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The hepatic circadian clock is preserved in a lipid-induced mouse model of non-alcoholic steatohepatitis

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ABSTRACT

Recent studies have correlated metabolic diseases, such as metabolic syndrome and non-alcoholic fatty liver disease, with the circadian clock. However, whether such metabolic changes *per se* affect the circadian clock remains controversial. To address this, we investigated the daily mRNA expression profiles of clock genes in the liver of a dietary mouse model of non-alcoholic steatohepatitis (NASH) using a custom-made, high-precision DNA chip. C57BL/6J mice fed an atherogenic diet for 5 weeks developed hypercholesterolemia, oxidative stress, and NASH. DNA chip analyses revealed that the atherogenic diet had a great influence on the mRNA expression of a wide range of genes linked to mitochondrial energy production, redox regulation, and carbohydrate and lipid metabolism. However, the rhythmic mRNA expression of the clock genes in the liver remained intact. Most of the circadianly expressed genes also showed 24-h rhythmicity. These findings suggest that the biological clock is protected against such a metabolic derangement as NASH.

Various behavioral and physiological processes, including feeding behavior and energy metabolism, exhibit circadian (i.e., 24-h) rhythmicity, which may play a role in maintaining functional homeostasis. Recent studies have revealed that the circadian clock system consists essentially of a set of clock genes [1,2]. In mammals, the circadian clock resides in the hypothalamic suprachiasmatic nucleus (SCN), which is recognized as being the master clock, and in almost all peripheral tissues [3]. The SCN appears to coordinate peripheral clocks, because it is not essential for driving peripheral oscillations [3].

Rhythmic transcriptional enhancement by two basic helix–loop–helix transcription factors, CLOCK and brain and muscle Arnt-like protein 1 (BMAL1), provides the basic drive for the intracellular clock [1,2]. In parallel, the heterodimer activates the transcription of various clock-controlled genes. 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]. For example, nearly half of the known nuclear receptors, including peroxisome proliferator-activated receptors (α, γ, δ) and thyroid hormone receptors (α, β), exhibit circadian expression in liver and adipose tissues, providing a possible explanation for the cyclical behavior of carbohydrate and lipid metabolism [4].

Recent studies have demonstrated relationships between circadian clock function and the development of metabolic diseases, such as type 2 diabetes, metabolic syndrome, and non-alcoholic fatty liver disease (NAFLD). In mice, homozygous mutations in the *Clock* gene lead to the development of metabolic syndrome [5]. Moreover, we showed that the rhythmic expression of clock genes is blunted in the liver and visceral adipose tissues in KK-A^y mice, a genetic model of obese diabetes [6]. In humans, a similar effect in type 2 diabetes was found in peripheral leukocytes [7]. Furthermore, genetic variations in the *BMAL1* gene are associated with susceptibility to type 2 diabetes and hypertension [8], and *CLOCK* haplotypes are associated with metabolic syndrome [9] and NAFLD [10]. Thus, impairment of the circadian clock appears to contribute to the development of metabolic diseases.

However, whether metabolic diseases *per se* affect the circadian clock remains controversial. High glucose down-regulates mRNA expression of the clock genes (*Per1* and *Per2*) in cultured fibroblasts [11]. Additionally, the DNA-binding activity of the CLOCK-BMAL1 heterodimer is regulated by the redox state, at least *in vitro* [12]. Kohsaka et al. [13] 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

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glucose, lipid, and energy metabolism; redox state; and/or the concentrations of humoral factors, such as plasma glucose, appear to influence the peripheral circadian clock. However, Oishi et al. [14] demonstrated that clock function was preserved, to a large degree, in the livers, hearts, and kidneys of mice with streptozotocin-induced insulinopenic diabetes. We also revealed that the circadian clock is hardly impaired in the liver and adipose tissues of non-obese, mild hyperglycemic Goto-Kakizaki rats [15]. Furthermore, we did not observe impairment of the circadian clock in the liver or adipose tissues of mice fed a high-fat diet, even though the mice developed metabolic syndrome, characterized by obesity, hyperlipidemia, and hyperglycemia [16]. Although the reasons for these discrepancies among the various studies are unknown, one reason might be differences in the severity of the pathological condition.

Non-alcoholic steatohepatitis (NASH) is an aggressive form of NAFLD, and the liver with steatosis and inflammation develops hepatic insulin resistance, lipotoxicity, oxidative stress, and mitochondrial abnormalities, which lead to hepatic fibrosis or cirrhosis [17]. We recently established a mouse model of NASH, induced by feeding an atherogenic diet [18]. In this model, the atherogenic diet induced steatosis, inflammation, cellular ballooning, stellate cell activation, hepatic insulin resistance, lipid peroxidation, and oxidative stress in the liver; it finally caused hepatic cirrhosis. Thus, the pathological conditions in the liver of this model are complex and quite severe compared with those of mice fed a simple high-fat diet [13,16]. Therefore, it is reasonable to expect that the hepatic circadian clock may be impaired in this model, if the alterations in metabolism and redox state affect the oscillator. To test this, we developed a custom-made, high-precision DNA chip useful for analyzing the metabolic status of the liver and investigated the rhythmic mRNA expression of clock genes and genes linked to carbohydrate and lipid metabolism, energy production, and redox regulation in the livers of mice fed an atherogenic diet.

