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ORIGINAL ARTICLE

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

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ABSTRACT

Aims/Introduction: Understanding morning–evening variation in metabolic state is critical for managing metabolic disorders. We aimed to characterize this variation from the viewpoints of insulin secretion and insulin sensitivity, including their relevance to the circadian rhythm.

Materials and Methods: A total of 14 and 10 people without diabetes were enrolled, and underwent a 75-g oral glucose tolerance test (OGTT) and hyperinsulinemic-euglycemic clamp study, respectively. Participants completed the OGTT or hyperinsulinemic-euglycemic clamp at 08.00 hours and 20.00 hours in random order. Before each study, hair follicles were collected. In mice, phosphorylation levels of protein kinase B were examined in the liver and muscle by western blotting.

Results: Glucose tolerance was better at 08 .00 hours, which was explained by the higher 1-h insulin secretion on OGTT and increased skeletal muscle insulin sensitivity on hyperinsulinemic-euglycemic clamp. Hepatic insulin sensitivity, estimated by the hepatic insulin resistance index on OGTT, was better at 20.00 hours. The 1-h insulin secretion and hepatic insulin resistance index correlated significantly with *Per2* messenger ribonucleic acid expression. The change (evening value – morning value) in the glucose infusion rate correlated significantly with the change in non-esterified fatty acid, but not with clock gene expressions. The change in non-esterified fatty acid correlated significantly with *E4bp4* messenger ribonucleic acid expression and the change in cortisol. In mice, phosphorylation of protein kinase B was decreased in the liver and increased in muscle in the beginning of the active period as, expected from the human study.

Conclusions: Glucose metabolism in each tissue differed between the morning and evening, partly reflecting lipid metabolism, clock genes and cortisol levels. Deeper knowledge of these associations might be useful for ameliorating metabolic disorders.

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 metabolism[1–3]. 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 functions[4]. 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 muscles[5]. Glucose tolerance testing in rats showed

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that whole-body glucose tolerance is higher at the start of an active period than at the start of an inactive period[6], with diurnal oscillations of systemic insulin sensitivity peaking in that period[7]. In addition, the daily rhythms of glucose stimulated insulin secretion (GSIS) are robust and persist even in isolated islets[8,9].

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[11–15]. 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[10]. Individuals with prediabetes also have poorer glucose tolerance in the evening, off-setting the evening decline in cortisol levels[15]. 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.*[16]

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[17]. 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[18]. 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 0.8.00 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)-AKTRelative 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 metabolic 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/m^2 (75-g OGTT) and 21.1 ± 0.29 kg/m^2 (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[19]. 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

