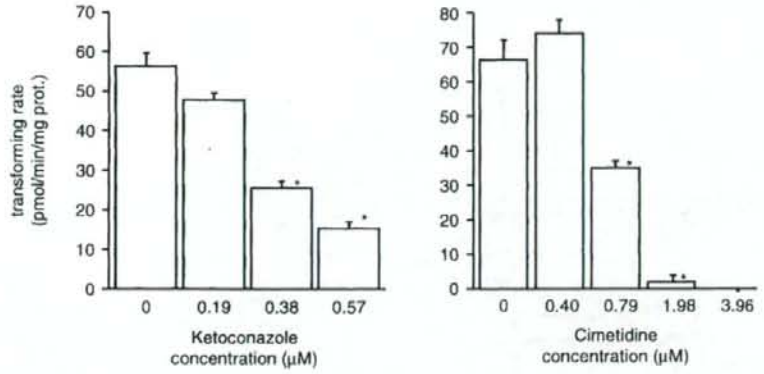

Figure 4. Inhibition of nifedipine hydroxylation by ketoconazole and cimetidine in HepG2-GS-3A4 cell. $^{*}p<0.0001$ *vs.* 0 μM. Data are the mean ± standard error (SE), $n=6$.

143.2; $n=4$), and 126.0 ± 10.2 (95% CI = 119.5 and 146.5; $n=4$) pmol min^{-1} mg^{-1} protein for vehicle, omeprazole, fluvoxamine, quinidine and sulfaphenazole, respectively.

Confirmation of human cytochrome P450 3A4 in HepG2-GS-3A4 cells

The HepG2-GS-3A4 cells had about a nine times larger amount of CYP3A4 protein than HepG2 in Western blot analysis. The densitometric value was 0.18 ± 0.02 (95% CI = 0.14 and 0.22) and 0.02 ± 0.01 (95% CI = 0.00 and 0.04) units for HepG2-GS-3A4 and HepG2, respectively (Figure 5A). Similar results were obtained in the immunohistochemistry. Representative findings are shown in Figure 5B.

NADPH reductase activity was determined in subfractions of the cells. The activity was mainly detected in microsomal fractions in both types of the cell and was significantly higher in HepG2-GS-3A4 cells than in the control (Figure 5C).

Discussion

It was found that the HepG2-GS-3A4 cell, which was originally designed for the possible treatment of hepatic failure and diazepam intoxication, can metabolize several substrates of CYP3A4 with very high capacity *in vitro*. K_m- and V_{max}-values for diazepam *N*-demethylation, diazepam 3-hydroxylation, nifedipine hydroxylation, and lidocaine monoethylglycylation in HepG2-GS-3A4 cells were comparable with the previous literature concerning human liver microsomes (Bargetzi et al. 1989; Niwa et al. 2003; Rawden et al. 2005). These results suggest that the capacity of drug metabolism in HepG2-GS-3A4 cells via CYP3A4 is comparable with that in microsomes of human hepatocytes. The high capacity of drug metabolism via CYP3A4 in HepG2-GS-3A4 cells was further supported by immunohistochemistry and Western blot in these cells. Furthermore, it was found by assay of β-nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome reductase activity that the CYP3A4 activity mostly existed in microsomal fractions, the same as with

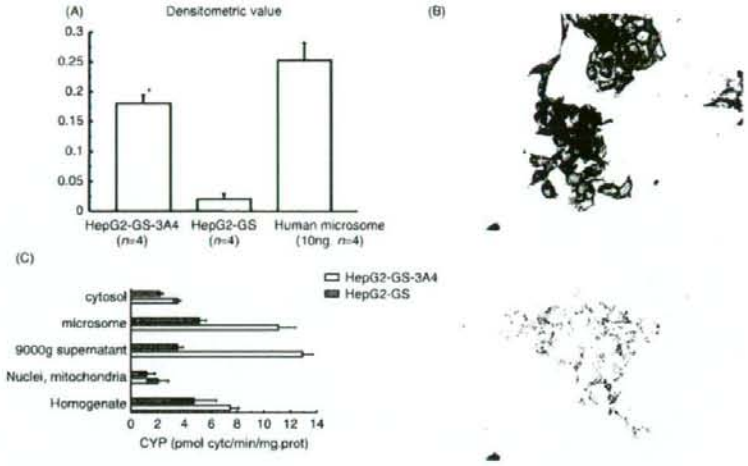

Figure 5. (A) Western blot of human CYP3A4 in HepG2-GS-3A4 and control (HepG2-GS) cells. $^{*}p<0.05$ versus HepG2-GS. (B) Immunohistochemistry of human CYP3A4 protein in HepG2-GS-3A4 (top) and control (HepG2-GS) cells (bottom). (C) Localization of β-nicotinamide adenine dinucleotide phosphate (NADPH) reductase activity in subfractions of the cells.

native hepatocytes. This finding also supported the results of immunohistochemistry that protein was mainly detected in cytosol rather than in the nucleus.

