Measuring Circulating Glucose and Insulin Concentrations

Blood glucose concentrations were measured using a Glutest Ace R diagnostic system (Sanwa Kagaku Kenkyusyo, Nagoya, Japan). The radioimmunoassay for serum insulin was performed using a commercial kit purchased from Linco Research (St. Charles, MO). The intra- and inter-assay coefficients of variation were less than 10%.

RNA Extraction and Real-Time Quantitative PCR

Isolation of total RNA was achieved using the RNeasy Mini Kit or the RNeasy Lipid Tissue Mini Kit, according to the manufacturer's instructions (Qiagen, Valencia, CA). Reverse transcription was performed with 1.2 μg of total RNA, random hexamer primers, and RevertAid M-MuLV reverse transcriptase (Fermentas, Hanover, MD). Gene expression was analyzed by real-time quantitative polymerase chain reaction (PCR) using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA), as previously described (11,20). All specific sets of primers and TaqMan probes were obtained from Applied Biosystems (TaqMan Gene Expression Assays and TaqMan Rodent GAPDH Control Reagents). To control for variation in the amount of DNA available for PCR in the different samples, gene expression levels of the target sequence were normalized in relation to the expression of an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data were analyzed using the comparative threshold cycle method.

Statistical Analysis

Data were analyzed using either a Kruskal–Wallis test or a Mann–Whitney *U*-test. Values are presented as the means ± SE, and p values of less than 0.05 were considered significant. All calculations were performed using SPSS version 12.0.2J for Windows (SPSS Japan Inc., Tokyo, Japan).

Results

At 10 weeks of age, GK rats were lean and mildly hyperglycemic compared to control Wistar rats (Figure 1). Although hyperglycemia was present, serum insulin concentrations were not elevated as expected, suggesting that glucose-stimulated insulin secretion is

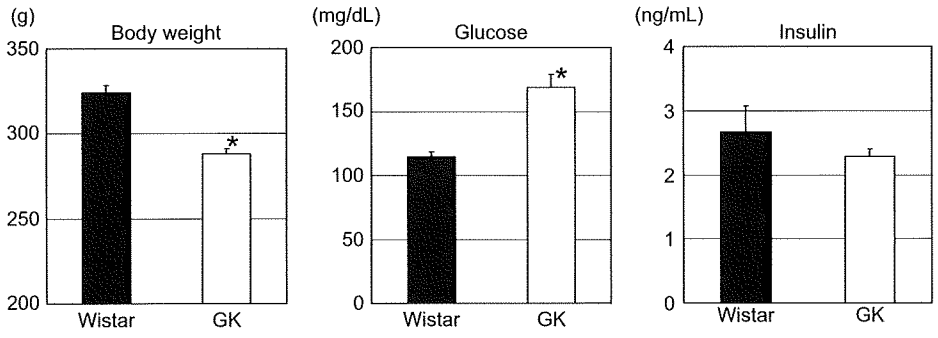

Figure 1. Biological characteristics of Wistar and GK rats at 10 weeks of age. Data are expressed as the means + SE of 16 rats in each group. *$P < 0.01$ vs. Wistar rats.

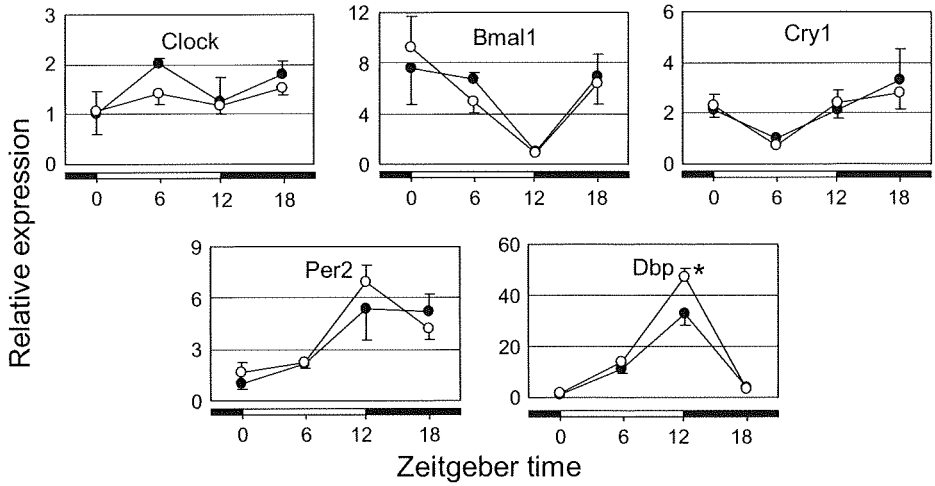

Figure 2. Daily mRNA expression profiles of clock genes in the visceral adipose tissues of Wistar (●—●) and GK (○—○) rats. Data are reported as the means and SE of four rats at each time point and are expressed as relative values (compared to the lowest values in Wistar rats) for each gene. *$P < 0.05$ vs. Wistar rats.

impaired in this strain. Thus, GK rats spontaneously developed glucose intolerance without obesity.

In the visceral adipose tissues of both Wistar and GK rats, mRNA expression of Bmal1, Per2, and Dbp showed significant 24-h rhythms (Figure 2; $\chi^2 = 8.5–14.1$, each $P < 0.05$, Kruskal–Wallis test). In a manner consistent with previous studies in mice (11), transcriptional levels of Per2 and Dbp peaked at ZT 12, whereas Bmal1 mRNA dropped to trough levels at that time. Conversely, rhythmic expression of Clock was not detected in either Wistar or GK rats (Figure 2). In contrast to obese, type 2 diabetic KK-A^y mice (11), peak levels of the clock genes Bmal1, Cry1, and Per2 were not diminished in GK rats. Instead, the rhythmic expression of Dbp, a marker of molecular clock function, appeared to be enhanced in GK rats. These results suggest that hyperglycemia without obesity does not impair the molecular clock system in visceral adipose tissue.

In the livers of both Wistar and GK rats, all of the clock and clock-controlled genes examined (including clock) exhibited significant 24-h rhythmicity (Figure 3; $\chi^2 = 10.9–14.1$, each $P < 0.05$, Kruskal–Wallis test). As in the visceral adipose tissue, the rhythmic mRNA expression of most of the genes examined (clock, Bmal1, Cry1, and Dbp) was not attenuated in GK rats. However, the peak level and amplitude of oscillation of Per2 was significantly reduced in GK rats.

Discussion

Previous studies performed in our lab have revealed that peak transcription levels of clock genes are mildly attenuated in the visceral adipose tissue of obese KK mice and are greatly attenuated in that of more obese, diabetic KK-A^y mice compared to control C57BL/6J mice (11). In contrast, this study demonstrated that rhythmic mRNA expression of clock genes is not dampened in the visceral adipose tissue of nonobese diabetic GK rats. Given

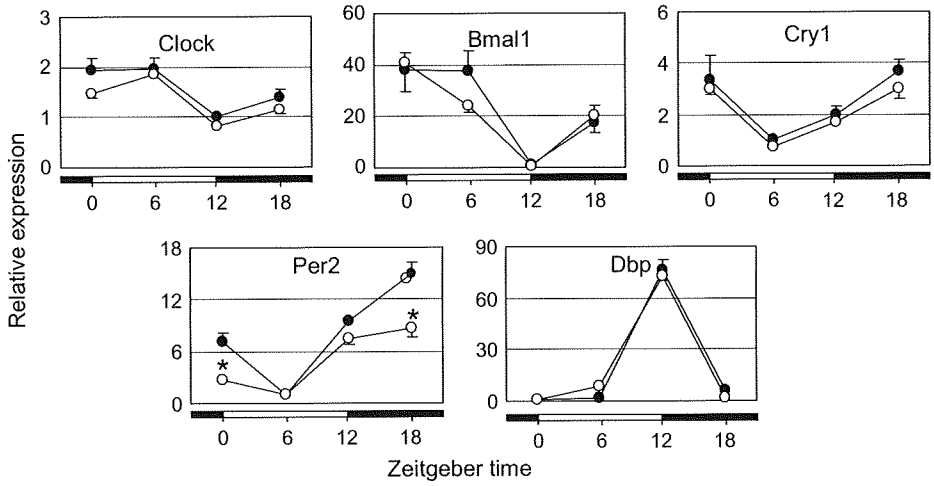

Figure 3. Daily mRNA expression profiles of clock genes in the liver of Wistar (●—●) and GK (○—○) rats. Data are reported as the means and SE of four rats at each time point and are expressed as relative values (compared to the lowest values in Wistar rats) for each gene. *$P < 0.05$ vs. Wistar rats.

that more than one clock gene is involved in the regulation of adipocyte differentiation (14,15), impairment of molecular clock function in the visceral adipose tissue may be related to obesity, but not directly to diabetes.