Materials and methods

Mice. Male C57BL/6J mice (Charles River Laboratories Japan, Yokohama, Japan) were obtained at 5 weeks of age and maintained under conditions of controlled temperature and humidity and a 12-h light (08:45–20:45 h)/12-h dark (20:45–08:45 h) cycle. Mice had free access to food and drinking water. After 3 days of acclimation, the mice were divided into two groups. Half of the mice ($n = 16$) were fed a standard laboratory diet (CRF-1, Oriental Yeast Co., Tokyo, Japan), whereas the others ($n = 16$) were given an atherogenic diet (Research Diets, New Brunswick, NJ) containing 34.3% fat (lard, soybean oil), 25.8% protein (casein, L-cystine), 24.6% carbohydrate (maltodextrin, sucrose), 1.3% cholesterol, 0.5% sodium cholate, 5.7% mineral mixture, 1.5% vitamin mixture, and 6.3% cellulose. After 5 weeks of feeding, animals were sacrificed to obtain blood and liver samples at the following zeitgeber times (ZT): 0, 6, 12, and 18, in which ZT 0 is defined as lights on and ZT 12 as lights off.

All animal procedures were performed in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi campus of Kanazawa University (Kanazawa, Japan).

Statistical analyses. Differences in the variables and mRNA levels between mice fed the atherogenic diet and control mice were evaluated using Student's t test. The rhythmicity of each gene was assessed using one-way ANOVA. The values are presented as the means ± SEM, and $P < 0.05$ was deemed to indicate statistical significance. All calculations were performed using SPSS software (version 11 for Windows, SPSS Japan, Tokyo, Japan).

Additional details on methods. For details on the blood chemistry, DNA chip analysis, and real-time quantitative PCR, see Supplemental Materials and methods.

Results

Development of a custom-made DNA chip suitable for metabolic research

We established a database of hepatic gene expression profiles in various human diseases, and rodent models of diabetes and/or obesity. The models include patients with type 2 diabetes, with or without obesity [19–24] and NAFLD [25]; genetic rodent models of type 2 diabetes and/or obesity [6,26]; diet-induced rodent models of obesity [27]; diet-induced rodent models of NAFLD [18,28,29]; and a rodent model of ischemic heart disease (manuscript submitted). We extracted the significantly altered genes in each metabolic pathway both in human diseases and animal models and selected 190 mouse genes linked to the circadian clock, energy production, redox regulation, ROS defense, MAPK cascade, energy and cholesterol metabolism, and protein degradation. Because expression of 70 of these genes was hardly detected in a liver sample (FirstChoice mouse liver total RNA, Applied Biosystems) or was determined differently from the results analyzed by real-time PCR, we used data for the other 120 genes for analyses in this study (Supplemental Table 1). The results of the 120 genes analyzed by the DNA chip strongly correlated with those obtained by real-time PCR (Pearson's correlation coefficient $r = 0.963$, $P < 0.0001$; Supplemental Fig. 2).

Mouse model of NASH induced by feeding an atherogenic diet

As reported previously [18], mice fed an atherogenic diet for 5 weeks developed NASH, diagnosed based on histology (Supplemental Fig. 3). Serum concentrations of ALT and total cholesterol in mice fed the atherogenic diet were significantly higher than those in control mice (Table 1). The concentration of d-ROMs was also elevated, suggesting that oxidative stress was induced in the mice on the atherogenic diet.

Global gene expression profile in the livers of mice fed an atherogenic diet

Consistent with the histological and biochemical findings, the DNA chip analyses revealed that the atherogenic diet had a wide influence on mRNA expression, affecting genes linked to energy production, redox regulation, ROS defense, the MAPK cascade, nuclear receptors, energy and cholesterol metabolism, and protein degradation (Supplemental Table 2). In most of the genes examined, the atherogenic diet decreased transcript levels. Specifically,

Table 1
Metabolic parameters in mice fed a regular or atherogenic diet.

Parameter	Control	Atherogenic	P
Body weight (g)	28.7 ± 0.8	23.2 ± 0.9	<0.01
Blood glucose (mg/dL)	166 ± 5	163 ± 8	0.73
Serum ALT (U/L)	18 ± 1	51 ± 7	<0.01
Serum total cholesterol (mg/dL)	98 ± 2	151 ± 7	<0.01
Serum HDL-cholesterol (mg/dL)	71 ± 2	71 ± 3	0.90
Serum triglyceride (mg/dL)	80 ± 13	14 ± 2	<0.01
d-ROMs (U)	20 ± 1	34 ± 3	<0.01

Blood samples were obtained from non-fasted mice at zeightgeber time 0 and 12 ($n = 4$ for each time point in both groups).
Data are means ± SEM of eight mice.
ALT, alanine aminotransferase; HDL, high-density lipoprotein; d-ROMs, derivatives of reactive oxygen metabolites.

the mRNA expression for 35 of 47 genes linked to energy production and redox regulation, 11 of 16 energy metabolism-related genes, and five of six cholesterol metabolism-related genes was significantly suppressed at one or more time points. However, there was no significant difference in the hepatic mRNA expression levels of clock genes between the mice fed the atherogenic diet and control mice at any time point (Supplemental Table 2). This finding was verified by real-time quantitative PCR (Fig. 1).

