Metabolic state switches between morning and evening in association with circadian clock in people without diabetes

Ruriko Fujimoto1, Ysuharu Ohta1,2*, Konosuke Masuda1, Akihiko Taguchi1, Masaru Akiyama1, Kaoru Yamamoto1, Hiroko Nakabayashi1, Yuko Nagao1, Takuro Matsumura1, Syunsuke Hiroshige1, Yasuko Kajimura1, Makoto Akashi3, Yukio Tanizawa1

1Department of Endocrinology, Metabolism, Hematological Science and Therapeutics, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi, Japan, 2Department of Diabetes Research, Yamaguchi University, School of Medicine, Ube, Yamaguchi, Japan, and 3The Research Institute for Time Studies, Yamaguchi University, Yamaguchi, Japan

Keywords
Glucose tolerance, Insulin secretion and sensitivity, Non-esterified fatty acid

*Correspondence
Yasuharu Ohta
Tel: +81-836-22-2251
Fax: +81-836-22-2342
E-mail address: yohta@yamaguchi-u.ac.jp

J Diabetes Investig 2022; 13: 1496--1505
doi: 10.1111/jdi.13810

INTRODUCTION

The circadian clock plays a critical role in many biological processes, including the sleep–wake cycle, hormone secretions, glucose and lipid metabolism, and body temperature regulation. Increased research attention during the past decade has focused on the role of the circadian clock as a factor contributing to the regulation of energy metabolism1–3. Most peripheral tissues throughout the body possess their own clock system, and most notably, circadian clocks in metabolically active tissues, such as the pancreas, liver and skeletal muscle, regulate tissue-specific functions4. For instance, during inactive/starvation periods, glucose is endogenously recruited from the liver to maintain the blood glucose level supplying fuel for other tissues, such as the brain and muscles5. Glucose tolerance testing in rats showed...
that whole-body glucose tolerance is higher at the start of an active period than at the start of an inactive period, with diurnal oscillations of systemic insulin sensitivity peaking in that period. In addition, the daily rhythms of glucose stimulated insulin secretion (GSIS) are robust and persist even in isolated islets.

Glucose tolerance in people without diabetes is better in the morning, just before the feeding/active period, than in the evening, just before the beginning of the fasted/inactive period. GSIS capacity seems to contribute to this observation, although daily variations in peripheral insulin sensitivity are also contributory. Rhythmic Ucp2 expression in mouse islets is required for the normal rhythms of GSIS capacity and glucose tolerance. Individuals with prediabetes also have poorer glucose tolerance in the evening, off-setting the evening decline in cortisol levels. However, the intrinsic regulators that control daily variations in glucose tolerance have not yet been identified. In addition, no human studies have examined the relationship between peripheral clocks and daily fluctuations in glucose tolerance, insulin secretion or insulin action.

The present study aimed to determine the difference between morning and evening metabolic states in people without diabetes, focusing especially on insulin secretion and insulin sensitivity in the liver and muscle. The findings were confirmed by mouse studies. We also explored the associations of clock gene expressions in hair follicles with the changes in metabolic indices obtained from oral glucose tolerance test (OGTT) or the hyperinsulinemic euglycemic (HE) clamp study. Here, we document remarkable changes in metabolic states between the morning and evening, which show associations with clock gene expressions.

MATERIALS AND METHODS
Participants
Study participants were required to be aged between 20 and 60 years at the time of screening, and to have no obvious diabetes or impaired glucose tolerance (fasting blood glucose ≤6.1 mmol/L). They were not regularly engaged in shift work, nor taking any medicine regularly. We did not enroll women because of the possible changes in glucose tolerance due to the menstrual cycle. The participants were not asked to regularize and standardize their sleep or their feeding schedules, except for a 10–13 h fast before each 75 g OGTT and HE clamp.

Study design
We confirmed that all of the enrolled participants did not have diabetes according to glycated hemoglobin (HbA1c) and fasting blood glucose values. Either OGTT or HE clamp was carried out twice on the same participant at 08.00 hours and 20.00 hours in random order at an interval of 5 days to 2 weeks. A total of 14 participants were enrolled for the OGTT, and 10 for the HE clamp study between January 2017 and November 2019. All study participants fasted for 10–13 h before each examination.