However, rhythmic mRNA expression of Per2 was mildly, but significantly, attenuated in the liver of GK rats. Kuriyama, Sasahara, Kudo, and Shibata (21) also reported that rhythmic Per2 expression is impaired in the liver, but not the SCN, of streptozotocin-induced, type 1 diabetic mice. In this model, treatment with insulin not only ameliorated hyperglycemia, but recovered impaired Per2 expression rhythms in the liver. These findings suggest that both type 1 and type 2 diabetes, even in the absence of obesity, could affect hepatic Per2 expression. Given that glucose can downregulate Per2 expression in Rat-1 fibroblasts *in vitro* (22), hyperglycemia may reduce peak mRNA levels of Per2 in the liver of diabetic animals.

In spite of the mild attenuation of Per2 expression, rhythmic mRNA expression of the other clock genes examined, and the representative clock-controlled gene Dbp, was not dampened in the liver of GK rats. These results seem to be consistent with findings suggesting that rhythmic Per1 expression is nearly normal in the SCN of heterozygous Per2 mutant mice, unlike in homozygous Per2 mutants (23). Moreover, it has recently been reported that hepatocyte-specific downregulation of Bmal1 expression has little influence on the rhythmic expression of Per2 in the liver of mice; however, the rhythmic expression of Per1, Rev-erbα, and Dbp is greatly attenuated (24). Therefore, hepatic expression of Per2, unlike Per1 and Rev-erbα, appears to be driven not only by the local molecular clock, but also by some other systemic cues. Again, further studies are needed to determine whether hepatic glucose concentrations regulate the rhythmic expression of Per2 in the liver.

In conclusion, the molecular clock was scarcely impaired in the visceral adipose tissue and liver of diabetic GK rats. Oishi, Kasamatsu, and Ishida (25) have also shown that the molecular clock function is preserved to a great extent in the liver, heart, and

kidney of mice with streptozotocin-induced insulinopenic diabetes. Because both GK rats and streptozotocin-treated mice are nonobese, molecular clock impairment in the peripheral tissues of obese diabetic animals seems to be caused by factors related to obesity or obese, type 2 diabetes, but not hyperglycemia. In this case, the mechanisms underlying the association between obese diabetes and molecular clock function remain to be elucidated. Additionally, it is possible that the impaired molecular clock is a cause, but not a consequence, of obese diabetes. Further studies are necessary to more fully clarify the role of the molecular clock in the development of obesity and type 2 diabetes.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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ARTICLE

Clock gene expression in peripheral leucocytes of patients with type 2 diabetes

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Abstract
Aim/hypothesis Recent studies have demonstrated relationships between circadian clock function and the development of metabolic diseases such as type 2 diabetes. We investigated whether the peripheral circadian clock is impaired in patients with type 2 diabetes.
Methods Peripheral leucocytes were obtained from eight patients with diabetes and six comparatively young non-diabetic volunteers at 09:00, 15:00, 21:00 and 03:00 hours (study 1) and from 12 male patients with diabetes and 14 age-matched men at 09:00 hours (study 2). Transcript levels of clock genes (*CLOCK*, *BMAL1* [also known as *ARNTL*], *PER1*, *PER2*, *PER3* and *CRY1*) were determined by real-time quantitative PCR.
Results In study 1, mRNA expression patterns of *BMAL1*, *PER1*, *PER2* and *PER3* exhibited 24 h rhythmicity in the leucocytes of all 14 individuals. The expression levels of these mRNAs were significantly ($p<0.05$) lower in patients with diabetes than in non-diabetic individuals at one or more time points. Moreover, the amplitudes of mRNA expression rhythms of *PER1* and *PER3* genes tended to diminish in patients with diabetes. In study 2, leucocytes obtained from patients with diabetes expressed significantly ($p<0.05$) lower transcript levels of *BMAL1*, *PER1* and *PER3* compared with leucocytes from control individuals, and transcript expression was inversely correlated with HbA_{1c} levels ($\rho=-0.47$ to -0.55, $p<0.05$).
Conclusions/interpretation These results suggest that rhythmic mRNA expression of clock genes is dampened in peripheral leucocytes of patients with type 2 diabetes. The impairment of the circadian clock appears to be closely associated with the pathophysiology of type 2 diabetes in humans.

Keywords Biological clock · Circadian rhythm · Clock gene · Type 2 diabetes

Abbreviations

BMAL1	brain and muscle Arnt-like protein 1
CLOCK	clock homologue (mouse)
HOMA-IR	homeostasis model assessment for insulin resistance
SCN	suprachiasmatic nucleus

Introduction

The circadian system is responsible for regulating a variety of physiological and behavioural processes, including

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feeding behaviour and energy metabolism [1, 2]. Recent studies revealed that the circadian clock system consists essentially of a set of clock genes [1, 2]. The circadian clock resides in the hypothalamic suprachiasmatic nucleus (SCN), which is recognised as being the master clock, and the same clock exists also in almost all peripheral tissues, including liver, heart, kidney [3–5] and leucocytes [6–8]. Although the SCN is not essential for driving peripheral oscillations, it appears to coordinate peripheral clocks [5].

In mammals, rhythmic transcriptional enhancement by two basic helix–loop–helix transcription factors, clock homologue (mouse) (CLOCK) and brain and muscle Arnt-like protein 1 (BMAL1), provides the basic drive for the intracellular circadian clock (Electronic supplementary material [ESM] Fig. 1) [9, 10]. The heterodimer activates the transcription of several other clock genes, including those for period (PER) and cryptochrome (CRY) [11–13]. The resultant PER and CRY proteins heterodimerise, translocate to the nucleus, and inhibit the activity of CLOCK–BMAL1, thus forming a transcriptional–translational feedback loop. In parallel, the CLOCK–BMAL1 heterodimer activates the transcription of various clock-controlled genes [1, 2]. Given that some clock-controlled genes also serve as transcription factors, the expression of numerous genes may be tied to the functions of the circadian clock [1, 2]. Moreover, nearly half of the known nuclear receptors, including peroxisome proliferator-activated receptors (α, γ and δ) and thyroid hormone receptors (α and β), exhibit circadian expression in the liver and adipose tissues, providing an explanation for the cyclic behaviour of glucose and lipid metabolism [14].

Recently, the link between circadian clock function and metabolic diseases has attracted attention. Turek et al. [15] demonstrated that *Clock* mutant mice are hyperphagic and develop metabolic syndrome, hyperglycaemia and hyperlipidaemia. In addition, we showed that the rhythmic expression of clock genes is blunted in the liver and visceral adipose tissues of KK-A^y mice, a genetic model of type 2 diabetes [16]. In humans, genetic variations in the *BMAL1* gene (also known as *ARNTL*) are reported to be associated with susceptibility to type 2 diabetes and hypertension [17]. Furthermore, *CLOCK* haplotypes are associated with metabolic syndrome [18] and non-alcoholic fatty liver disease [19]. These findings strongly indicate that dysfunction of the circadian clock contributes to the development of type 2 diabetes and metabolic syndrome. However, whether clock function is impaired in human patients with these metabolic diseases, as has been shown in mice, remains to be determined. To address this issue, we obtained peripheral leucocytes from patients with type 2 diabetes and from non-diabetic volunteers and compared their mRNA expression rhythms of clock genes.

Methods

Participants Studies 1 and 2 were approved by the ethics committees of Kanazawa University (Kanazawa, Japan) and Medical Co. LTA (Fukuoka, Japan), respectively, and were conducted in accordance with the Declaration of Helsinki as revised in 2000. All individuals were Japanese and participated in the study after giving their written informed consent. We excluded the following individuals: those who had experienced either jet lag or shift work during the 2 weeks preceding the study, those who took psychotrophic drugs in the preceding month, and those with sleep disorder, inflammatory disease, malignancy or anaemia. Additional information about the lifestyles (habits, mealtimes and sleep time) was collected from all participants on the day of the study.

Study 1 The first study was performed from October to December 2006. Eight inpatients with type 2 diabetes and two non-diabetic inpatients with fatty liver were recruited from Kanazawa University Hospital (Kanazawa). All patients with diabetes met the American Diabetes Association's diagnostic criteria for diabetes [20], whereas the other two were classified as having normal glucose tolerance and impaired fasting glucose, respectively, based on a 75 g OGTT. Four healthy men were also enrolled in this study. All of the 14 individuals kept regular hours for at least 2 weeks until the study day or hospital admission. Most individuals usually had three meals a day, whereas one healthy individual always skipped breakfast (ESM Fig. 2). As shown in Table 1, fasting glucose and HbA_{1c} levels in patients with diabetes were significantly higher than those in non-diabetic individuals. In six of eight patients with diabetes, the disease was poorly controlled ($HbA_{1c} \geq 7.0\%$). Three of the six patients were treated with oral agents (pioglitazone, glimepiride + metformin and glibenclamide + metformin + acarbose, respectively). Additionally, patients with diabetes were older than the non-diabetic individuals. The other variables did not differ between the groups.