In control mice, the DNA chip analyses detected rhythmic mRNA expression in 31 genes, in addition to the clock genes (Fig. 2, Supplemental Fig. 4 and Supplemental Table 1). As reported previously [16], daily expression profiles of *Cyp7a1* gene were opposite in phase between the groups (Fig. 2D). Additionally, the atherogenic diet dampened the mRNA expression rhythms in two of two genes related to ROS defense and seven of eight genes involved in protein degradation (Fig. 2E, Supplemental Fig. 4A and Supplemental Table 1). However, transcript levels of most of the genes related to energy production, redox regulation, MAPK cascade, nuclear receptors, and energy and cholesterol metabolism, as well as the clock genes, showed significant 24-h rhythmicity in mice fed the atherogenic diet and in control mice (Fig. 2A–D, Supplemental Fig. 4B and Supplemental Table 1). These results suggest that the circadian clock function is maintained in the livers of mice with NASH, probably due to compensating alterations in the expression of various genes, including ROS defense- and protein degradation-associated genes.

Discussion

Accumulating evidence shows that the circadian clock regulates many physiological functions, such as carbohydrate and lipid metabolism [4], mitochondrial energy production, redox regulation, ROS defense [30,31], and MAPK activity [32]. Thus, it is not surprising that dysfunction in the circadian clock can cause various disorders, including metabolic syndrome [5] and malignancies [33]. However, whether these pathological conditions *per se* cause impairment of clock function remains to be clarified. In particular, our previous finding [16] that simple fatty liver induced by high-fat feeding had little effect on the hepatic circadian clock in mice differs considerably from the results of Kohsaka et al. [13]. To address this issue, we developed a severe NASH model, with oxidative stress and drastic metabolic changes, and investigated the expression rhythms of the clock genes and metabolism- and inflammation-associated genes in the liver of this animal model.

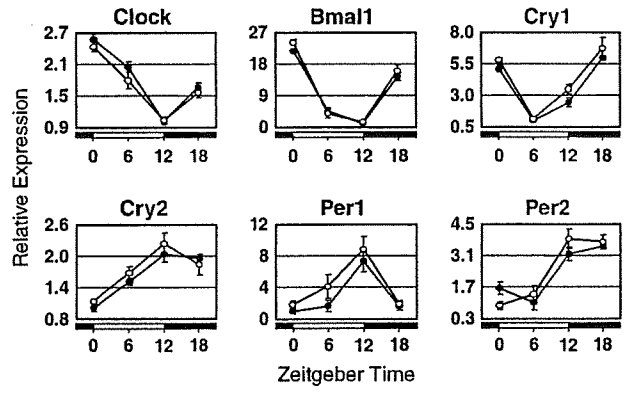

Fig. 1. Daily mRNA expression profiles of clock genes in the livers of mice fed a regular (black circles) or an atherogenic (white circles) diet. Transcript levels of the clock genes were determined by real-time quantitative PCR. Data are means ± SEM of four mice at each time point and are expressed as relative values to the lowest values in control mice for each gene.

As expected, the atherogenic diet altered the mRNA expression of various genes related to energy production, redox regulation, the MAPK cascade, and carbohydrate and lipid metabolism. Additionally, these effects on mRNA expression exhibited daily variation; they became marked during the dark/active phase. Because the light condition and daily feeding profile did not differ between mice fed the atherogenic diet and control mice, the daily variation in the intake of the atherogenic diet components may have caused the difference between mRNA expression profiles in the dark and light phases. However, the intracellular clock remained intact under these drastically altered conditions. These results suggest that the circadian clock is protected against, or not susceptible to, alterations in the intracellular environment, including redox state and metabolism.

Light and dietary intake strongly entrain the master and hepatic clocks, respectively [2,31]. The master clock in the SCN may synchronize the peripheral oscillators, at least partly via the autonomic nervous system [2]. In this study, the mice with NASH were maintained on a well-regulated 12-h light/12-h dark cycle. Additionally, their daily feeding rhythm did not differ from that of control mice (data not shown). Under this condition, the hepatic clock ticked normally. Kohsaka et al. [13] reported that a high-fat diet lengthened the period of locomotor activity rhythm under constant darkness in mice, but the effect was not detected under a 12-h light/12-h dark cycle. Moreover, night-time restricted feeding can normalize the impaired circadian clock in the livers of db/db mice [34]. These results suggest that the signals induced by light and feeding can entrain the hepatic circadian clock, even in the face of the alterations of metabolism and redox state. The influence of a high-fat diet on the hepatic clock may have been observed by Kohsaka et al. [13], but not us [16], due to differences in daily feeding rhythm, which was dampened in their study but not in ours.