OGTT in humans
At 08.00 hours or at 20.00 hours, participants underwent a 75-g OGTT according to the recommendations of the World Health Organization. Venous blood samples were obtained before and during the OGTT (30, 60, 90 and 120 min) for measurements of plasma glucose and serum insulin concentrations. These parameters were measured by SRL (Tokyo, Japan) using standard methods. The hepatic insulin resistance index was the product of the total areas under the curve (AUC) for glucose and insulin during the first 30 min of the OGTT (glucose AUC 0–30 min × insulin AUC 0–30 min), as defined by Abdul-Ghani et al.

HE clamp
The HE clamp was carried out at 08.00 hours or 20.00 hours with the use of an artificial endocrine pancreas (STG-55; Nikkiso, Shizuoka, Japan). The clamp technique was previously described in detail. In brief, during the HE clamp period, participants were given a constant infusion of regular insulin (Humulin R; Eli Lilly Japan KK, Kobe, Japan; 1.25 mU kg⁻¹ min⁻¹) with variable-rate exogenous infusion of 10% glucose to maintain blood glucose at 5.3 mmol/L. When the rate of exogenous glucose infusion reached a steady-state level, insulin sensitivity during the final 30 min of the glucose clamp (approximately 90–120 min after starting the infusion of insulin) was evaluated as the average glucose infusion rate (GIR).

Clock gene expressions in hair follicle cells
The procedures were previously described in detail. In brief, hair follicle cells were collected before each examination by pulling out the roots of scalp hairs. Two to 10 hair follicles were quickly soaked in lysis buffer (RNAqueous-Micro Kit; Thermo Fisher Scientific, Waltham, MA, USA). Total ribonucleic acid (RNA) was extracted and purified using an RNAqueous-Micro Kit according to the manufacturer’s instructions, and a 100 ng quantity of total RNA was reverse-transcribed using a SuperScript VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA). The messenger RNA (mRNA) of clock genes (Dbp, E4bp4, Bmal1, Clock, Nr1d1 and Per2) was quantified by real-time polymerase chain reaction using a Taqman MGB probe (Applied Biosystems, Waltham, MA, USA) and a 1/20 volume of the reverse transcription product. mRNA expressions were normalized using 18S rRNA expression. The relative expression value of each gene at 20.00 hours was calculated by dividing the value at 20.00 hours by the value at 0.80 hours for each individual.

Animals
C57BL6j mice were housed in a temperature-controlled (22° ± 1°C) room under a 12-h light : 12-h dark cycle (LD 12:12). Zeitgeber time (ZT) 0 is usually designated as lights on and ZT12 as lights off. Half of the male mice (ZT1 mice) were housed under a normal light–dark cycle (turn on the light at 08.00 hours, off at 20.00 hours). The other half (ZT13 mice)
from the same litter were transferred to a reversed cycle (turn on the light at 20.00 hours, off at 08.00 hours) at 8 weeks-of-age.

Insulin tolerance tests were carried out on randomly fed 13-week-old male mice. Insulin (0.75 units/kg) was injected intraperitoneally at 09.00 hours, tail blood was collected at 0, 30, 60, 90 and 120 min, and blood glucose concentrations were determined using ANTSENCE II (Horiba Industry, Kyoto, Japan).

Tail blood of 13-week-old randomly-fed male mice was collected 15 min after insulin injection (0.75 units/kg) at 09.00 hours. Plasma non-esterified fatty acid (NEFA) levels were measured by enzyme assay kit (FUJIFILM Wako, Osaka, Japan).

**Western blotting**

Total cellular protein of the liver and gastrocnemius muscle was extracted using Cell Lysis Buffer (CST) after homogenization by GentleMACS Dissociator according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Protein concentrations were determined using a BCA Protein Assay kit (Thermo Fisher Scientific). Two to 20 mg of nuclear protein samples or 15 mg of total cellular protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA). The membranes were then incubated with primary antibodies. Antibodies used in western blot analysis included anti-protein kinase B (Akt; CST), anti-phospho (p)-AKT, Relative clock gene expressions in hair follicles (Ser473; CST), anti-PEPCK (Santa-Cruz Biotechnology, Dallas, TX, USA) and anti-α-tubulin (CST).