On the day of the study, blood samples were taken from the forearm vein at 09:00, 15:00, 21:00 and 03:00 hours beginning at 09:00 hours. We chose these time points because *BMAL1* and *PER2* mRNA levels have been reported to peak at about 15:00 and 08:00 hours, respectively [21]. The healthy individuals were asked to assume their everyday routines and sleep in a dim room at their usual times. For the inpatients, sampling commenced within 48 h after admission and was conducted in their hospital room. Fasting blood samples for clinical chemistry were obtained from the inpatients in the early morning on the day after the admission and from the healthy individuals at least 2 weeks before the study day.

Table 1 Characteristics of participants in study 1

Characteristic	Non-diabetic individuals	Patients with type 2 diabetes
N	6	8
Men (*n*, %)	5 (83)	5 (63)
Age (years)	28±7	60±10**
BMI (kg/m^2)	21.4±2.5	26.3±8.9
Current smoker (*n*, %)	1 (17)	2 (25)
Current drinker (*n*, %)	1 (17)	3 (38)
Diabetes treatment (*n*, %)		
Diet alone	–	5 (63)
Oral agents	–	3 (38)
Insulin	–	0
Fasting glucose (mmol/l)	4.7±0.7	7.8±1.9**
Fasting insulin (pmol/l)	49±14	46±28
HOMA-IR	1.5±0.6	2.3±1.5
HbA_{1c} (%)	4.9±0.4	8.1±1.8**
Aspartate aminotransferase (U/l)	16±2	21±9
Alanine aminotransferase (U/l)	16±6	21±10
Total cholesterol (mmol/l)	4.6±1.1	5.0±1.0
Triacylglycerol (mmol/l)	0.9±0.5	1.1±0.7
HDL-cholesterol (mmol/l)	1.5±0.5	1.2±0.2

Values are *n* or means±SD
**$p<0.01$ vs non-diabetic individuals

Table 2 Characteristics of participants in study 2

Characteristic	Healthy individuals	Patients with type 2 diabetes
N	14	12
Age (years)	59±6	58±6
BMI (kg/m^2)	23.1±2.6	24.8±2.4
Current smoker (*n*, %)	8 (57)	3 (25)
Current drinker (*n*, %)	12 (86)	11 (92)
Diabetes treatment (*n*, %)		
Diet alone	–	5 (42)
Oral agents	–	6 (50)
Oral agents + insulin	–	1 (8)
Fasting glucose (mmol/l)	5.6±0.6	9.6±2.2**
Fasting insulin (pmol/l)	45±27	55±27
HOMA-IR	1.7±1.1	3.4±1.9**
HbA_{1c} (%)	5.0±0.3	7.3±1.0**
Aspartate aminotransferase (U/l)	22±7	25±6
Alanine aminotransferase (U/l)	23±9	31±13
Total cholesterol (mmol/l)	5.4±0.6	5.7±0.7
Triacylglycerol (mmol/l)	1.4±0.8	1.5±0.8
HDL-cholesterol (mmol/l)	1.5±0.3	1.5±0.3

Values are *n* or means±SD
**$p<0.01$ vs healthy individuals

Study 2 The next study was carried out from November 2007 to January 2008. Twenty-six men with ages in their 50s to 60s were recruited from LTA PS Clinic (Fukuoka). Twelve individuals were outpatients with type 2 diabetes, whereas 14 individuals were healthy volunteers. All of the participants kept regular hours for at least 2 weeks until the study day. Most of them usually awoke between 05:00 and 07:00 hours and went to bed between 22:00 and 24:00 hours. One healthy participant consumed four meals a day, another healthy individual did not eat breakfast, and the other participants usually ate three meals a day. As shown in Table 2, seven patients were treated for type 2 diabetes with medications (glimepiride + metformin, $n=4$; glimepiride + an α-glucosidase inhibitor, $n=2$; glibenclamide + human insulin, $n=1$). In addition, five and three patients were on medication to treat hypertension (an angiotensin II receptor antagonist, $n=4$; amlodipine + olmesartan, $n=1$) and hyperlipidaemia (pravastatin, $n=2$; bezafibrate, $n=1$), respectively. Venous blood samples for RNA isolation and blood chemistry were collected between 08:30 and 09:30 hours at the LTA PS Clinic after an overnight fast.

Isolation of leucocytes and purification of RNA Immediately after blood sampling, leucocytes were isolated and stabilised using the LeukoLOCK Fractionation and Stabilization Kit (Applied Biosystems, Foster City, CA, USA). Briefly, 10 ml EDTA-anticoagulated blood was passed through a LeukoLOCK filter that captured only leucocytes, and the filter was flushed with PBS to remove residual erythrocytes. The filter was then filled with RNAlater to stabilise leucocyte RNA. The stabilised cells were stored on the filter at −20°C until RNA extraction. The isolation of total RNA was achieved using the LeukoLOCK Total RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions.

cDNA synthesis and real-time quantitative PCR cDNA was synthesised from 1 μg total RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). Gene expression was analysed by real-time quantitative PCR using the Applied Biosystems 7900HT real-time PCR system, as previously described [16, 22]. All specific sets of primers and TaqMan probes (TaqMan gene expression assays) were obtained from Applied Biosystems. To control for variation in the amount of cDNA available for PCR in the different samples, gene expression levels of the target sequences were normalised to the expression of an endogenous control gene (*GAPDH*). The GenBank accession numbers, assay ID, and the target exons were NM_004898.2, Hs00231857_m1, 18-19 for *CLOCK*; NM_001178.4, Hs00154147_m1, 9-10 for *BMAL1*; NM_002616.1, Hs00242988_m1, 22-23 for *PER1*; NM_022817.1, Hs00256143_m1, 8-9 for *PER2*; NM_016831.1, Hs00213466_m1, 15-16 for *PER3*; NM_004075.2, Hs00172734_m1, 2-3 for *CRY1*; NM_001001928.2, Hs00947538_m1, 6-7 for *PPARA*; NM_138711.3, Hs01115512_m1, 4-5 for *PPARG*; NM_006238.3, Hs00602622_m1, 3-4 for *PPARD*; NM_002046.3, Hs99999905_m1, 3-3 for *GAPDH*; and NM_001002.3,

Hs99999902_m1, 3-3 for ribosomal protein, large, P0 (*RPLP0*), respectively. Data were analysed using the comparative threshold cycle method.

Blood chemistry Samples obtained after an overnight fast were assayed for plasma glucose, serum insulin, total cholesterol, HDL-cholesterol, triacylglycerols, aspartate aminotransferase, alanine aminotransferase and HbA_{1c}. Each variable was measured using a commercial kit. Insulin sensitivity was estimated using the homeostasis model assessment for insulin resistance (HOMA-IR) [23].

Statistical analysis Differences in the variables and mRNA levels between patients with diabetes and control individuals were evaluated using the Mann–Whitney test. The rhythmicity of each gene was assessed using the Friedman test. The values are presented as means±SD, and $p<0.05$ was considered significant. All calculations were performed using SPSS version 11 for Windows (SPSS Japan, Tokyo, Japan).

Results

Study 1 Because biological clock function in leucocytes is controversial [24], we first analysed the daily variation in mRNA expression of the clock genes in all 14 individuals. As shown in Fig. 1b–e, the mRNA expression of *BMAL1*, *PER1*, *PER2* and *PER3* exhibited slight but significant 24 h rhythmicity ($\chi^2=12.9$, $p<0.01$ for *BMAL1*; $\chi^2=22.9$, $p<0.001$ for *PER1*; $\chi^2=22.0$, $p<0.001$ for *PER2*; $\chi^2=25.0$, $p<0.001$ for *PER3*; Friedman test to evaluate rhythmicity). Similarly to previous reports [6–8, 21], the levels of *PER1*, *PER2* and *PER3* peaked in the early morning and dropped to a trough level in the evening. On the other hand, the mRNA levels of *CLOCK*, *CRY1* (Fig. 1a,f) and another endogenous control gene (*RPLP0*) remained constant throughout the day ($\chi^2=6.8$, $p=0.08$ for *CLOCK*; $\chi^2=4.5$, $p=0.21$ for *CRY1*; $\chi^2=3.3$, $p=0.34$ for *RPLP0*; Friedman test). When the mRNA levels of clock genes were normalised to the expression of *RPLP0*, *CRY1* ($\chi^2=11.4$, $p<0.01$) as well as *BMAL1*, *PER1*, *PER2* and *PER3* ($\chi^2=13.0$–25.1, each $p<0.01$) showed significant 24 h rhythms with a peak in the morning. These results support the idea that the circadian clock functions in leucocytes.