Consistent with the intact intracellular clock, the daily expression rhythms of most circadianly expressed genes examined were preserved in the livers of mice with NASH. However, the 24-h expression rhythms of some genes were blunted or changed by the atherogenic diet. It is interesting that the expression rhythms of genes involved in protein degradation were markedly changed in the mice with NASH. The clock proteins, as well as the other short-lived proteins, are degraded by the ubiquitin-proteasome system [2]. Degradation rates of the clock proteins are controlled by their phosphorylation [2] and binding to an F-box protein [35]. These post-translational regulation mechanisms may account for the fact that Cry2 protein accumulates with a markedly higher circadian amplitude than *Cry2* mRNA [36]. Further studies are needed to determine whether the degradation rates of clock proteins are altered to compensate for the effects of the atherogenic diet.

In conclusion, the atherogenic diet caused NASH and alterations in the intracellular environment, affecting energy metabolism, protein degradation, and redox state. However, these conditions did not impair the circadian clock or the expression rhythms of most of the genes examined in the liver. These findings provide evidence that the circadian clock is protected against alterations in the intracellular environment, including metabolism and redox state. The impairment of biological clock appears to be important as a cause of metabolic disease.

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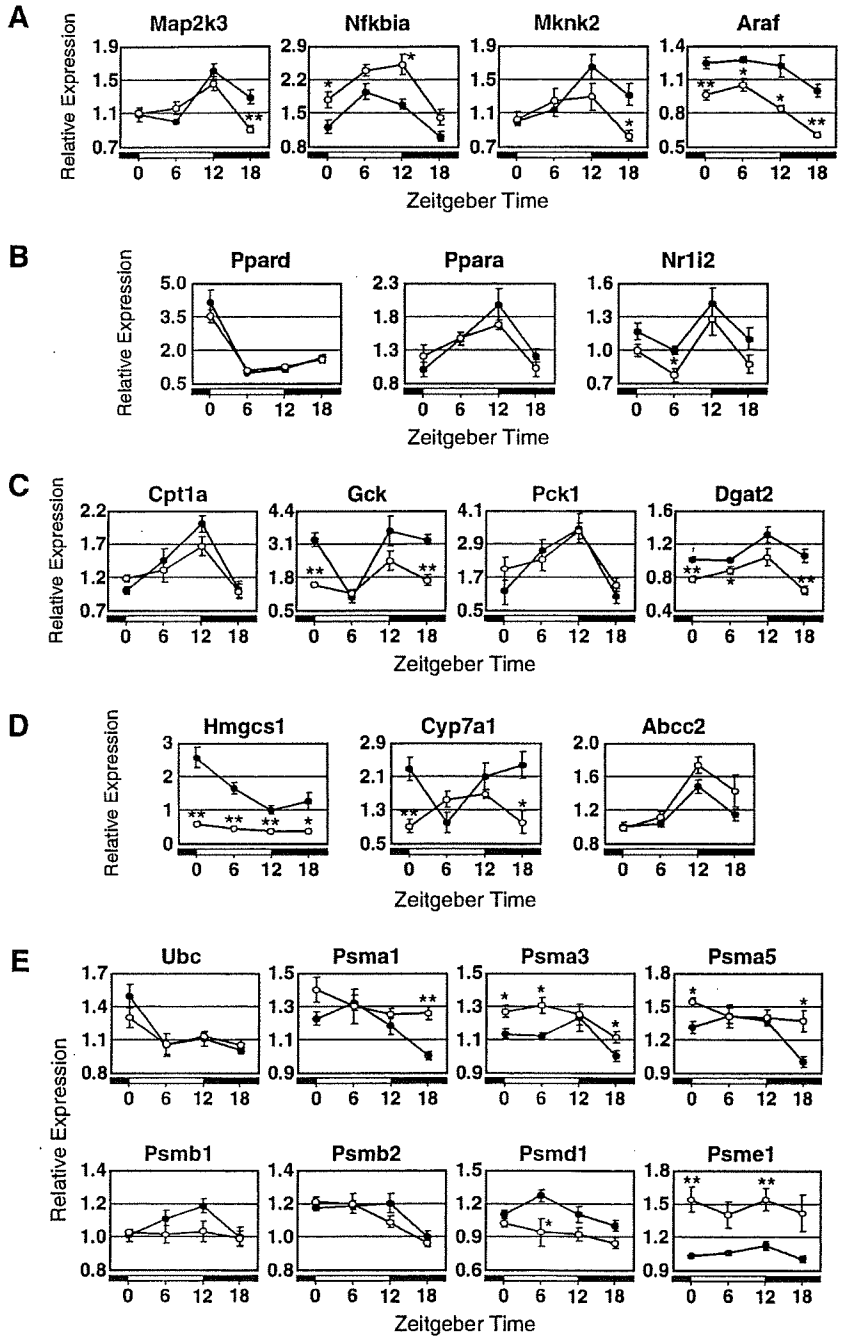

Fig. 2. Daily mRNA expression profiles of the circadianly expressed genes related to the MAPK cascade (A), nuclear receptors (B), energy metabolism (C), cholesterol metabolism (D), and protein degradation (E) in the livers of mice fed a regular (black circles) or an atherogenic (white circles) diet. Transcript levels of the clock genes were determined by the custom-made, high-precision DNA chip. Data are means ± SEM of four mice at each time point and are expressed as relative values to the lowest value in control mice for each gene. $^{*}P < 0.05$, $^{**}P < 0.01$, *vs.* control mice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.150.