**Statistical analysis**

We defined the diurnal variation in all variables of the test as Δ = evening value – morning value. To reach 80% power and a significance level of 0.05, a minimal calculated sample size of n = 8 was required in the human study. Data are presented as the mean ± standard error of the mean throughout, unless otherwise stated. Statistical significance was set at P < 0.05. The 08.00 hours and 20.00 hours values for glucose and insulin levels, the hepatic insulin resistance index, GIR, and NEFA levels were compared using one-tailed paired t-tests. The relationships between relative clock gene expressions at 20.00 hours and various metabolobic factors were assessed by applying the Pearson or Spearman correlation coefficient, as appropriate. The other relationships were also assessed, using the same method.

**RESULTS**

**Participant characteristics**

In total, 14 and 10 participants without diabetes were enrolled for the 75-g OGTT and the HE clamp study, respectively. Of these, five participants were enrolled in both studies. All participants were Japanese men. The mean ages were 38.4 years (75-g OGTT) and 33.5 years (HE clamp). None of the participants were obese, with a mean BMI of 21.9 ± 0.41 kg/m² (75-g OGTT) and 21.1 ± 0.29 kg/m² (HE clamp), and all were without diabetes with a mean HbA1c of 5.25 ± 0.07% (75-g OGTT) and 5.33 ± 0.07% (HE clamp; Table S1).

**Glucose tolerance and insulin secretion during 75-g OGTT at 08.00 hours and 20.00 hours**

As shown in Figure 1, there were significant differences in both glucose and insulin levels during OGTT between the morning and the evening. Post-oral glucose load plasma glucose levels were significantly higher during OGTT at 20.00 hours (Figure 1a): at 60 min (08.00 hours 8.44 ± 0.69 vs 20.00 hours 10.77 ± 0.65 mmol/L, P = 0.0028), 90 min (08.00 hours 7.11 ± 0.53 vs 20.00 hours 10.34 ± 0.65 mmol/L, P = 0.0001) and 120 min (08.00 hours 6.95 ± 0.40 vs 20.00 hours 9.04 ± 0.52 mmol/L, P = 0.0005). Accordingly, glucose AUC 0–120 min was smaller at 08.00 hours (08.00 hours 15.39 ± 0.77 vs 20.00 hours 18.54 ± 0.90 mmol h/L, P = 0.0007; Figure 1b). These results show that glucose tolerance in people without diabetes is much better at 08.00 hours than at 20.00 hours. The fasting insulin level was higher at 08.00 hours than at 20.00 hours (08.00 hours 29.7 ± 2.4 vs 20.00 hours 19.7 ± 2.1 pmol/L, P = 0.0001). Although the insulin level 30 min post-oral glucose load tended to be higher at 08.00 hours (08.00 hours 284.4 ± 59.0 vs 20.00 hours 181.2 ± 18.0 pmol/L, P = 0.0565), the insulin level 90 min post-oral glucose load was significantly lower at 08.00 hours (08.00 hours 211.0 ± 34.0 vs 20.00 hours 306.0 ± 39.2 pmol/L, P = 0.0438). The time to reach the mean insulin peak was greatly accelerated during the OGTT at 08.00 hours, as compared with that at 20.00 hours (08.00 hours 30 vs 20.00 hours 90 min; Figure 1c). Although there was no significant difference in insulin AUC 0–120 min between 08.00 hours and 20.00 hours (429.8 ± 49.9 vs 411.4 ± 34.5 pmol/L, P = 0.6186), AUC insulin 0–60 min was greater during OGTT at 08.00 hours than at 20.00 hours (08.00 hours 215.8 ± 31.7 vs 20.00 hours 148.5 ± 12.2 pmol/L, P = 0.0216; Figure 1d). These results suggest the early phase of insulin secretion to be greater in the morning than in the evening, although there is no diurnal variation in total (0–120 min) insulin secretion during OGTT. Akashi et al. showed that qualitative evaluation of clock gene expressions in hair follicle cells is a potentially effective approach to studying the human circadian clock. Therefore, we investigated the relationships between clock gene expressions in hair follicles (Table 1) and the early phase of insulin secretion (AUC insulin 0–60 min). We found the relative expression of Per2 at 20.00 hours to be significantly associated with the Δinsulin AUC 0–60 min (r = −0.685, P = 0.007; Figure 1e).