Next, we compared the mRNA expression rhythms of the clock genes in patients with diabetes with those in non-diabetic individuals (Fig. 1a–f). Interestingly, *BMAL1* mRNA peaked in the evening in non-diabetic individuals ($\chi^2=8.0$, $p<0.05$; Friedman test to evaluate rhythmicity), whereas that peaked in the morning in patients with diabetes ($\chi^2=11.9$, $p<0.01$). In addition, the transcript levels of *BMAL1*, *PER1*, *PER2* and *PER3* were significantly lower in patients with diabetes than in non-diabetic individuals at

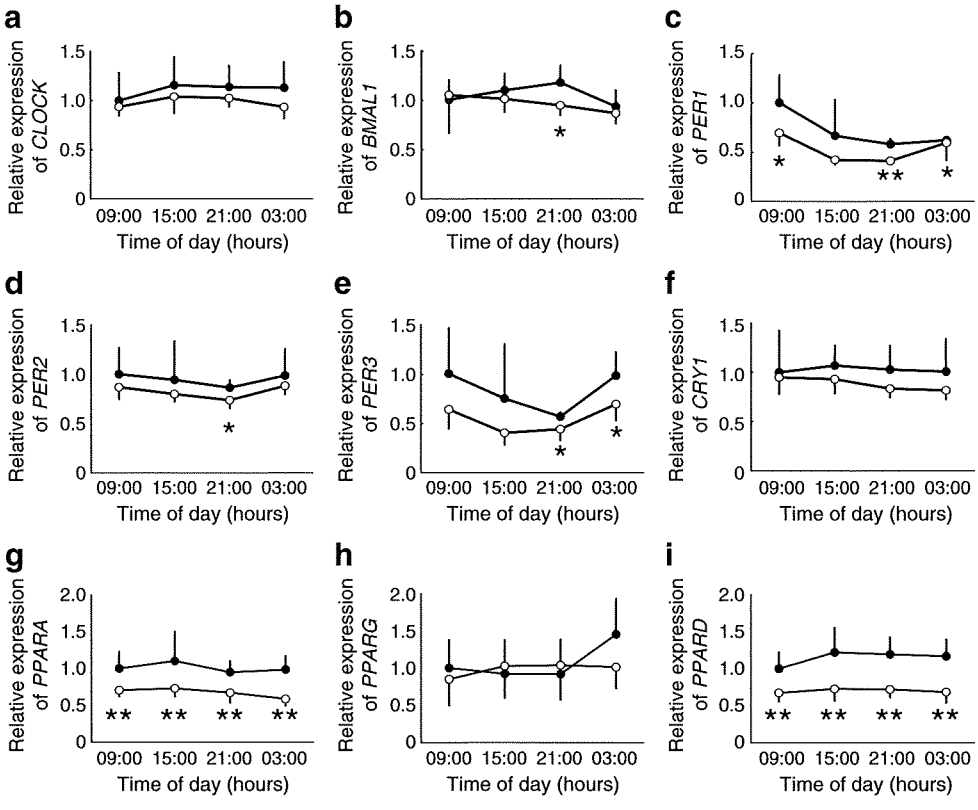

Fig. 1 Daily mRNA expression profiles of clock genes in the peripheral leucocytes of patients with diabetes and non-diabetic individuals. **a** *CLOCK*; **b** *BMAL1*; **c** *PER1*; **d** *PER2*; **e** *PER3*; **f** *CRY1*; **g** *PPARA*; **h** *PPARG*; **i** *PPARD*. Peripheral leucocytes were obtained from six non-diabetic individuals (black circles) and eight patients with type 2 diabetes (white circles) at 09:00, 15:00, 21:00 and 03:00 hours. Transcript levels of the clock genes were determined by real-time quantitative PCR. The mean value of non-diabetic individuals at 09:00 hours was set to 1 for each gene. Means±SD. *$p<0.05$, **$p<0.01$ vs non-diabetic individuals

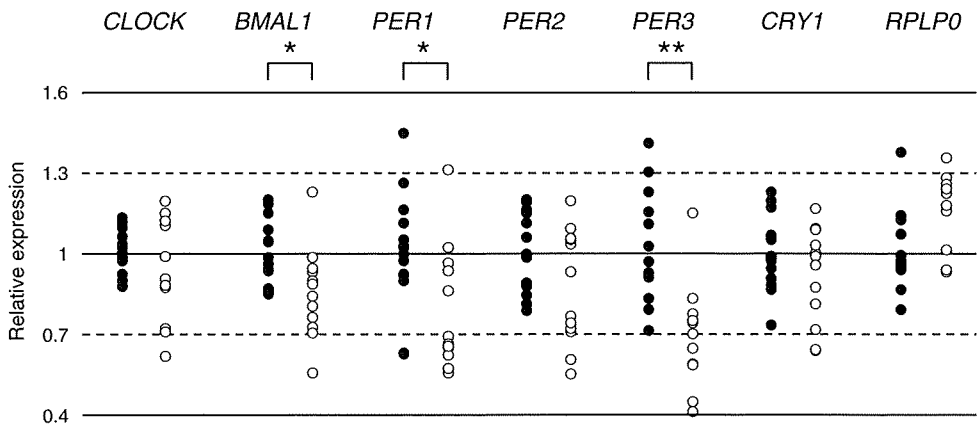

Fig. 2 Transcript levels of the clock genes and *RPLP0* in peripheral leucocytes of patients with diabetes and control individuals. Peripheral leucocytes were obtained from 14 healthy individuals (black circles) and 12 patients with type 2 diabetes (white circles) at 09:00 hours. Transcript levels of the target genes were determined by real-time quantitative PCR. The mean value of healthy individuals was set to 1 for each gene. *$p<0.05$, **$p<0.01$

one or more observation points. A multiple regression analysis identified that both *PER1* and *PER3* mRNA levels at 21:00 hours were correlated with the presence or absence of type 2 diabetes, but not with age (data not shown). Furthermore, the amplitudes of *PER1* and *PER3* were diminished in patients with diabetes (56% and 38% of those in non-diabetic patients; $p=0.06$ and $p<0.01$, respectively). The amplitude of *PER3* determined using *RPLP0* as an internal control also tended to be lower in patients with diabetes (72% of that in non-diabetic patients; $p=0.09$). These results suggest that the function of the circadian clock was impaired in peripheral leucocytes of the patients with diabetes examined in this study.

Because peroxisome proliferator-activated receptors are known to exhibit circadian expression in liver and adipose tissues [14], their daily mRNA expression profiles in the leucocytes were determined as an indicator of the circadian clock function. The mRNA expression levels of *PPARA* and *PPARD* were maintained constant throughout the day (Fig. 1g–i; $\chi^2=5.5$, $p=0.14$ for *PPARA*; $\chi^2=4.7$, $p=0.19$ for *PPARD*), although those of patients with diabetes were significantly lower than those of non-diabetic individuals. On the other hand, *PPARG* mRNA showed a significant 24 h rhythm ($\chi^2=9.6$, $p<0.05$) with a peak in the small hours. This rhythmicity disappeared in patients with diabetes ($\chi^2=5.0$, $p=0.18$) as was expected. This result supports the view that the clock function was diminished in patients with diabetes.

Study 2 The patients with diabetes were older than the non-diabetic individuals in study 1. Because senescence might impair the circadian clock [25], the possibility exists that the age differences in part affected the results. Therefore, we next compared the transcript levels of the clock genes in patients with type 2 diabetes with those from age-matched healthy individuals. Moreover, we recruited only men for this study to exclude a sex effect suggested previously [16, 26]. As shown in Table 2, fasting glucose, HOMA-IR and HbA_{1c} levels were significantly higher in patients with diabetes, but the other variables were similar between the two groups.

Consistent with the findings of study 1, the mRNA levels of *PER1* at 09:00 hours were significantly lower in patients with diabetes than in control individuals (Fig. 2). Moreover, *BMAL1* and *PER3* mRNA levels were also lower in the patients. Conversely, no differences were observed between the two groups in the transcript levels of *CLOCK*, *PER2*, *CRY1* or the endogenous control gene *RPLP0*. Note that *BMAL1*, *PER1* and *PER3* mRNA levels were inversely correlated with HbA_{1c} levels (Spearman's rank correlation coefficient $\rho=-0.47$, $p<0.05$ for *BMAL1*; $\rho=-0.52$, $p<0.01$ for *PER1*; $\rho=-0.55$, $p<0.01$ for *PER3*; Fig. 3). Thus, this study provides evidence that mRNA expression of a subset of clock genes is diminished in patients with type 2 diabetes, especially those with poorly controlled blood glucose.

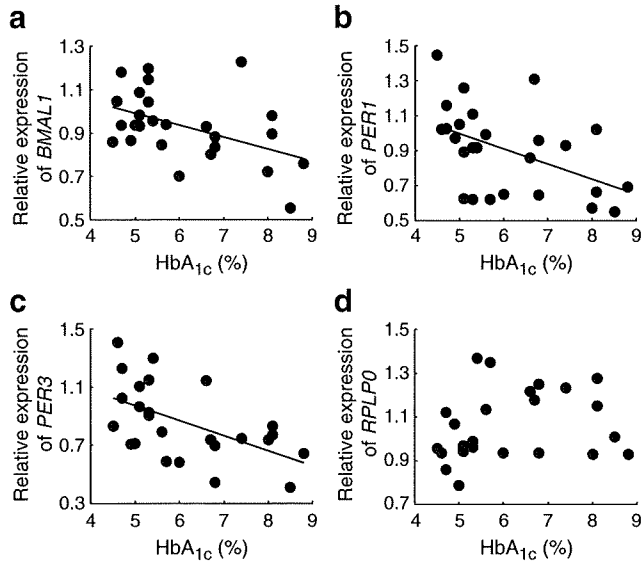

Fig. 3 Relationships between HbA_{1c} levels and mRNA levels of *BMAL1* (**a**), *PER1* (**b**), *PER3* (**c**) and *RPLP0* (**d**)

Discussion

Recent studies have correlated metabolic diseases such as metabolic syndrome and type 2 diabetes with the circadian clock. Our previous study [16] revealed that the rhythmic expression of clock genes was slightly diminished in the peripheral tissues of mildly diabetic KK mice and was greatly blunted in severely diabetic KK-A^y mice. In this study, we demonstrated for the first time that the circadian clock of peripheral leucocytes is diminished in patients with type 2 diabetes, particularly in those with poorly controlled blood glucose.