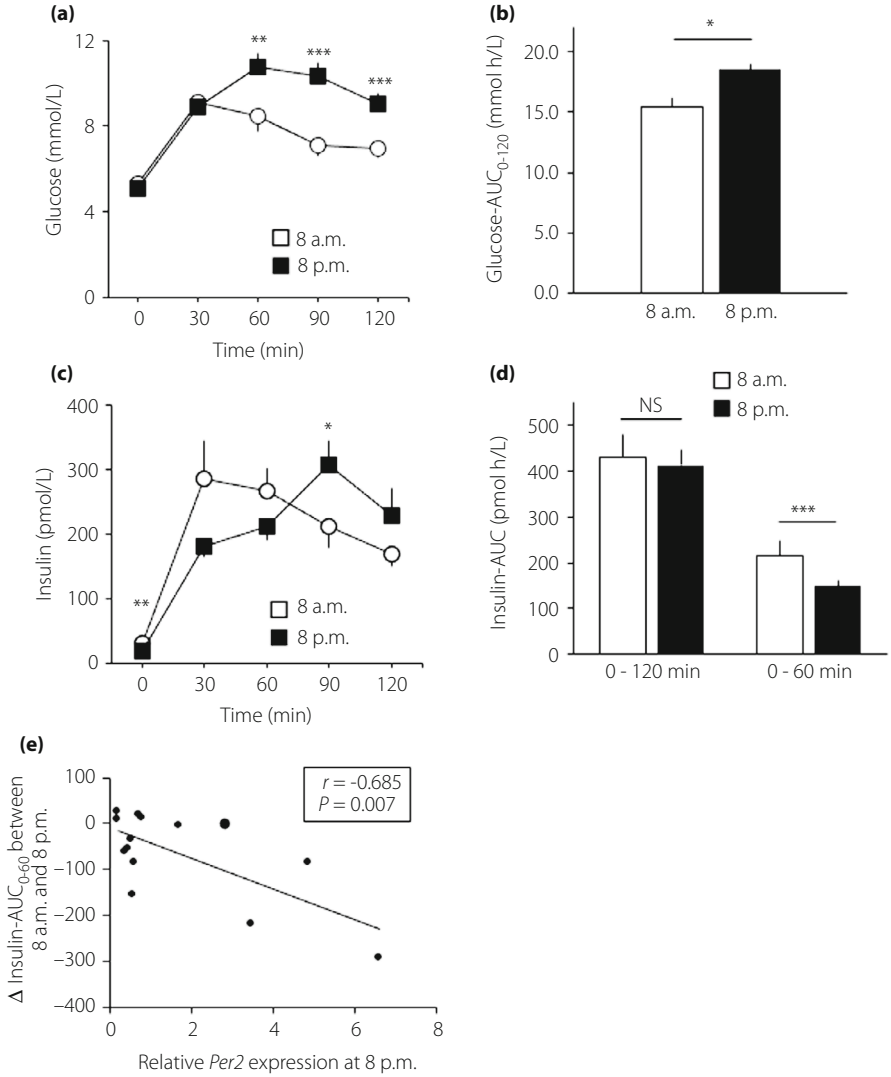

Figure 1 | Comparison of the results of 75 g oral glucose tolerance tests (OGTT) at 8 a.m. and 8 p.m. (n = 14). (a) Plasma glucose levels during 75 g OGTT. (b) Plasma glucose-area under the curve (AUC) $_{0–120\ min}$ during OGTT. (c) Plasma Insulin levels during OGTT. (d) Plasma insulin-AUC $_{0–120\ min}$ and insulin-AUC $_{0–60\ min}$ during OGTT. Data are shown as the mean ± standard error of the mean and were obtained by one-tailed paired t-test at the respective time point. *P < 0.05, **P < 0.01 and ***P < 0.001. (e) Scatterplots showing a linear relationship between Δ insulin-AUC $_{0–60\ min}$ (evening value – morning value) and the relative *Per2* mRNA in hair follicle cells (value at 8 p.m. / value 8 a.m.). NS, not significant.

the first 30 min of the OGTT (glucose AUC $_{0–30\ min}$ × insulin AUC $_{0–30\ min}$)[16]. The hepatic insulin resistance index was significantly greater at 08.00 hours than at 20.00 hours (08.00 hours 285.0 ± 55.0 vs 20.00 hours 175.9 ± 16.3, P = 0.0336; Figure 2a), and we found that it was significantly associated with relative expression of *Per2* in hair follicles at 20.00 hours (r = –0.690, P = 0.006; Figure 2b). In addition, the plasma concentrations of some hormones showed significant diurnal changes in the study participants. In particular, the cortisol level considerably changed (08.00 hours 342.6 ± 18.1 vs 20.00 hours 131.8 ± 20.0 mmol/L, P < 0.00001). Although high cortisol levels are known to decrease glucose tolerance, the Δcortisol (value at 20.00 hours – value at 08.00 hours) was not associated with the Δhepatic insulin resistance index in our regression analysis (Figure 2c).

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 ± 55.0 vs 20.00 hours 6.69 ± 16.3, P = 0.026; Figure 3a). However, we did not find that ΔGIR 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.