**Hepatic insulin resistance and the correlation with Per2 expression in hair follicles**

Abdul-Ghani et al. defined the hepatic insulin resistance index as the product of AUC for glucose and insulin during
the first 30 min of the OGTT (glucose AUC \(\text{0-30 min} \times \text{insulin AUC } \text{0-30 min} \))\(^6\). The hepatic insulin resistance index was significantly greater at 08.00 hours than at 20.00 hours (08.00 hours \(285.0 \pm 55.0 \) vs 20.00 hours \(175.9 \pm 16.3, P = 0.0336\); Figure 2a), and we found that it was significantly associated with relative expression of \(\text{Per2}\) in hair follicle cells (value at 8 p.m. / value 8 a.m.). (e) Scatterplots showing a linear relationship between \(\Delta \text{insulin-AUC } \text{0-60 min}\) (evening value – morning value) and the relative \(\text{Per2}\) mRNA in hair follicle cells (value at 8 p.m. / value 8 a.m.). NS, not significant.

GIR in the HE clamp study and the correlation with NEFA

The GIR in the HE clamp study was significantly greater at 08.00 hours than at 20.00 hours (08.00 hours \(8.93 \pm 55.0 \) vs 20.00 hours \(6.69 \pm 16.3, P = 0.026\); Figure 3a). However, we did not find that \(\Delta\text{GIR}\) was significantly associated with the
Table 1 | Relative clock gene expressions in hair follicles at 8 p.m. (8 p.m. / 8 a.m. before 75 g oral glucose tolerance test)

| Participant/gene | Dbp | E4bp4 | Bmal1 | Nr1d1 | Per2 |
|------------------|-----|-------|-------|-------|------|
| 1                | 4.14| 0.081 | 0.62  | 1.04  | 0.81 |
| 2                | 0.82| 1.24  | 1.89  | 0.81  | 0.4  |
| 3                | 0.22| 0.7   | 0.28  | 1.08  | 0.22 |
| 4                | 0.1 | 0.27  | 0.49  | 0.7   | 0.63 |
| 5                | 1.28| 0.79  | 1.44  | 2.35  | 0.46 |
| 6                | 3.81| 1.05  | 1.9   | 0.62  | 4.87 |
| 7                | 9.98| 1.17  | 0.76  | 1.66  | 1.73 |
| 8                | 0.71| 0.56  | 1.02  | 0.69  | 0.58 |
| 9                | 3.33| 4.62  | 3.61  | 0.75  | 6.64 |
| 10               | 1.48| 8.5   | 10.22 | 0.92  | 2.82 |
| 11               | 2.11| 0.73  | 2.06  | 0.8   | 0.19 |
| 12               | 0.98| 1.29  | 2.28  | 1.29  | 0.54 |
| 13               | 0.42| 1.38  | 2.16  | 1.38  | 0.72 |
| 14               | 3.6 | 10.92 | 4.27  | 3.72  | 3.49 |

relative expression of any of the clock genes at 20.00 hours (Table 2 and data not shown). In addition, ΔGIR was not associated with Δcortisol ($r = 0.622, P = 0.055$). Interestingly, we found a significant association between ΔGIR and ΔNEFA ($r = -0.639, P = 0.047$; Figure 3b).

Serum NEFA levels and the correlation with E4bp4 expression in hair follicles

We measured serum NEFA concentrations in 19 participants undergoing either OGTT or HE clamp. For those undergoing both OGTT and HE clamp (5 individuals), the data provided by the first examination was used in the following analysis. The serum NEFA level was significantly higher at 20.00 hours than at 08.00 hours (08.00 hours 0.583 – 0.076 vs 20.00 hours 0.857 – 0.058 mmol/L, $P = 0.0007$; Figure 4a).

ΔNEFA was significantly associated with relative expression of E4bp4 in hair follicles collected at 20.00 hours ($r = 0.574, P = 0.01$; Figure 4b), although none of the clock genes had relative expressions directly associated with ΔGIR. In addition, we found a significant association between ΔNEFA and Δcortisol ($r = -0.311, P = 0.003$; Figure 4c), suggesting that both clock gene and cortisol regulate skeletal muscle insulin sensitivity partly through serum NEFA.