High glucose has been shown to downregulate *Per1* and *Per2* mRNA expression in cultured fibroblasts [27]. In addition, Kohsaka et al. [26] reported that a high-fat diet affected the rhythmic mRNA expression of *Clock*, *Bmal1* and *Per2* in the liver and adipose tissues of mice. Considering these findings, alterations in glucose, lipid and energy metabolism and/or changes in the concentrations of humoral factors such as plasma glucose appear to influence the peripheral clock.

As shown in Fig. 1, human leucocytes exhibited very weak clock gene oscillations compared with the clocks of the liver and adipose tissues in mice [16]. The peripheral clocks are thought to be synchronised by the SCN through neural and humoral signals [2]. Because peripheral blood leucocytes, unlike the other peripheral tissues, are not controlled directly by neural signals, their oscillators might be easily desynchronised and greatly affected by humoral signals. The observation that the mRNA expression rhythms of *PPARA* and *PPARD* disappeared in leucocytes (Fig. 1g,i) supports this hypothesis. However, *PPARG*, as well as the clock genes, exhibited weak, but significant 24 h rhythmicity, suggesting that the each circadian clock in leucocytes works the same way as those in liver and adipose tissues. Whether each intracellular clock of individual leucocytes is impaired in patients with diabetes remains to be determined; however, attenuation of overall rhythmicity in peripheral leucocytes may cause leucocyte dysfunction. Leucocyte function is known to be depressed in patients with diabetes, which may contribute to their increased susceptibility to infection [28].

That the mutation and genotypes of core clock genes are associated with metabolic diseases [15, 17–19] leads us to speculate that the circadian clock contributes to the development of diabetes. Oishi et al. [29] demonstrated that clock function is preserved to a great extent in the livers, hearts and kidneys of mice with streptozotocin-induced insulinopenic diabetes. We could not confirm impairment of the circadian clock in the liver and adipose tissues of mice fed a high-fat diet, which was reported by Kohsaka et al. [26], even though the mice developed metabolic syndrome characterised by obesity, hyperlipidaemia and hyperglycaemia [30]. Thus, impairment of the circadian clock by type 2 diabetes or metabolic syndrome remains controversial. Further studies are needed to determine whether impaired clock function can be improved by glycaemic control in patients with type 2 diabetes.

Sedentary lifestyles and high dietary fat intake are thought to be instigators of metabolic diseases such as type 2 diabetes and metabolic syndrome. Additionally, it is not uncommon for modern people to keep irregular hours, live rather nocturnal lives or eat late-night snacks. Because light and dietary intake strongly entrain the master and peripheral clocks, respectively [2], these lifestyle features could cause malfunction of peripheral oscillators. Almost all of the individuals enrolled in this study kept regular hours and ordinary lifestyles. Nevertheless, their lives were rather nocturnal compared with the lives of ancient peoples. Therefore, the possibility exists that life in modern society could affect the biological clock, especially in highly susceptible individuals. Although the genotypes of the clock genes were not determined in this study, patients with type 2 diabetes might have defective or fragile circadian clocks.

In summary, rhythmic mRNA expression of clock genes was dampened in peripheral leucocytes of patients with type 2 diabetes. The impairment of the circadian clock appears to be closely associated with the pathophysiology of type 2 diabetes in humans.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Influence of Age on Clock Gene Expression in Peripheral Blood Cells of Healthy Women

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Recent studies have demonstrated a close relationship between circadian clock function and the development of obesity and various age-related diseases. In this study, we investigated whether messenger RNA (mRNA) levels of clock genes are associated with age, body mass index, blood pressures, fasting plasma glucose, or shift work. Peripheral blood cells were obtained from 70 healthy women, including 25 shift workers, at approximately 9:00 AM. Transcript levels of clock genes (*CLOCK*, *BMAL1*, *PER1*, and *PER3*) were determined by real-time quantitative polymerase chain reaction. Stepwise multiple regression analysis demonstrated that *BMAL1* mRNA levels were correlated only with age ($\beta = -.50$, $p < .001$). In contrast, *PER3* levels were correlated with fasting plasma glucose ($\beta = -.29$, $p < .05$) and shift work ($\beta = .31$, $p < .05$). These results suggest that increased age, glucose intolerance, and irregular hours independently affect the intracellular clock in humans.

Key Words: Aging—Biological clock—Circadian rhythm—Shift work.

BIOLOGICAL clocks represent an adaptation to daily 24-hour changes in the environment and enable organisms to maintain physiological homeostasis (1,2). The circadian clock resides in the hypothalamic suprachiasmatic nucleus (SCN), which is recognized as being the master clock. The same clock also exists in almost all peripheral tissues, including liver, heart, kidney (3,4), and blood cells (5,6). Although the SCN is not essential for driving peripheral oscillations, it appears to coordinate peripheral clocks (3).

The intracellular clock consists of autoregulatory transcriptional–translational feedback loops that have both positive and negative elements (1,2). The positive components are two basic helix–loop–helix transcription factors called CLOCK and brain and muscle Arnt-like protein 1 (BMAL1) (7,8). The heterodimer activates the transcription of several other clock genes, including Period (*PER*) and Cryptochrome (*CRY*) (9–11). The resultant PER and CRY proteins form a heterodimer, translocate to the nucleus, and inhibit the activity of CLOCK–BMAL1, thus forming a negative feedback loop. The intracellular clock is thought to directly and/or indirectly regulate the expression of numerous genes (1,2).

It has become evident that circadian clock function is important to the preservation of health. For example, a certain mutation in the *CLOCK* gene induces obesity and metabolic syndrome in mice (12), and genetic variations in the *BMAL1* and *CLOCK* genes are associated with susceptibility to obesity, hypertension, and type 2 diabetes in humans (13–15). Additionally, the *Per2* gene plays an important role in tumor suppression and DNA damage responses: *Per2* mutant mice are known to be cancer prone (16). Interestingly, several epidemiological studies have shown that shift work is associated with obesity, hypertriglyceridemia, glucose intolerance, hypertension, and breast cancer (17–20). Moreover, aging is known to be associated with a variety of alterations in circadian rhythms, which may contribute to various age-related diseases (21,22). It was previously shown that cellular senescence impairs circadian expression of clock genes in vitro (22). These findings raise the possibility that shift work and aging may cause various pathological conditions (e.g., obesity and cancer) through the impairment of biological clock function.

To date, the effects of aging and shift work on intracellular clocks remain to be determined. To address this issue, we measured the messenger RNA (mRNA) levels of *BMAL1*, *PER1*, and *PER3* in peripheral blood cells obtained from healthy women at approximately 9:00 AM, as indicators of overall clock function (6). We investigated the associations between the mRNA levels and age, shift work, body mass index (BMI), blood pressure, and fasting plasma glucose concentration.

Methods

Participants and Sampling

We recruited 70 healthy women (age range 20–79 years, median 53.5 years; BMI range 17.1–31.5 kg/m^2, median 22.2 kg/m^2) from the registered healthy volunteers at the clinical trial unit of LTA PS Clinic (Fukuoka, Japan) between December 2007 and April 2008 and the staff of Moka Hospital (Moka, Japan) between September and November 2008. Because mRNA levels of a subset of clock genes in

Table 1. Characteristics of Participants

	All Participants		Participants Who Kept Regular Hours		Shift Workers	
	n	*M* ± *SD* or %	*n*	*M* ± *SD* or %	*n*	*M* ± *SD* or %
Total	70		45		25	
Age (y)	70	52 ± 14	45	59 ± 11	25	40 ± 12**
Participants aged 65 y or older	15	21	14	31	1	4**
Postmenopausal women	40	57	36	80	4	16**
BMI (kg/m^2)	70	22.5 ± 3.0	45	22.4 ± 2.9	25	22.6 ± 3.2
Participants with BMI ≥25 kg/m^2	14	20	10	22	4	16
Systolic blood pressure (mmHg)	69	120 ± 12	44	121 ± 13	25	118 ± 10
Diastolic blood pressure (mmHg)	69	70 ± 9	44	71 ± 8	25	68 ± 9
Participants with blood pressure ≥140/90 mmHg	3	4	2	5	1	4
Fasting plasma glucose (mg/dL)	59	93 ± 9	44	94 ± 9	15	93 ± 10
Relative messenger RNA levels of clock genes						
CLOCK	70	1 ± 0.20	45	1.04 ± 0.21	25	0.93 ± 0.16*
BMAL1	70	1 ± 0.17	45	0.98 ± 0.16	25	1.04 ± 0.18
PER1	70	1 ± 0.51	45	0.92 ± 0.44	25	1.15 ± 0.58
PER3	70	1 ± 0.33	45	0.89 ± 0.23	25	1.19 ± 0.40**

Notes: BMI = body mass index.
*p < .05, **p < .01 versus participants who kept regular hours.

peripheral blood cells differ between men and women (unpublished data, 2009), only women were included. The following participants were excluded from the study: those who took any medications during the 2 weeks preceding the study; those who experienced jet lag in the preceding month; and those with a sleep disorder, inflammatory disease, malignancy, severe anemia (hemoglobin level <8.0 g/dL), diagnosed hypertension, or diabetes. This information was obtained by interviewing participants and by reviewing the medical records if available. Additional information about the lifestyles (sleep time and mealtimes) was also collected from all participants on the day of the study.