Formation of atorvastain lactone by HepG2-GS-3A4 cells was not different from that by control cells. Metabolism of ammonia was also unchanged between the two cells. This result indicated that the capacity of metabolism is specifically increased in CYP3A4, and the non-CYP3A4-related metabolism was comparable with control cells. Because atorvastain lactone was produced by several pathways, including formation of hydroxyl-atorvastatin via CYP3A4, its formation, which is not related with hydroxyl-atorvastatin, might increase in control cells.

It was shown that the cells can be used for the screening of chemicals to determine whether or not they inhibit CYP3A4 activity. The fact that inhibitors specific to isoforms, other than CYP3A4 such as omeprazole, did not affect the formation of hydroxyl-nifedipine also supported this finding. This is the most important finding of this study. Because CYP3A4 is the most common isoform of P450, many xenobiotics are metabolized by this enzyme. Metabolism of these xenobiotics is reduced when a co-administration drug inhibits CYP3A4 activity, and subsequently leads to overdosing of the drug. Therefore, a drug that does not inhibit CYP3A4 activity is favourable to avoid such drug–drug interactions. To develop a new drug, screening to determine whether or not it inhibits CYP3A4 activity is important (Spilker 1997). *In vivo* screening and *in vitro* screening by human hepatocyte or microsome are usually performed; however, these take time and labour, and simple and easy screening methods are needed. Recombinant protein from *Escherichia coli* or other cells is recently available. However, it also needs to establish specific condition(s) to obtain a good quality assay (Yun et al. 2006; Polasek and Miners 2007) and the data derived from recombinant P450s are not always consistent with those generated from human tissue

preparations (Tang et al. 2005). Therefore, it is not perfect and still needs some improvement before its use. Because the HepG2-GS-3A4 cells possess very high activity of the enzyme, we did not need to extract cells and just measured metabolite concentration in the culture medium, which could not be done in previous methods. Furthermore, the data in this report are much more consistent with that of recombinant CYP3A4 (Yun et al. 2006). We think these are advantages of the HepG2-GS-3A4 cells for evaluation of CYP3A4 activity.

The incidence of pharmacokinetic adverse drug reactions is increased. When physicians find an unexplained overdose of drugs which are a substrate of CYP3A4, the present system can be used to evaluate possible mechanisms. This is the clinical and toxicological significance of this study. One cannot eliminate the contribution of background expression of CYP3A4 and 3A5 in this cell, which may affect to the results in this study. This is a limitation of the present study.

It was confirmed in this study that the inhibitor of CYP3A4, such as ketoconazole, significantly inhibited the formation of nifedipine metabolites, which can be detected in the medium. Therefore, this cell may be used as an easy screening system of new chemicals for future drug development. Ono et al. (1996) previously reported on similar cells into which the CYP3A4 gene was introduced. However, their cells needed to extract a microsomal fraction for measurement of metabolites, which indicated that the expression and activity of CYP3A4 are significantly higher in our cells. Therefore, we think that an assay with our cells is more sensitive and favourable for the future screening of new compounds for inhibition of CYP3A4 activity. Although CYP3A4 is the most common isoform of P450, other isoforms, such as CYP2D6 and 2C19, are also important for drug metabolism. Novel cell lines with the enhanced expression of these isoforms are needed because the present system uses living cells and always need ideal conditions for the cells (such as temperature, humidity, nutrition, etc.). Although these conditions are easily obtained in the usual laboratory, slight changes might affect the sensitivity of the assay, which has to be evaluated in the future.

In summary, the character of HepG2-GS-3A4 cells was determined, which we previously developed for the treatment of hepatic failure and diazepam intoxication. It was found that the expression of CYP3A4 was mainly detected in microsomal fractions and cytosol. It was also found that these cells selectively metabolized several substrates of CYP3A4, and their metabolites were easily detected in the culture medium without any extraction of the microsome. By using this cell, it was found that the pretreatment of ketoconazole and cimetidine, the selective and non-selective inhibitor of CYP3A4 activity, respectively, inhibited the formation of hydroxylated nifedipine. These results indicate that this cell might be applied for the screening of new compounds to evaluate an inhibitory effect on CYP3A4 activity.