Figure 2 | Analysis of the results of oral glucose tolerance test-derived hepatic insulin resistance index. The hepatic insulin resistance index is defined as glucose-area under the curve (AUC$_{0-30\text{ min}}$ × insulin-AUC$_{0-30\text{ min}}$) on oral glucose tolerance test ($n = 14$). (a) The difference in hepatic insulin resistance between 08.00 hours and 20.00 hours. Data are shown as means and were obtained by one-tailed paired t-test. *$P < 0.05$. (b,c) Scatterplots showing linear relationships of Δhepatic insulin resistance index (evening value – morning value) with (b) relative Per2 mRNA expression in hair follicle cells (value at 8 a.m. / value at 8 p.m.) or with (c) Δcortisol (evening value – morning value). Data were obtained by single linear regression analysis.
Liver and muscle insulin sensitivity in mice at the start of the active and inactive period

ZT1 is the early inactive phase of ZT1 mice and the early active phase of ZT13 mice (Figure 5a). In insulin tolerance test, ZT13 mice showed lower plasma glucose 90 and 120 min after insulin administration compared to ZT1 mice, suggesting greater systemic or muscle insulin sensitivity during active phase (Figure 5b). Serum NEFA 15 min after insulin injection at ZT1 (09.00 hours) was significantly lower in ZT13 mice compared with ZT1 mice (Figure 5c). We assessed p-AKT-to-AKT ratios in the liver and gastrocnemius muscle 15 min after insulin injection as a marker of liver and muscle insulin sensitivity. AKT phosphorylation was significantly decreased in ZT13 mice livers, whereas it was significantly increased in ZT13 mice muscle compared with ZT1 mice (Figure 5d). PEPCK protein level increased in ZT13 mice livers, suggesting increased glucose production (Figure 5e). These findings demonstrate that mice show increased muscle insulin sensitivity and decreased liver insulin sensitivity during the active phase compared with the inactive phase, in agreement with the observations in people without diabetes.

DISCUSSION

We investigated diurnal metabolic changes in people without diabetes, focusing on glucose metabolism. We recognized significant variations in glucose metabolism in these participants with increased GSIS and skeletal muscle insulin sensitivity in the morning, resulting in better glucose tolerance. These results are generally compatible with those described in previous reports, although the participants’ racial and dietary backgrounds differed among studies. Enhanced GSIS and greater muscle insulin sensitivity undoubtedly contribute to better glucose tolerance in the morning. The hepatic insulin resistance index, however, appears to be in the opposite direction, with higher levels in the morning. In agreement with these findings in humans and mice, insulin-stimulated AKT phosphorylation is better in the early active phase in the skeletal muscle, and better during the early inactive phases in the liver. No prior studies have shown diurnal changes in hepatic glucose metabolism in people without diabetes, although elevated hepatic glucose production is known to be the major contributor to the morning hyperglycemia characteristic of type 2 diabetes. From an evolutionary perspective, in the skeletal muscle, the rapid increase in insulin-mediated glucose uptake and glycogen synthesis after exercise benefits survival in ‘fight or flight’ situations.

A previous mouse study suggested the cell-autonomous circadian rhythm in skeletal muscle to increase insulin sensitivity during the dark/active period. Circadian clock genes have been reported to intrinsically regulate hepatic gluconeogenesis.

Table 2 | Relative clock gene expressions in hair follicles at 8 p.m. (8 p.m. / 8 a.m.) before hyperinsulinemic-euglycemic clamp

| Participant/Gene | Dbp | E4bp4 | Bmal1 | Nr1d1 | Per2 |
|------------------|-----|-------|-------|-------|------|
| 1                | 0.14| 0.32  | 0.8   | 0.49  | 0.2  |
| 2                | 0.53| 0.82  | 0.52  | 0.75  | 0.57 |
| 3                | 2.81| 1.17  | 1.52  | 1.53  | 0.93 |
| 4                | 0.59| 1.23  | 1.46  | 0.43  | 0.6  |
| 5                | 0.6 | 1.05  | 1.65  | 1.16  | 0.64 |
| 6                | 1.4 | 1.22  | 1.14  | 1.28  | 1.22 |
| 7                | 0.62| 0.45  | 0.44  | 0.94  | 0.89 |
| 8                | 0.56| 0.76  | 1.4   | 4.21  | 0.33 |
| 9                | 0.4 | 0.26  | 0.28  | 0.33  | 0.23 |
| 10               | 0.73| 0.79  | 1.03  | 0.57  | 0.27 |
by suppressing glucocorticoid receptor-dependent gene expressions. We hypothesize that the increase in liver glucose production and supply to muscle at the beginning of the active period provides a survival benefit, based on preparing for increases in physical activity and nutritional requirements.