Physical measurements and venous blood sampling were performed between 8:30 and 9:30 AM following an overnight fast. Systolic and diastolic blood pressures were measured in a standardized manner. The samples for RNA isolation were collected into PAXgene Blood RNA tubes (Becton, Dickinson and Company Japan, Tokyo, Japan), and the tubes were incubated at room temperature for at least 4 hours and then stored at −80°C until RNA extraction. In all the 56 participants aged 40 years and older and 3 of 14 participants younger than 40 years, plasma glucose levels were measured using a commercial kit.

As shown in Table 1, 40 of the 70 participants were postmenopausal women. Fourteen participants were overweight or obese, three participants had mild hypertension (systolic and diastolic blood pressures: 143–145 and 86–87 mmHg, respectively), but none of the participants had diabetes (fasting plasma glucose ≥126 mg/dL). Twenty-five participants at Moka Hospital were shift workers and had worked through the night several times during the preceding month. The other 45 women had kept regular hours for at least 2 weeks prior to the beginning of the study, usually waking between 5:00 and 8:00 AM and retiring between 9:00 PM and 1:00 AM. Four participants did not eat breakfast, and three participants did not eat lunch. The other 38 typically consumed three meals per day.

This study was approved by the ethics committees of Jichi Medical University (Shimotsuke, Japan) and Medical Co. LTA (Fukuoka) and was conducted in accordance with the Declaration of Helsinki. Participants participated in the study only after providing their written informed consent.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

Total RNA isolation was achieved using a PAXgene Blood RNA kit (Qiagen Japan, Tokyo, Japan) according to the manufacturer's instruction. Complementary DNA (cDNA) was synthesized from 300 ng of total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems Japan, Tokyo, Japan). Gene expression was analyzed by real-time quantitative polymerase chain reaction (PCR) using an Applied Biosystems' StepOnePlus Real-Time PCR system. All specific sets of primers and TaqMan probes (TaqMan Gene Expression Assays) were obtained from Applied Biosystems. To control for variation in the amount of cDNA available for PCR in the different samples, gene expression levels of the target sequences were normalized to the expression of an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The GenBank accession numbers, assay ID, and target exons were NM_004898.2, Hs00231857_m1, and 18-19 for *CLOCK*; NM_001178.4, Hs00154147_m1, and 9-10 for *BMAL1*; NM_002616.1, Hs00242988_m1, and 22-23 for *PER1*; NM_016831.1, Hs00213466_m1, and 15-16 for *PER3*; and NM_002046.3, Hs99999905_m1, and 3-3 for *GAPDH*, respectively. Data were analyzed using the comparative threshold cycle method.

Table 2. Correlation Coefficients Between Clock Gene Messenger RNA Levels and Variables

	CLOCK	*BMAL1*	*PER1*	*PER3*
All participants				
Age	0.15	−0.29*	−0.13	−0.41**
BMI	0.16	−0.05	−0.06	0.21
Systolic blood pressure	−0.03	−0.03	−0.07	−0.02
Diastolic blood pressure	0.19	0.18	−0.04	0.05
Fasting plasma glucose	−0.25	−0.10	0.01	−0.31*
Participants who kept regular hours				
Age	−0.06	−0.46**	0.24	−0.11
BMI	0.20	−0.05	0.05	0.26
Systolic blood pressure	−0.04	−0.12	−0.11	0.10
Diastolic blood pressure	0.19	0.12	−0.04	0.22
Fasting plasma glucose	−0.25	−0.07	0.17	−0.28
Shift workers				
Age	0.01	0.07	−0.27	−0.29
BMI	0.15	−0.06	−0.22	0.20
Systolic blood pressure	−0.12	0.19	0.06	−0.06
Diastolic blood pressure	0.07	0.35	0.04	0.06
Fasting plasma glucose	−0.42	−0.16	−0.49	−0.39

Notes: BMI = body mass index.
*$p < .05$, **$p < .01$.

Statistical Analysis

Associations between clock gene mRNA levels and age, BMI, and shift work were assessed using Pearson's correlation coefficient and stepwise multiple regression analysis. Differences between participants who kept regular hours and shift workers were assessed by chi-square and *t* tests. Statistical significance was defined as $p < .05$. All calculations were performed using SPSS version 11 for Windows (SPSS Japan, Tokyo, Japan).

Results

As shown in Table 1, the shift workers were significantly younger than the participants who kept regular hours. Consequently, there were fewer postmenopausal women in the shift worker group. However, BMI, blood pressures, and fasting plasma glucose concentration did not differ between the groups. The mRNA levels of *CLOCK* were slightly but significantly lower in the shift workers, whereas their *PER3* levels were higher than that in the other participants.

In the 45 women who were not shift workers, there was significant correlation between the mRNA levels of *BMAL1* and age (Table 2; $r = -.46, p < .01$). This negative correlation was also detected in all participants (Table 2 and Figure 1A; $r = -.29, p = .01$). *PER3*, but not the other clock genes examined, also correlated with age ($r = -.41, p < .001$). In addition, *PER3* negatively correlated with fasting plasma glucose ($r = -.31, p < .05$) in accord with our previous results (6). However, these correlations were not observed in the shift workers (Table 2). Contrary to expectation, none of the clock genes examined correlated with BMI and blood pressures in either the 45 participants who kept regular hours or the entire group of 70 participants (Table 2 and Figure 1B).

Because the shift workers were significantly younger than the others, we next performed stepwise multiple regression analysis with age, BMI, blood pressures, fasting plasma glucose, and shift work as independent variables and clock gene transcript levels as dependent variables. This analysis further demonstrated that *BMAL1* was correlated only with age ($R = .50, p < .001$; age, $\beta = -.50, p < .001$). Moreover, *PER3* mRNA levels were correlated with fasting

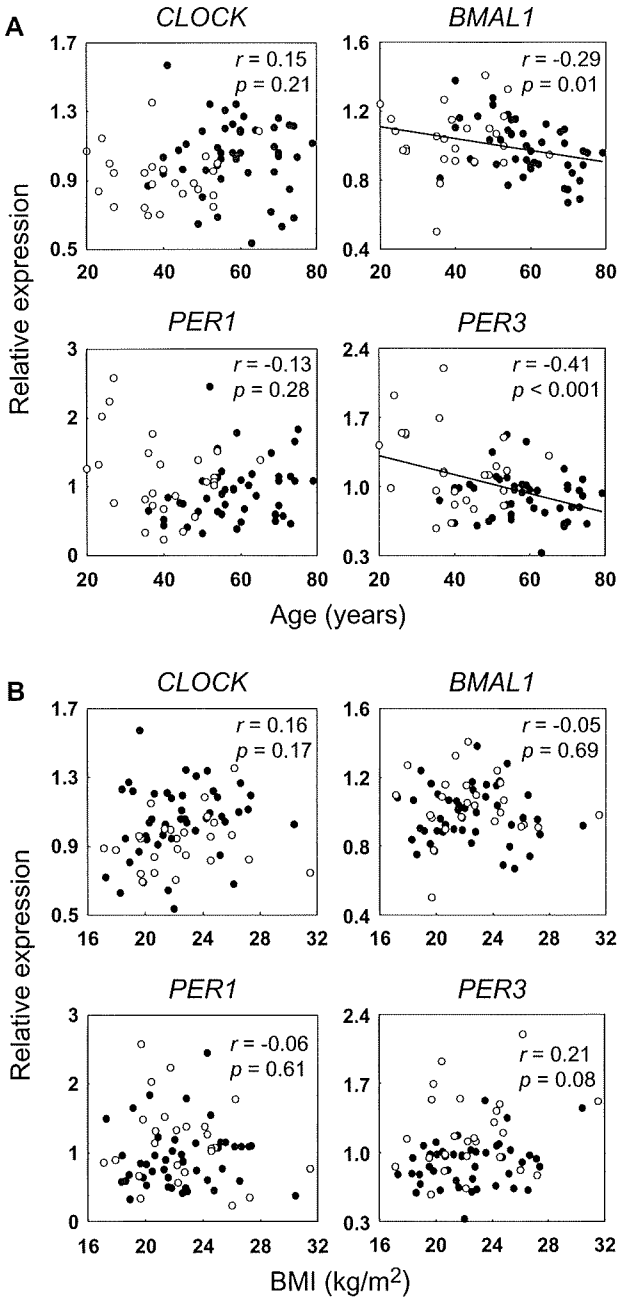

Figure 1. Relationship between messenger RNA levels of clock genes (*CLOCK*, *BMAL1*, *PER1*, and *PER3*) and age (**A**) or body mass index (BMI) (**B**) assessed using Pearson's correlation coefficient. Peripheral blood cells were obtained from 70 healthy women at approximately 9:00 AM. Twenty-five participants (white circles) were shift workers, and the other 45 women (black circles) kept regular hours for at least 2 weeks prior to the study. The transcript levels of clock genes were determined by real-time quantitative polymerase chain reaction. The mean value of all participants was set to 1 for each gene.