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Clock Gene Expression in the Liver and Adipose Tissues of Non-Obese Type 2 Diabetic Goto-Kakizaki Rats

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Recent studies have revealed a close relationship between the pathophysiology of metabolic syndrome, which is characterized by obesity and hyperglycemia, and the functioning of internal molecular clocks. In this study, we show that the rhythmic mRNA expression of clock genes (Clock, Bmal1, Cry1, and Dbp) is not attenuated in the liver and visceral adipose tissues of Goto-Kakizaki rats, a model of nonobese, type 2 diabetes, as compared to control Wistar rats. Our results suggest that molecular clock impairment in peripheral tissues of obese diabetic animals may be either caused by obesity-related factor(s), but not hyperglycemia, or be a cause, but not a consequence, of hyperglycemia.

Keywords circadian rhythm, clock gene, type 2 diabetes, metabolic syndrome, liver

Introduction

Various physiological and behavioral processes exhibit circadian (i.e., 24 h) rhythmicity, which in turn may play a pivotal role in maintaining functional homeostasis. Recent studies have revealed that these endogenous rhythms are generated at the cellular level by circadian core oscillators, composed of transcriptional/translational feedback loops involving a set of clock genes (1,2). In mammals, rhythmic transcriptional enhancement by two basic helix–loop–helix Per–Arnt–Sim domain-containing transcription factors, that is, CLOCK and brain and muscle Arnt-like protein 1 (BMAL1), provides the basic drive

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for the intracellular clock system. Specifically, the CLOCK-BMAL1 heterodimer activates the transcription of various clock-controlled genes (3,4). Given that some clock-controlled genes, including the albumin D-site binding protein (Dbp), also serve as transcription factors, the expression of numerous genes may be tied to the functions of the molecular clock (1,2). In parallel, the heterodimer activates the transcription of several clock genes, including Period (Per) and Cryptochorome (Cry) (5–7). The resultant PER and CRY proteins translocate back into the nucleus and inhibit the activity of CLOCK-BMAL1, thus forming the negative feedback loop (1,2).

The molecular clock system resides not only in the hypothalamic suprachiasmatic nucleus (SCN), which is recognized as the central mammalian clock, but also in various peripheral tissues (8–11). The SCN is not essential for driving peripheral oscillations but acts to synchronize peripheral oscillators (10). Therefore, local molecular clocks may directly control the physiological rhythmicity of peripheral tissues.

Recently, type 2 diabetes mellitus has reached epidemic proportions (12). Pancreatic β-cell dysfunction and insulin resistance are key elements in the pathogenesis of type 2 diabetes and both contribute to the presence of hyperglycemia in this disease. In particular, insulin resistance is strongly associated with obesity, and several mechanisms mediating this interaction have been identified (12). For example, various humoral factors, so-called adipocytokines, which originate in adipose tissue, have been shown to modulate insulin action and may be involved in the development of various diseases including type 2 diabetes, hypertension, dyslipidemia, and cardiovascular disease (11,12). Interestingly, it has been demonstrated that clock mutant mice have an attenuated 24 h feeding rhythm, are hyperphagic and obese, and develop metabolic syndrome, including hyperglycemia and hyperlipidemia (13). In addition, BMAL1 and the clock gene Rev-erbα are known to be involved in the regulation of adipocyte differentiation (14,15). Impaired adipocyte differentiation, as well as increased adipocyte hypertrophy, plays major roles in the development of metabolic syndrome (16). Furthermore, previous studies have revealed that the rhythmic expression of clock genes is attenuated in the visceral adipose tissue and liver of genetically obese, diabetic mice (KK-A^y, ob/ob, and db/db mice) (11,17). Thus, a close relationship may exist between molecular clock functioning and the pathophysiology of obesity and/or type 2 diabetes. At this time, however, it remains to be elucidated whether type 2 diabetes, without obesity, is also associated with impaired molecular clock function. To address this issue, we investigated the rhythmic mRNA expression of clock genes in the visceral adipose tissue and liver of Goto-Kakizaki (GK) rats, a model of nonobese type 2 diabetes (18,19).

Materials and Methods

Rats

Eight-week-old male GK/Jcl and Wistar rats were obtained from CLEA Japan (Tokyo, Japan). All rats were maintained under specific pathogen-free conditions, controlled temperature, controlled humidity, and a 12-h light (07:00–19:00 h)/12-h dark (19:00–07:00 h) cycle. Rats were provided with a standard laboratory diet (CE-2, CLEA, Japan) and water *ad libitum*. After 2 weeks, animals were killed to obtain blood, liver, and epididymal fat samples at the following zeitgeber times (ZT): 0, 6, 12, and 18, where ZT 0 is defined as lights on and ZT 12 as lights off. All animal procedures were performed in accordance with the guidelines for animal research at Jichi Medical University, Japan.