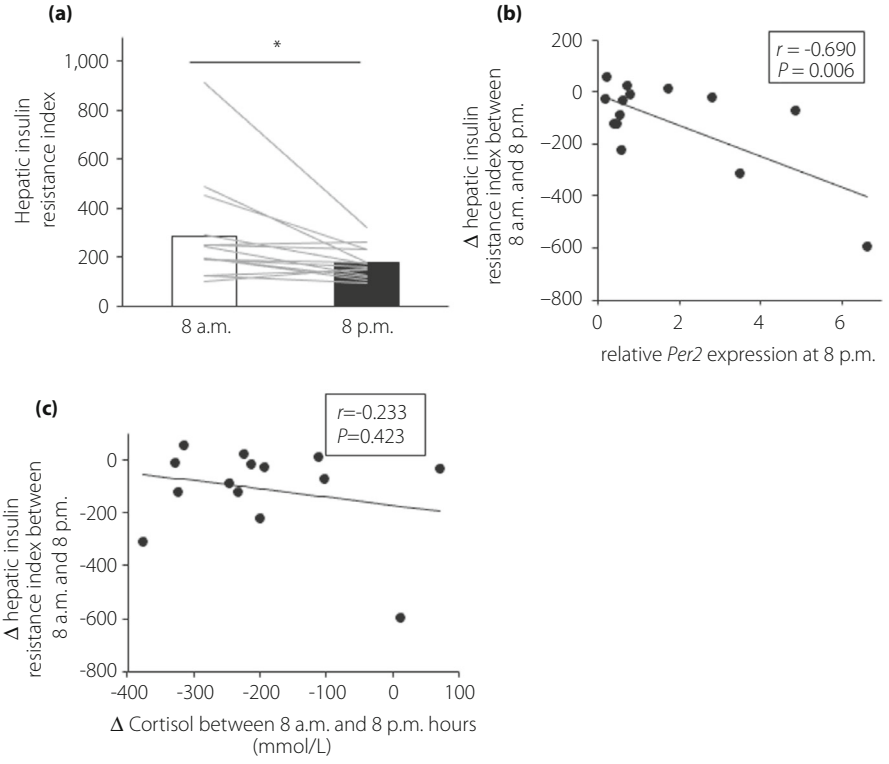

Figure 2 | Analysis of the results of oral glucose tolerance test-derived hepatic insulin resistance index. The hepatic insulin resistance index is defined as glucose-area under the curve $(AUC)_{0–30\ min}$ × insulin-$AUC_{0–30\ min}$ on oral glucose tolerance test (n = 14). (a) The difference in hepatic 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 the (b) relative *Per2* mRNA expression in hair follicle cells (value at 8 a.m. / value at 8 p.m.) or with (c) Δ cortisol (evening value – morning value). Data were obtained by single linear regression analysis.

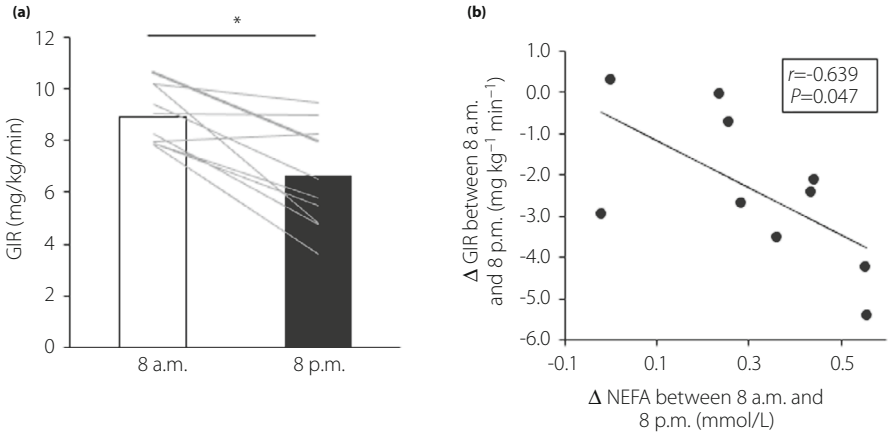

Figure 3 | Analysis of the results of hyperinsulinemic-euglycemic clamp (n = 10). (a) The glucose infusion rate (GIR) difference in hyperinsulinemic-euglycemic clamp between 8 a.m. and 8 p.m. Data are shown as means and were obtained by one-tailed paired t-test. *$P < 0.05$. (b) Scatterplots showing the linear relationship between Δ GIR and Δ serum non-esterified fatty acid (NEFA; evening value − morning value). Data were obtained by single linear regression analysis.