plasma glucose and shift work ($R = .44$, $p < .01$; fasting plasma glucose, $\beta = -.29$, $p < .05$; shift work, $\beta = .31$, $p < .05$). These results suggest that both age and shift work affect mRNA expression of a subset of clock genes.

Discussion

Here, we report for the first time a clear association between age and mRNA levels of *BMAL1*, which is a core element of the circadian clock, in humans. Although the rhythm of *BMAL1* expression in peripheral blood cells varies among individuals, *BMAL1* transcript levels often reach a trough level at the time specifically investigated in this study (5,6). Therefore, aging appears to affect *BMAL1* levels per se, rather than the phase of expression rhythms. Aging is known to influence the endocrine circadian system and the amplitudes of rhythms in particular. For example, secretion levels of various hormones, including cortisol, generally decrease in the elderly participants (23). It is speculated that the glucocorticoid signal is important for the oscillation of peripheral clocks as dexamethasone can reset them (24). In addition, age-associated decreases in nitric oxide production may also cause reversible impairment of the circadian clock (25). Furthermore, in this study, *BMAL1* levels in the postmenopausal participants were significantly lower than those in the menstruating women (data not shown). Thus, the age-related alterations in the endocrine circadian system may cause a reduction in the clock gene levels. Because at least part of the age-dependent disturbances of the circadian system can be reversed (26), the molecular machinery of intracellular clocks appears to be maintained even in advanced age. Further studies are needed to determine whether *BMAL1* levels correlate with circulating levels of some humoral factors including cortisol and nitric oxide or whether susceptibility to humoral cues decreases in the peripheral clocks of the elderly participants.

Previous studies from our laboratory have shown that the rhythmic expression of clock genes is blunted in both the liver and the visceral adipose tissues of obese diabetic mice (27) and in the peripheral leukocytes of patients with type 2 diabetes (6). In human leukocytes, the transcript levels of *BMAL1*, *PER1*, and *PER3* are inversely correlated with glycosylated hemoglobin levels (6). Given that several obesity-related humoral factors, including glucose (28), tumor necrosis factor α (29), and angiotensin II (30), can affect the circadian clock in vitro, it is possible that obesity alters the rhythmic expression of clock genes. Nevertheless, a significant association between BMI and clock gene expression was not detected in this study. The effect of mild simple obesity (without diabetes or hypertension) on circadian clock function in peripheral blood cells appears to be negligible.

Findings from recent studies suggest that circadian clock function plays a role in the development of obesity (12,14,15). However, the biological systems involved in body weight regulation are extremely complex, and many genes and chromosomal regions may contribute to defining the common obese phenotype (31). These genes are implicated in a wide variety of biological functions, including the regulation of food intake, energy expenditure, lipid and glucose metabolisms, and adipose tissue development (31). Therefore, it is not surprising that the association between BMI and clock gene expression was not detected in our small-scale study, even though such functions may be regulated by the biological clock.

Sleep–wake cycles have been shown to influence the rhythmic mRNA expression of clock genes in peripheral blood cells of healthy participants (32,33). Archer and colleagues (33) reported that *PER3* and *BMAL1* mRNA levels in peripheral blood cells of healthy participants were positively and negatively correlated with sleep time, respectively. The present study identified similar effects in people performing shift work. Several epidemiological studies suggest that shift work increases the risk of obesity, hypertriglyceridemia, glucose intolerance, hypertension, and breast cancer (17–20). As clock gene dysfunction is thought to cause these pathological conditions (12–16), impairment of the circadian clock may contribute to their subsequent development in shift workers. Further studies are needed to determine the degree of impairment in the clock oscillation system in shift workers.

In this study, we measured the transcript levels of clock genes at only one time point. In addition, daily rhythms of biological functions, such as core body temperature, and rhythmic expression of clock-controlled genes were not investigated. Therefore, it is not known exactly what the altered levels of clock genes indicate. However, the fact that altered mRNA levels of clock genes in peripheral blood cells obtained at an appropriate time point often indicate impaired expression rhythms of them has been revealed in patients with various diseases, including type 2 diabetes (6), circadian rhythm sleep disorder (34), and obstructive sleep apnea syndrome (35). In addition, the clock gene transcript levels in peripheral blood cells (36) and adipose tissues (37) obtained at a time point were reported to be correlated with the metabolic syndrome parameters. Therefore, the alternate measurement approach used in this study may be useful to assess the rhythm and/or function of circadian clock at least in peripheral tissues. Whether the altered clock gene levels in peripheral blood cells are associated with the development of various diseases should be determined in future studies.

In summary, the mRNA levels of a subset of clock genes in the peripheral blood cells of healthy women were found to correlate with age, fasting plasma glucose, and shift work. These results suggest that aging, like glucose intolerance and irregular hours, affects the rhythmic expression of clock genes in humans.

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Histological Course of Nonalcoholic Fatty Liver Disease in Japanese Patients

Tight glycemic control, rather than weight reduction, ameliorates liver fibrosis

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OBJECTIVE — The goal of this study was to examine whether metabolic abnormalities are responsible for the histological changes observed in Japanese patients with nonalcoholic fatty liver disease (NAFLD) who have undergone serial liver biopsies.

RESEARCH DESIGN AND METHODS — In total, 39 patients had undergone consecutive liver biopsies. Changes in their clinical data were analyzed, and biopsy specimens were scored histologically for stage.

RESULTS — The median follow-up time was 2.4 years (range 1.0–8.5). Liver fibrosis had improved in 12 patients (30.7%), progressed in 11 patients (28.2%), and remained unchanged in 16 patients (41%). In a Cox proportional hazard model, decrease in A1C and use of insulin were associated with improvement of liver fibrosis independent of age, sex, and BMI. However, ΔA1C was more strongly associated with the improvement of liver fibrosis than use of insulin after adjustment for each other (χ^2; 7.97 vs. 4.58, respectively).

CONCLUSIONS — Tight glycemic control may prevent histological progression in Japanese patients with NAFLD.

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Accumulating trans-sectional evidence suggests that the presence of multiple metabolic disorders, including obesity, diabetes, dyslipidemia, hypertension, and ultimately metabolic syndrome, are associated with nonalcoholic fatty liver disease (NAFLD) (1). However, it remains unclear which metabolic abnormalities are responsible for the pathological progression of NAFLD, especially in Japanese patients, who generally are not severely obese compared with Western patients.

We retrospectively compared clinical features with the histological changes in the livers of Japanese patients with NAFLD who had undergone serial liver biopsies.

RESEARCH DESIGN AND METHODS

— We recruited 195 patients with clinically suspected NAFLD who had undergone liver biopsies at Kanazawa University Hospital from 1997 through 2008. For details about the study subjects and the exclusion criteria, see supplementary Fig. 1 in the online appendix, available at http://care.diabetesjournals.org/cgi/content/full/dc09-0148/DC1. Of 178 patients diagnosed histologically as having NAFLD, 39 had undergone serial liver biopsies.

Data collection

Clinical information, including age, sex, body measurements, and prevalence of metabolic abnormalities, was obtained for each patient. Venous blood samples drawn for laboratory testing before the liver biopsies were obtained. All subjects had been administered a 75-g oral glucose tolerance test at baseline and at follow-up.

Liver biopsies

Biopsies were obtained after a thorough clinical evaluation and receipt of signed informed consent from each patient. All biopsies were analyzed twice and at separate times randomly by a single pathologist who was blinded to the clinical information and the order in which the biopsies were obtained. The biopsied tissues were scored for steatosis, stage, and grade as described (2), according to the standard criteria for grading and staging of nonalcoholic steatohepatitis proposed by Brunt et al. (3).

For additional details on subjects, data collection methods, liver pathology, and statistical analyses, see supplementary Methods in the online appendix.

