely attenuated and the peak time was advanced from ZT 18 to 12 in the liver of STZ-treated mice. Whereas,amplitude mPER2 expression in the SCN was unaffected by STZ injection. These results suggest that oscillation of the peripheral oscillator but not central SCN oscillator is impaired in STZ-treated diabetic mouse.

Similar results were obtained from study of the STZ-treated rat heart [3] and mouse liver [4]. Our present finding supports the fact that a dampened oscillation in the STZ-injected mouse liver was observed in not only *mPer2* but also mPER2 expression. Daily oscillation of mPER2, not only in the SCN but also in the cerebral cortex, did not differ between vehicle- and STZ-injected mice. In the constant darkness condition, STZ-injection mice showed an almost similar free-running period as control mice (data not shown). These results lead us to believe that the central oscillator in the SCN and cerebral cortex is not altered in STZ-treated diabetic mice and that the diabetes-induced dampened oscillation of the peripheral clock does not result from change in the central clock oscillation. The mechanism of damping oscillation in the liver of STZ-treated mouse is still unknown. Recent evidence suggests that the binding of NPAS2 and CLOCK to the E-box gene depends on the ratio of NAD to NADH [2]. These molecules are essential components of the respiratory enzyme chain and the ratio between them fluctuates according to changes in cellular metabolism.

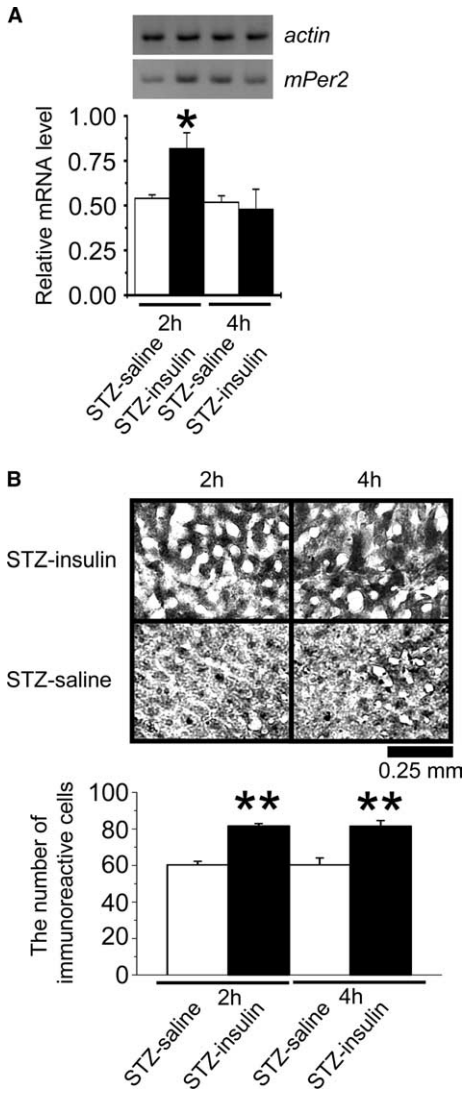

Fig. 5. Acute effect of insulin injection on expression of *mPer2* and mPER2-ir cells in the liver of STZ-treated mice. Mice were injected with STZ (200 mg/kg) and housed for next 5 days (day 1–5). Insulin (5 IU/kg) or saline were injected into mice at ZT 11.5 on day 5. Then mice were sacrificed 2 or 4 h after injection. (A) The *mPer2* mRNA expression demonstration by RT-PCR. Time course of *mPer2* mRNA expression in the mice liver after saline (control group; white box) or insulin (insulin group; black box) injected at ZT 11.5 ($n = 3$–4). Relative mRNA levels of *mPer2* are shown as ratios to *β-actin*. *, $P < 0.05$ STZ-saline vs, STZ-insulin (Student's *t* test). (B) Representative PER2 immunostained sections containing the liver of each group. Time course of mPER2-ir cells after saline (control group; white box) or insulin (insulin group, black box) injected at ZT 11.5 mice liver ($n = 3$–4). **, $P < 0.01$ STZ-saline vs. STZ-insulin (Student's *t* test).

Diaz-Munoz et al. [8] reported that the NAD:NADH ratio was affected by restricted feeding condition. Such a ratio change in STZ-treated mouse may contribute to impairment of *mPer2* and mPER2 expression in the liver. Further studies are needed to examine whether the change in the ratio of NAD:NADH can rescue abnormality of peripheral clock gene expression in STZ-treated mice.

The change of *mPer2* and mPER2 in the STZ-treated mouse liver suggests that insulin, leptin [9] or other factors known to be altered by both types of diabetes such as corticosterone [10,11] or growth hormone [12] may modulate synchronization

of the peripheral oscillator. Actually, corticosterone is reported to be a possible factor for entrainment of peripheral clock [13]. Further studies are needed to elucidate the interactions between peripheral clock gene function and diabetic functional deficits.

Daily injection of insulin may recover the impairment of *mPer2* and mPER2 expression rhythm in the STZ-treated mouse liver. In the SCN, however insulin injection at activity onset or offset did not affect mPER2 expression. The present result demonstrated an importance of injection-time difference in recovery effect on impaired clock of the liver. Daily injection at activity onset time, in which feeding behavior usually initiates, normalized the impairment of *mPer2* and mPER2 expression rhythm in the liver. This result strongly suggests that daily insulin injection not only attenuated the diabetic syndrome but also recovered the peripheral circadian clock function. It was reported that insulin acutely increased *Per1* and *Per2* gene expression in the Rat-1 fibroblast [14]. Therefore, injection of insulin may directly increase *mPer2* gene expression and mPER2 expression to the normal level.

Alternately, food intake after insulin injection may increase *mPer2* gene and mPER2 expression in STZ-treated mouse. In the present experiment, we actually observed that both *mPer2* expression and mPER2-ir cell increase 2 and 4 h after insulin injection, along with increase of food intake (data not shown). In order to decide whether the effect of insulin on the clock gene expression is specific effect or just a consequence of the altered glucose concentration, *mPer2* expression was examined after insulin and insulin + glucose (5 g/kg) injection. As there are similar increases of *mPer2* gene expression in both groups (data not shown), insulin-induced *mPer2* gene expression prefers a specific effect. Recent in vitro experiment suggests the above possibility, because glucose administration decreased *Per1* and *Per2* gene expression in Rat-1 fibroblast [15]. Although locomotor activity level was slightly decreased by STZ injection, insulin injection unaffected activity level (data not shown). Thus, the insulin recovered the impairment of *mPer2* and mPER2 expression without affecting activity.

As single injection of insulin is enough to recover the deficit of mPER2 and *mPer2* gene expression,daily injection of insulin facilitated the recovery of this deficit. Thus, present result shows the impact of insulin treatment on clock gene expression in the liver of diabetic mice.

In summary, we demonstrated that *mPer2* as well as mPER2 expression rhythm was impaired in STZ-treated mouse liver without affecting SCN mPER2 expression rhythm, and that insulin injection at appropriate clock time could recover the impaired peripheral clock function in diabetic mice.

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