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

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[11-13,15]. 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[20]. From an evolutional 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[21].

A previous mouse study suggested the cell-autonomous circadian rhythm in skeletal muscle to increase insulin sensitivity during the dark/active period[22]. Circadian clock genes have been reported to intrinsically regulate hepatic gluconeogenesis

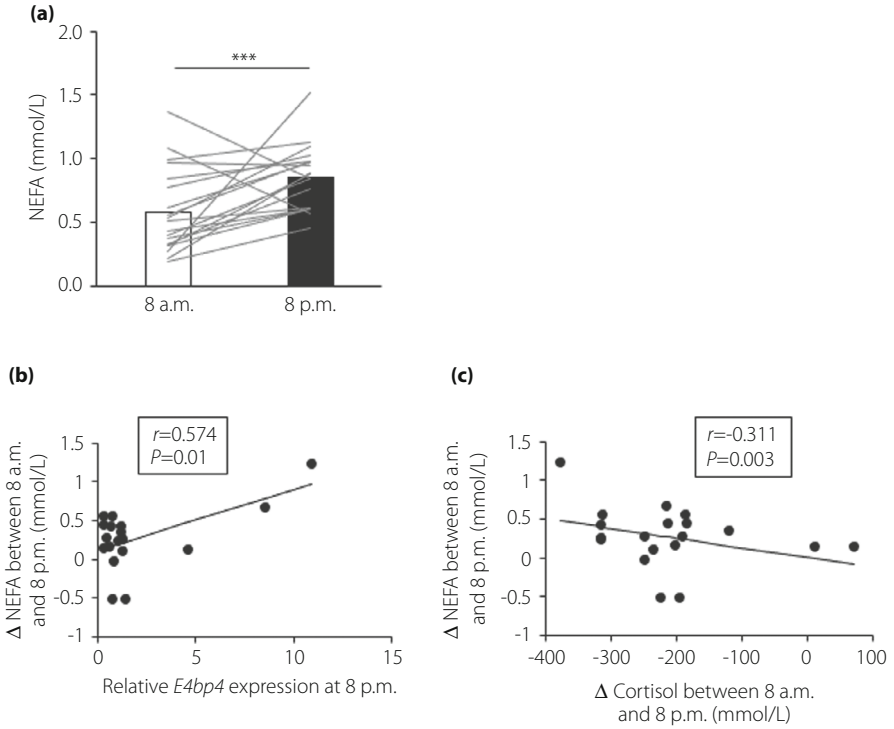

Figure 4 | Analysis of serum non-esterified fatty acid (NEFA) levels. (a) The difference in serum NEFA levels between 8 a.m. and 8 p.m. (n = 19). Data are shown as means and were obtained by one-tailed paired t-test. ***P < 0.001. (b,c) Scatterplots showing linear relationships of Δ serum NEFA (evening value − morning value) with (b) the relative *E4bp4* messenger ribonucleic acid expression in hair follicle cells (value at 8 p.m. / value at 8 a.m.) and with (c) Δ cortisol (evening value − morning value). Data were obtained by single linear regression analysis.

by suppressing glucocorticoid receptor-dependent gene expressions[23]. 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[24,25]. Liver-specific *Bmal1* knockout mice had blunted insulin sensitivity during the fasting phase[26]. 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[27]. Skeletal muscle-specific *Bmal1* knockout reduces insulin-dependent glucose uptake in isolated muscles without changing glucose tolerance[28]. These studies suggest that circadian clocks within individual organs (pancreatic β-cell, liver and skeletal muscle) differently affects 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[29]. 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[30]. 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.