RESULTS

— The basal clinical and biochemical data from 39 patients with NAFLD are described in supplementary Table 1. Prevalence of type 2 diabetes, hypertension, and dyslipidemia were 77, 36, and 64%, respectively. The median follow-up period was 2.4 years (range 1.0–8.5). Medications for diabetes and medication changes during the follow-up period are described in supplementary Table 2. Seventeen patients treated with oral diabetic agents were switched to insulin therapy after the initial biopsy. No patients initiated pioglitazone during follow-up.

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Table 1—*Baseline and follow-up clinical features and gradients of laboratory markers associated with changes in liver fibrosis in 39 patients with NAFLD*

	Baseline				Follow-up			
	Improved	Stable	Progressed	*P*	Improved	Stable	Progressed	*P*
n	12	16	11		12	16	11	—
Simple fatty liver:nonalcoholic steatohepatitis (*n*)	3:9	9:7	10:1		10:2	9:7	6:5	
Age (years)	51.5 (29–66)	48.5 (20–79)	51.5 (29–66)	0.97				
Sex (M:F)	5:7	12:4	5:7	0.17				
BMI (kg/m^2)	27.5 (23.2–34.1)	27.7 (22.5–44.4)	30.9 (23.4–37.7)	0.74	26.9 (22.8–31.2)	29.1 (24.3–44.8)	30.7 (24.1–36.3)	0.13
Aspartate transaminase (IU/l)	70 (11–106)	29 (14–86)	32 (13–83)	0.05	23 (11–28)	26 (15–71)	24 (14–164)	0.20
Alanine transaminase (IU/l)	71 (10–209)	48 (23–81)	40 (11–162)	0.13	21 (11–53)	36 (21–66)	31 (12–202)	0.10
Fasting plasma glucose (mg/dl)	133 (96–207)	143 (87–414)	111 (76–167)	0.20	103 (93–220)	121 (83–198)	116 (88–199)	0.51
A1C (%)	8.2 (4.7–11.6)	8.0 (4.9–13.6)	6.2 (5.1–9.5)	0.27	6.0 (5.0–9.0)	6.2 (5.0–10.0)	7.0 (6.0–11.0)	0.10
HOMA-IR	3.9 (0.7–5.5)	3.4 (1.9–7.7)	3.9 (1.6–11.1)	0.91	3.1 (1.5–8.5)	3.4 (1.9–7.7)	3.9 (1.6–11.1)	0.76
QUICKI	0.32 (0.29–0.40)	0.31 (0.27–0.34)	0.31 (0.29–0.35)	0.32	0.33 (0.28–0.37)	0.32 (0.30–0.35)	0.31 (0.29–0.34)	0.82
Muscle insulin resistance	2.1 (1.5–4.0)	1.7 (0.3–3.3)	3.0 (2.1–4.4)	0.20	2.0 (1.3–5.9)	2.4 (1.6–4.5)	1.9 (1.3–4.5)	0.80
Hepatic insulin resistance ($\times 10^6$)	5.3 (2.3–10.2)	5.0 (2.3–10.0)	3.7 (1.4–10.6)	0.66	3.9 (1.4–9.8)	4.3 (1.9–15.9)	4.5 (2.3–8.8)	0.75
Total cholesterol (mg/dl)	191 (128–276)	187 (129–252)	206 (163–244)	0.57	192 (114–224)	195 (136–273)	194 (162–234)	0.74
Triglycerides (mg/dl)	111 (28–224)	114 (36–204)	96 (36–521)	0.87	104 (22–241)	115 (57–241)	131 (36–173)	0.68
HDL cholesterol (mg/dl)	47 (35–82)	51 (31–73)	48 (20–74)	0.68	53 (40–71)	52 (39–64)	52 (36–79)	0.92
Platelets ($\times 10^4/\mu$l)	21.1 (9.4–30.8)	23.0 (7.0–38.2)	24.3 (20.2–41.2)	0.29	23.3 (14.5–27.6)	21.5 (6.3–31.8)	24.0 (15.2–32.6)	0.60
Ferritin (μg/dl)	185 (13–452)	397 (190–604)	46 (10–347)	0.14	74 (16–211)	162 (110–614)	62 (10–171)	0.05
hs-CRP	0.40 (0.08–7.53)	0.14 (0.02–0.61)	0.06 (0.00–0.30)	0.23	0.09 (0.04–0.23)	0.10 (0.00–0.24)	0.09 (0.00–0.89)	0.89
Type IV collagen 7S (ng/dl)	5.1 (2.7–10.0)	4.1 (3.1–7.2)	3.7 (3.3–4.5)	0.27	3.5 (2.3–3.9)	8.3 (3.2–14.0)	4.0 (3.2–5.0)	0.21
HA (ng/dl)	20.6 (0.0–144.7)	25.5 (11.5–299)	30.4 (0.0–61.7)	0.66	32.8 (0.0–117.2)	24.5 (0.0–570)	24.3 (0.0–140.3)	0.63
P-III-P (U/ml)	0.6 (0.5–1.2)	0.6 (0.4–45.0)	0.5 (0.4–0.6)	0.07	0.6 (0.3–0.8)	0.5 (0.5–233.0)	0.6 (0.4–1.0)	0.96
Diabetes (%)	82	69	64	0.59	82	75	64	0.56
Dyslipidemia (%)	73	63	73	0.95	73	63	73	0.86
Hypertension (%)	64	18	36	0.03	64	18	36	0.10
Metabolic syndrome (%)	73	38	27	0.18	67	50	45	0.43
ΔA1C					−1.9 (−6.0 to 0.4)	−1.2 (−6.1 to 4.4)	0.3 (−1.8 to 7.1)	0.02
ΔBody weight					−4.7 (−10.6 to 10.2)	2.2 (−9.4 to 13.4)	−0.9 (−12.7 to 9.6)	0.04
ΔHOMA-IR					−1.3 (−4.4 to 1.2)	−0.3 (−4.3 to 3.3)	−0.7 (−6.1 to 1.8)	0.81

Data are medians (range) or %. A Kruskal-Wallis test and a χ^2 test were used to compare the continuous and categorical variables among three groups. HA, hyaluronic acid; hs-CRP, high-sensitivity C-reactive protein; P-III-P, procollagen III peptide.

Liver fibrosis improved in 12 patients (30.7%), progressed in 11 patients (28.2%), and remained unchanged in 16 patients (41%). As shown in Table 1, fasting plasma glucose, A1C, insulin resistance indicators, and prevalence of metabolic disorders were not significantly different among the three liver fibrosis groups. In the Cox proportional hazard model (supplementary Table 3), although some of the confidence intervals were very wide because of the small sample size, improvement of liver fibrosis was significantly associated with changes in A1C between the initial and final liver biopsies (ΔA1C) ($P = 0.005$) and use of insulin for the treatment of diabetes ($P = 0.019$). Both ΔA1C and use of insulin were independently associated with the improvement of liver fibrosis after adjusted for each other. However, ΔA1C was more strongly associated with the improvement of liver fibrosis than use of insulin (χ^2; 7.97 vs. 4.58, respectively; supplementary Table 3).

CONCLUSIONS— In the present study, we showed that a change in glycemic control (ΔA1C), but not changes in insulin resistance indicators, was an independent predictor of the progression of liver fibrosis in Japanese patients with NAFLD. This is the first report identifying a change in A1C as a predictor of the histological course in the liver of patients with NAFLD. Two of five previous longitudinal studies have identified obesity, higher BMI, and homeostasis model assessment of insulin resistance (HOMA-IR) as predictors of liver fibrosis progression in Western populations (4,5). The difference between those results and the results of the present study may be due in part to differences in the assessed severity of obesity and insulin resistance between the populations. We previously demonstrated that diabetes is an independent risk factor for the progression of liver fibrosis in hepatitis C (6) and that diabetes accelerates the pathology of nonalcoholic steatohepatitis in the type 2 diabetic rat model OLETF (7).

Liver fibrosis is closely associated with two regulators of fibrosis: transforming growth factor (TGF)-β (8,9) and plasminogen activator inhibitor type 1 (PAI-1) (8,10). High glucose levels induce the expression of TGF-β (11) and PAI-1 (12). We previously reported that the expression of TGF family member genes, PAI-1, and genes involved in fibrogenesis are upregulated in the livers of patients with type 2 diabetes (13,14), suggesting that a diabetic state increases the risk for liver fibrosis.

In the present study, only ΔA1C was associated with the progression of liver fibrosis, but not liver inflammation (data not shown). We speculate that the reduction of A1C inhibits the expression of master genes such as *TGF-β* and *PAI-1* that are involved in the regulation of fibrogenesis, rather than genes involved in liver inflammation, and thereby improves liver fibrosis in NAFLD.

The major limitation of this study was small population size. We could not evaluate the changes of liver histology according to the difference in detail characteristics such as treatment of diabetes. Lower statistical power of this study should be consider when we evaluate the results.

In conclusion, our study suggested that ΔA1C could predict liver fibrosis progression in Japanese patients with NAFLD, and tight glycemic control may ameliorate liver fibrosis. Future large-scale prospective studies are needed to confirm our results.

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No potential conflicts of interest relevant to this article were reported.

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