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

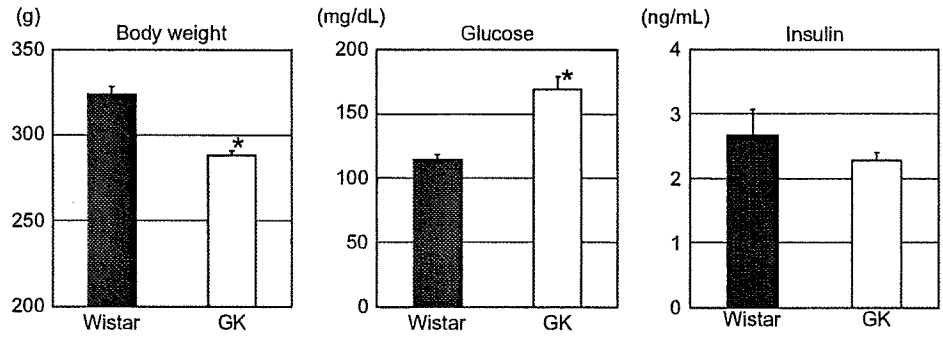

Figure 1. Biological characteristics of Wistar and GK rats at 10 weeks of age. Data are expressed as the means + SE of 16 rats in each group. *$P < 0.01$ vs. Wistar rats.

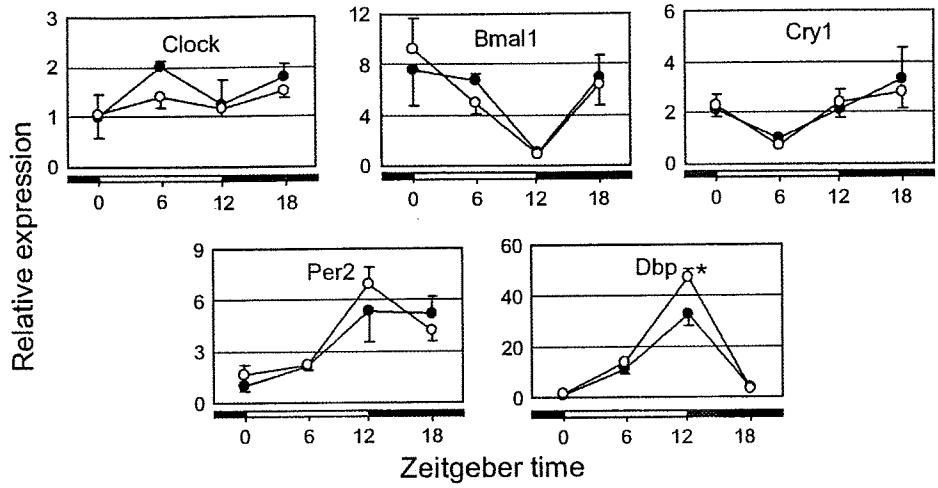

Figure 2. Daily mRNA expression profiles of clock genes in the visceral adipose tissues of Wistar (●—●) and GK (○—○) rats. Data are reported as the means and SE of four rats at each time point and are expressed as relative values (compared to the lowest values in Wistar rats) for each gene. *$P < 0.05$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

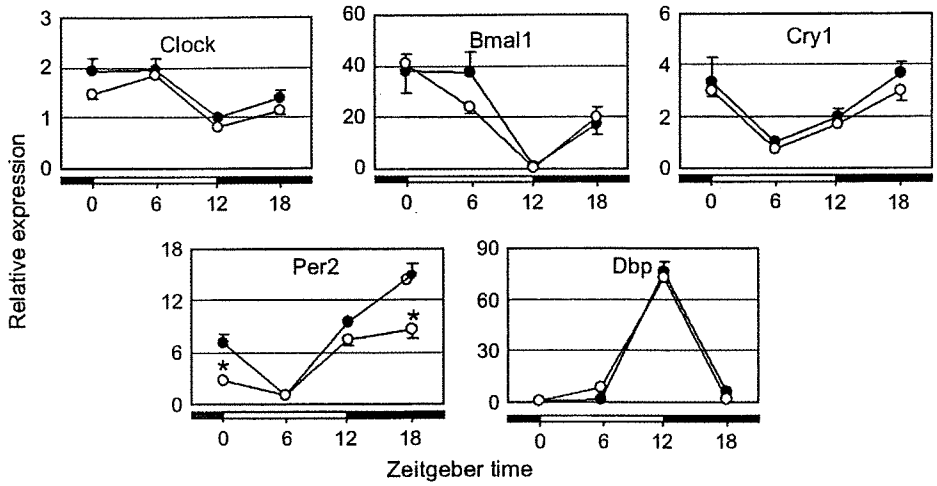

Figure 3. Daily mRNA expression profiles of clock genes in the liver of Wistar (●—●) and GK (○—○) rats. Data are reported as the means and SE of four rats at each time point and are expressed as relative values (compared to the lowest values in Wistar rats) for each gene. *$P < 0.05$ vs. Wistar rats.