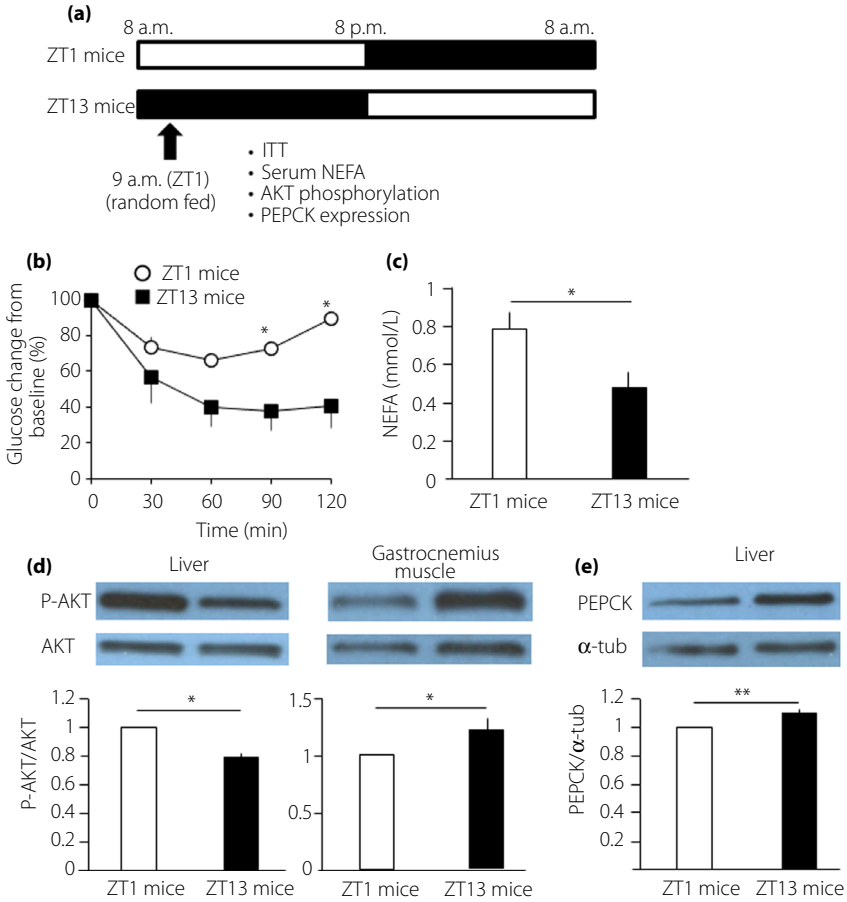

Figure 5 | Insulin sensitivity in mice at the start of the active and inactive period. (a) Experimental conditions and groups. All mice were kept under a light/dark (LD) cycle (light on from 8 a.m. to 8 p.m.) until eight weeks-of-age. After eight weeks-of-age, ZT1 mice were kept under the same LD cycles, 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 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 (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/α-tubulin ratio (mean ± standard error of the mean, n = 5) are presented. *P < 0.05 and **P < 0.01 (one-tailed paired t-test).

NEFA is recognized as one of the major factors contributing to systemic (mainly skeletal muscle) insulin resistance[31,32], and excess exposure to NEFA is associated with the muscle insulin resistance. We observed a diurnal rhythm in plasma NEFA levels to be significantly associated with one of the clock gene (*E4bp4*) expressions and Δcortisol. 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. In this context, of note is that *E4bp4* overexpression in the liver indirectly causes muscle insulin resistance possibly through NEFA[27].

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[33]. Glucagon is speculated to activate hepatic glucose production following insulin secretion after the first meal[34]. 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

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.

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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.

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SUPPORTING INFORMATION

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

Table S1 | Participant characteristics.