Internal molecular clocks play an important role in glucose and lipid metabolism through tissue-specific mechanisms. Pancreas-specific Bmal1 knockout and β-cell-specific E4bp4 overexpression impairs GSIS. Liver-specific Bmal1 knockout mice had blunted insulin sensitivity during the fasting phase. Liver-specific E4bp4 overexpression induced marked insulin resistance not only in the liver, but also in skeletal muscle, associated with reduced fatty acid oxidation during inactive phases. Skeletal muscle-specific Bmal1 knockout reduces insulin-dependent glucose uptake in isolated muscles without changing glucose tolerance. These studies suggest that circadian clocks within individual organs (pancreatic β-cell, liver and skeletal muscle) differently affect whole-body metabolism. They are likely to be orchestrated by interorgan communications to maintain systemic homeostasis.

Here, we investigated the associations of circadian gene expressions in hair follicle cells with GSIS, GIR (skeletal muscle insulin sensitivity), hepatic insulin resistance index (liver glucose production) and plasma NEFA levels in human participants. We showed the variations in GSIS and hepatic insulin resistance to be significantly associated with clock gene (Per2) expressions, and plasma NEFA levels with E4bp4 in human participants.

Cortisol has been recognized as a determinant of hepatic glucose production by stimulating PEPCK gene expression. In humans, cortisol levels are high in the morning and low in the evening/night, and the cortisol diurnal variation is expected to correlate with hepatic insulin resistance. However, in the present study, the amplitude of cortisol diurnal variation (ΔCortisol) did not appear to be associated with the hepatic insulin resistance index, suggesting that cortisol is, unexpectedly, not a major determinant of the diurnal variation of the hepatic insulin sensitivity/resistance. Rather, other mechanisms, such as clock gene (Per2) could be working.

In skeletal muscle, cortisol is also known to be a potent antagonist of insulin action. However, the present findings show that GIR, representing muscle insulin sensitivity, is lower in the evening, despite a greater decline in cortisol levels. The cortisol diurnal variation (ΔCortisol) showed no correlation with ΔGIR (r = −0.062, P = 0.864). In this regard, the effect of cortisol on skeletal muscle insulin sensitivity appears to be offset by other factors. In contrast, diurnal variation of GIR correlated significantly with that of NEFA.
NEFA is recognized as one of the major factors contributing to systemic (mainly skeletal muscle) insulin resistance\(^3\), and excess exposure to NEFA is associated with muscle insulin resistance. We observed a diurnal rhythm in plasma NEFA levels to be significantly associated with one of the clock gene expression and \(\Delta\)cortisol. Therefore, NEFA is one of the possible regulators of the diurnal rhythm in muscle insulin sensitivity, and muscle insulin sensitivity could be under the indirect control of clock genes through NEFA\(^2\). In this context, of note is that \(E4bp4\) overexpression in the liver indirectly causes muscle insulin resistance possibly through NEFA\(^2\). In the present study, we could not measure glucagon-like peptide-1, gastric inhibitory polypeptide and glucagon, which should be the limitation. In humans, glucagon-like peptide-1 and gastric inhibitory polypeptide are likely to contribute to rapid insulin response after the first meal\(^3\). Glucagon is speculated to activate hepatic glucose production following insulin secretion after the first meal\(^4\). It is an important point to analyze the circadian secretion pattern of them and the association with clock genes.

In summary, the present study analyzed diurnal changes in whole-body glucose homeostasis in men without diabetes, and discussed the factors contributing to these changes at the tissue-specific level. The diurnal patterns were partly reflected by the rhythmicity of insulin secretion and skeletal muscle insulin sensitivity. Furthermore, we showed that hepatic and muscle insulin sensitivity changed in an opposite direction during a day. Hepatic glucose production appears to increase in the