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

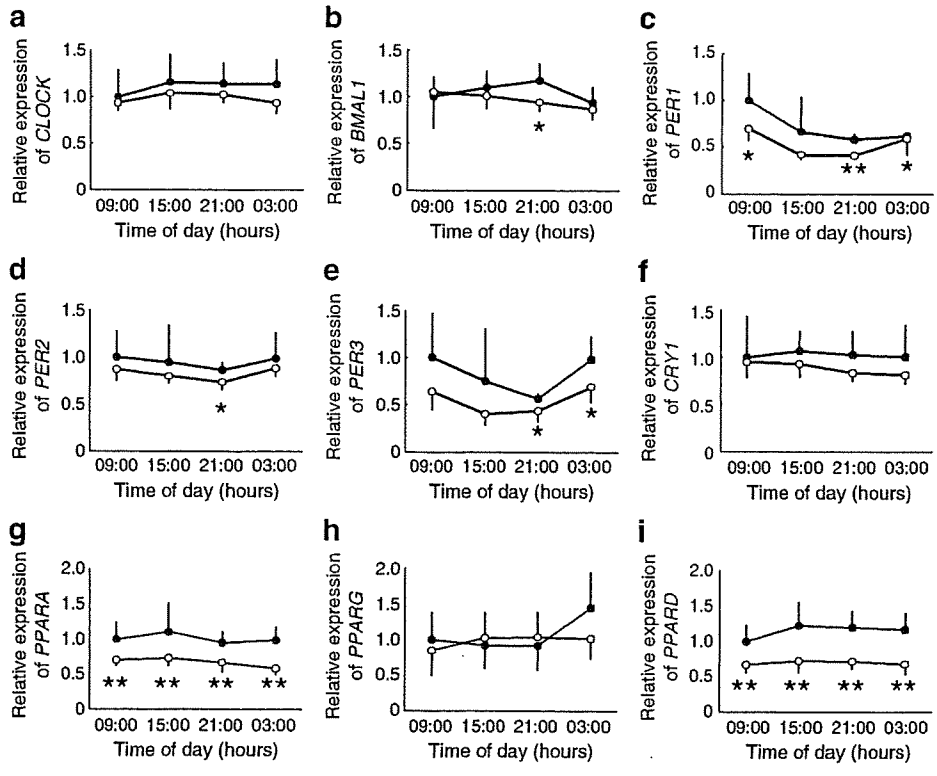

Fig. 1 Daily mRNA expression profiles of clock genes in the peripheral leucocytes of patients with diabetes and non-diabetic individuals. **a** *CLOCK*; **b** *BMAL1*; **c** *PER1*; **d** *PER2*; **e** *PER3*; **f** *CRY1*; **g** *PPARA*; **h** *PPARG*; **i** *PPARD*. Peripheral leucocytes were obtained 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

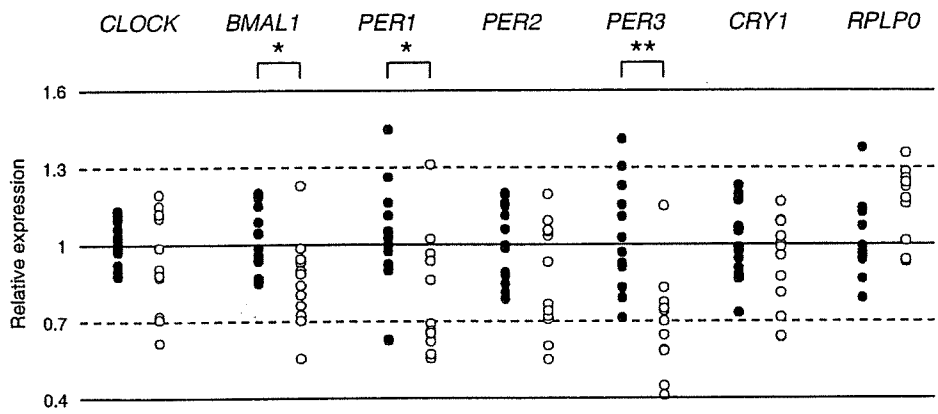

Fig. 2 Transcript levels of the clock genes and *RPLP0* in peripheral leucocytes of patients with diabetes and control individuals. Peripheral leucocytes were obtained from 14 healthy individuals (black circles) and 12 patients with type 2 diabetes (white circles) at 09:00 hours. Transcript levels of 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.