---

**Figure 5** | Insulin sensitivity in mice at the start of the active and inactive period. (a) Experimental conditions and groups. All mice were kept under a light/dark (LD) cycle (light on from 8 a.m. to 8 p.m.) until eight weeks-of-age. After eight weeks-of-age, ZT1 mice were kept under the same LD cycles, and ZT13 mice were housed under the reverse LD cycle (light on from 8 p.m. to 8 a.m.). After an overnight fast, plasma glucose and insulin level were measured. (b) Relative plasma glucose levels from the baseline in insulin tolerance test \((n = 4)\). (c) Serum non-esterified fatty acid (NEFA) levels 15 min after insulin \((0.75 \text{ U/kg})\) injection \((n = 4)\). (d) Western blot of phospho-protein kinase B (P-AKT) and AKT in the liver and gastrocnemius muscle isolated from ZT1 and ZT13 mice, 15 min after insulin injection. Representative blot and P-AKT/AKT ratio \((\text{mean ± standard error of the mean, } n = 5)\) are presented. (e) Western blot of PEPCK in the liver isolated from ZT1 and ZT13 mice, 15 min after insulin injection. Representative blot and PEPCK/\(\alpha\)-tubulin ratio \((\text{mean ± standard error of the mean, } n = 5)\) are presented. *\(P < 0.05\) and **\(P < 0.01\) (one-tailed paired \(t\)-test).
morning, whereas muscle insulin sensitivity is better in the morning and correlated with plasma NEFA concentrations, associated in turn with clock gene expressions and Δcortisol. In human studies, it is difficult to examine diurnal changes in clock genes in each such organ. Although analysis of hair follicles is useful for human studies\(^{19}\), further studies are required to verify the present results. A deeper mechanistic understanding of the communication between the circadian clock and metabolic states might lead to strategies aimed at preventing or treating metabolic disorders.

**ACKNOWLEDGMENTS**

We thank Y Wada for skilled technical assistance. We also thank all study participants. This study was funded by The Japan Diabetes Society Young Investigator Grant and Manpei Suzuki Diabetes Foundation. This research was also supported by grants from the Japan Society for the Promotion of Science (grant no.19 K09006, 18 K081517, 19H03710 and 15H04849).

**DISCLOSURE**

The authors declare no conflict of interest.

Approval of the research protocol: The research protocol was reviewed and approved by the Institutional Ethics Committee of Yamaguchi University Hospital and registered with the clinical trial registry (UMIN 000026015).

Informed consent: All participants were fully informed as to the aims and conduct of the study, and we obtained written consent from each of the participants before their participation. Approval date of registry and the registration no. of the study/trial: H28-122, the date on which the approval was granted: 13 December 2016.

Animal study: All mouse experimental protocols were approved by the Ethics of Animal Experimentation Committee at Yamaguchi University School of Medicine.

**REFERENCES**

1. Huang W, Ramsey KM, Marcheva B, et al. Circadian rhythms, sleep, and metabolism. *J Clin Invest* 2011; 121: 141.

2. Shi M, Zheng X. Interactions between the circadian clock and metabolism: there are good times and bad times. *Acta Biochim Biophys Sin* 2013; 45: 61–69.

3. Dollet L, Pendergrast LA, Zierath JR. The role of the molecular circadian clock in human energy homeostasis. *Curr Opin Lipidol* 2021; 32: 16–23.

4. Cagampang FR, Bruce KD. The role of the circadian clock system in nutrition and metabolism. *Br J Nutr* 2012; 108: 381–392.

5. Han HS, Kang G, Kim JS, et al. Regulation of glucose metabolism from a liver-centric perspective. *Exp Mol Med* 2016; 48: e218.

6. La Fleur SE, Kalsbeek A, Wortel J, et al. A daily rhythm in glucose tolerance: a role for the suprachiasmatic nucleus. *Diabetes* 2001; 50: 243.

7. Ma X, Zhou Z, Chen Y, et al. RBP4 functions as a hepatokine in the regulation of glucose metabolism by the circadian clock in mice. *Diabetologia* 2016; 59: 354–362.

8. Picinato MC, Haber EP, Carpinelli AR, et al. Daily rhythm of glucose-induced insulin secretion by isolated islets from intact and pinealectomized rat. *J Pineal Res* 2002; 33: 172–177.

9. Delattre E, Cipolla-Neto J, Boscher AC. Diurnal variations in insulin secretion and K+ permeability in isolated rat islets. *Clin Exp Pharmacol Physiol* 1999; 26: 505–510.

10. Seshadri N, Jonasson ME, Hunt KL, et al. Uncoupling protein 2 regulates daily rhythms of insulin secretion capacity in MIN6 cells and isolated islets from male mice. *Mol Metab* 2017; 6: 760–769.

11. Saad A, Dalla Man C, Nandy DK, et al. Diurnal pattern to insulin secretion and insulin action in healthy individuals. *Diabetes* 2012; 61: 700.

12. Carroll KF, Nestel PJ. Diurnal variation in glucose tolerance and in insulin secretion in man. *Diabetes* 1973; 22: 333–348.

13. Van Cauter E, Désir D, Decoster C, et al. Nocturnal decrease in glucose tolerance during constant glucose infusion. *J Clin Endocrinol Metab* 1989; 69: 604–611.

14. Van Cauter E, Blackman JD, Roland D, et al. Modulation of glucose regulation and insulin secretion by circadian rhythmicity and sleep. *J Clin Invest* 1991; 88: 934–942.

15. Sonnier T, Rood J, Gimble JM, et al. Glycemic control is impaired in the evening in prediabetes through multiple diurnal rhythms. *J Diabetes Complications* 2014; 28: 836–843.

16. Abdul-Ghani MA, Matsuda M, Balas B, et al. Muscle and liver insulin resistance indexes derived from the oral glucose tolerance test. *Diabetes Care* 2007; 30: 89–94.

17. Okuno Y, Komada H, Sakaguchi K, et al. Postprandial serum C-peptide to plasma glucose concentration ratio correlates with oral glucose tolerance test- and glucose clamp-based disposition indexes. *Metabolism* 2013; 62: 476.

18. Yamaguchi A, Tatsumoto M, Matsumura R, et al. Normal peripheral circadian phase in the old-old with abnormal circadian behavior. *Genes Cells* 2018; 23: 849–859.

19. Akashi M, Soma H, Yamamoto T, et al. Noninvasive method for assessing the human circadian clock using hair follicle cells. *Proc Natl Acad Sci USA* 2010; 107: 648.

20. Radziuk J, Pye S. Diurnal rhythm in endogenous glucose production is a major contributor to fasting hyperglycaemia in type 2 diabetes. Suprachiasmatic deficit or limit cycle behaviour? *Diabetologia* 2006; 49: 628.

21. Jensen J, Rustad PI, Kolnes AJ, et al. The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise. *Front Physiol* 2011; 2: 112.

22. Basse AL, Dalbram E, Larson L, et al. Skeletal muscle insulin sensitivity show circadian rhythmicity which is independent of exercise training status. *Front Physiol* 2018; 9: 1198.

23. Lamia KA, Papp SJ, Yu RT, et al. Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature* 2011; 480: 552–556.
24. Marcheva B, Ramsey KM, Buhr ED, et al. Disruption of the CLOCK components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. Nature 2010; 466: 627–631.
25. Ohta Y, Taguchi A, Matsumura T, et al. Clock gene dysregulation induced by chronic ER stress disrupts β-cell function. EBioMedicine 2017; 18: 146–156.
26. Lamia KA, Storch KF, Weitz CJ. Physiological significance of a peripheral tissue circadian clock. Proc Natl Acad Sci USA 2008; 105: 177.
27. Matsumura T, Ohta Y, Taguchi A, et al. Liver-specific dysregulation of clock-controlled output signal impairs energy metabolism in liver and muscle. Biochem Biophys Res Commun 2020; 534: 415–421.
28. Dyar KA, Ciciliot S, Wright LE, et al. Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock. Mol Metab 2013; 3: 29–41.
29. Beaufere C, Liboz A, Feve B, et al. Molecular mechanisms of glucocorticoid-induced insulin resistance. Int J Mol Sci 2021; 22: 623.
30. Harffmann BD, Schroder EA, Esser KA. Circadian rhythms, the molecular clock, and skeletal muscle. J Biol Rhythms 2015; 30: 84–94.
31. Rachek L. Free fatty acids and skeletal muscle insulin resistance. Prog Mol Biol Transl Sci 2014; 121: 267–292.
32. Kraegen EW, Cooney GJ. Free fatty acids and skeletal muscle insulin resistance. Curr Opin Lipidol 2008; 19: 235–241.
33. Lindgren O, Mari A, Deacon CF, et al. Differential islet and incretin hormone responses in morning versus afternoon after standardized meal in healthy men. J Clin Endocrinol Metab 2009; 94: 892.
34. Petrenko V, Dibner C. Circadian orchestration of insulin and glucagon release. Cell Cycle 2017; 16: 142.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | Participant characteristics.