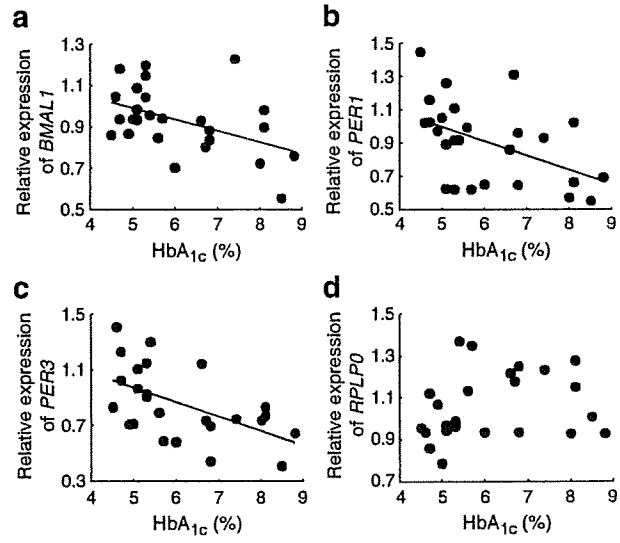

Fig. 3 Relationships between HbA_{1c} levels and mRNA levels of *BMAL1* (**a**), *PER1* (**b**), *PER3* (**c**) and *RPLP0* (**d**)

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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Palmitate Induces Insulin Resistance in H4IIEC3 Hepatocytes through Reactive Oxygen Species Produced by Mitochondria*[S]

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Visceral adiposity in obesity causes excessive free fatty acid (FFA) flux into the liver via the portal vein and may cause fatty liver disease and hepatic insulin resistance. However, because animal models of insulin resistance induced by lipid infusion or a high fat diet are complex and may be accompanied by alterations not restricted to the liver, it is difficult to determine the contribution of FFAs to hepatic insulin resistance. Therefore, we treated H4IIEC3 cells, a rat hepatocyte cell line, with a monounsaturated fatty acid (oleate) and a saturated fatty acid (palmitate) to investigate the direct and initial effects of FFAs on hepatocytes. We show that palmitate, but not oleate, inhibited insulin-stimulated tyrosine phosphorylation of insulin receptor substrate 2 and serine phosphorylation of Akt, through c-Jun NH_2-terminal kinase (JNK) activation. Among the well established stimuli for JNK activation, reactive oxygen species (ROS) played a causal role in palmitate-induced JNK activation. In addition, etomoxir, an inhibitor of carnitine palmitoyltransferase-1, which is the rate-limiting enzyme in mitochondrial fatty acid β-oxidation, as well as inhibitors of the mitochondrial respiratory chain complex (thenoyltrifluoroacetone and carbonyl cyanide *m*-chlorophenylhydrazone) decreased palmitate-induced ROS production. Together, our findings in hepatocytes indicate that palmitate inhibited insulin signal transduction through JNK activation and that accelerated β-oxidation of palmitate caused excess electron flux in the mitochondrial respiratory chain, resulting in increased ROS generation. Thus, mitochondria-derived ROS induced by palmitate may be major contributors to JNK activation and cellular insulin resistance.

Insulin is the major hormone that inhibits gluconeogenesis in the liver. Visceral adiposity in obesity causes hepatic steatosis and insulin resistance. In an insulin-resistant state, impaired insulin action allows enhancement of glucose production in the liver, resulting in systemic hyperglycemia (1) and contributing to the development of type 2 diabetes. In addition, we have demonstrated experimentally that insulin resistance accelerated the pathology of steatohepatitis in genetically obese diabetic OLETF rats (2). In contrast, lipid-induced oxidative stress caused steatohepatitis and hepatic insulin resistance in mice (3). In fact, steatosis of the liver is an independent predictor of insulin resistance in patients with nonalcoholic fatty liver disease (4).

It remains unclear whether hepatic steatosis causally contributes to insulin resistance or whether it is merely a resulting pathology. Excessive dietary free fatty acid (FFA)[2] flux into the liver via the portal vein may cause fatty liver disease and hepatic insulin resistance. Indeed, elevated plasma FFA concentrations correlate with obesity and decreased target tissue insulin sensitivity (5).

Experimentally, lipid infusion or a high fat diet that increases circulating FFA levels promotes insulin resistance in the liver. Candidate events linking FFA to insulin resistance *in vivo* are the up-regulation of SREBP-1c (6), inflammation caused by activation of c-Jun amino-terminal kinase (JNK) (7) or IKKβ (8), endoplasmic reticulum (ER) stress (9), ceramide (10, 11), and TRB3 (12).

However, which event is the direct and initial target of FFA in the liver is unclear. Insulin resistance induced by lipid infusion or a high fat diet is complex and may be accompanied by alterations not restricted to the liver, making it difficult to determine the contribution of FFAs to hepatic insulin resistance. For example, hyperinsulinemia and hyperglycemia secondary to the initial event also may contribute to the development of diet-induced insulin resistance *in vivo* (6).

To address the early event(s) triggering the development of high fat diet- or obesity-induced insulin resistance, we investigated the molecular mechanism(s) underlying the direct action of FFA on hepatocytes to cause insulin resistance *in vitro*, using the rat hepatocyte cell line H4IIEC3. We found that mitochondria-derived reactive oxygen species (ROS) were a cause of palmitate-induced insulin resistance in hepatocytes.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–8.
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[2] The abbreviations used are: FFA, free fatty acid; IRS, insulin receptor substrate; JNK, c-Jun NH_2-terminal kinase; ER, endoplasmic reticulum; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; H2DCFDA, 2′,7′-dichlorofluorescin diacetate; OXPHOS, oxidative phosphorylation; PVDF, polyvinylidene difluoride.