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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

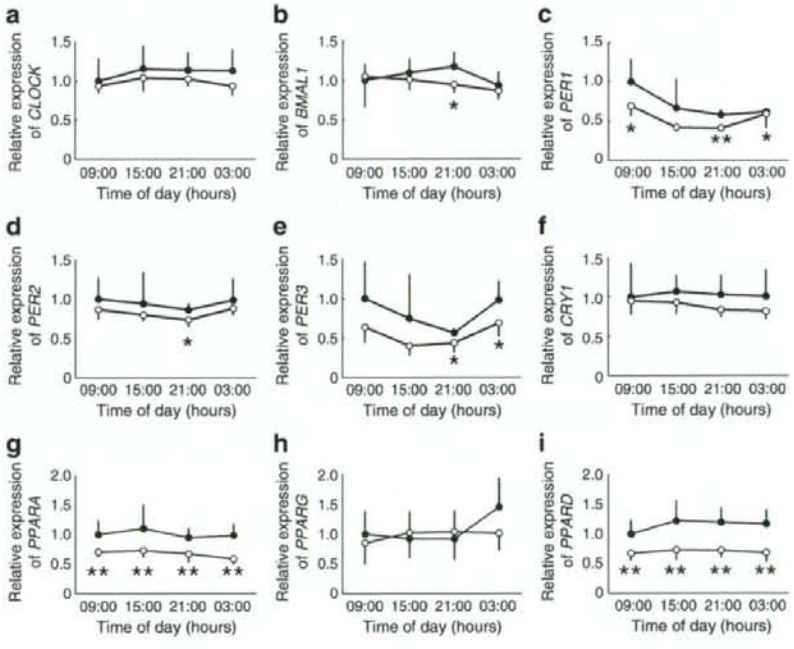

Fig. 1 Daily mRNA expression profiles of clock genes in the peripheral leucocytes of patients with diabetes and non-diabetic individuals. **a** *CLOCK*; **b** *BMAL1*; **c** *PER1*; **d** *PER2*; **e** *PER3*; **f** *CRY1*; **g** *PPARA*; **h** *PPARG*; **i** *PPARD*. Peripheral leucocytes were obtained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

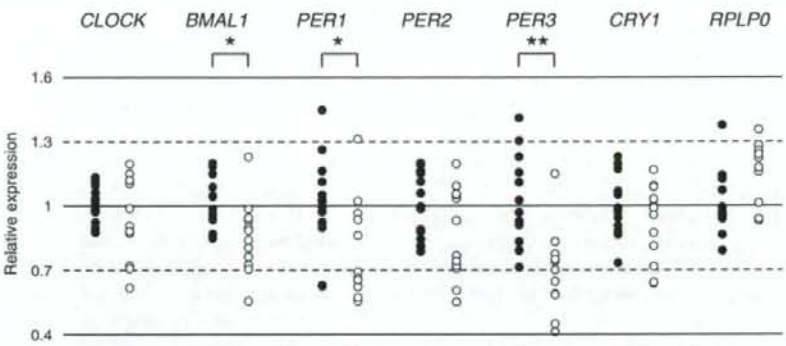

Fig. 2 Transcript levels of the clock genes and *RPLP0* in peripheral leucocytes of patients with diabetes and control individuals. Peripheral leucocytes were obtained from 14 healthy individuals (black circles) and 12 patients with type 2 diabetes (white circles) at 09:00 hours. Transcript levels of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

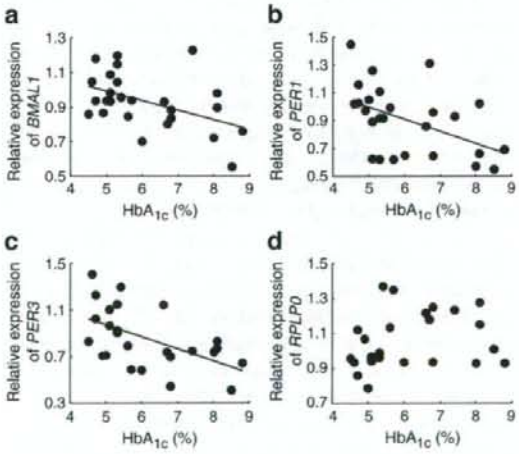

Fig. 3 Relationships between HbA_{1c} levels and mRNA levels of *BMAL1* (**a**), *PER1* (**b**), *PER3* (**c**) and *RPLP0* (**d**)

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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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.

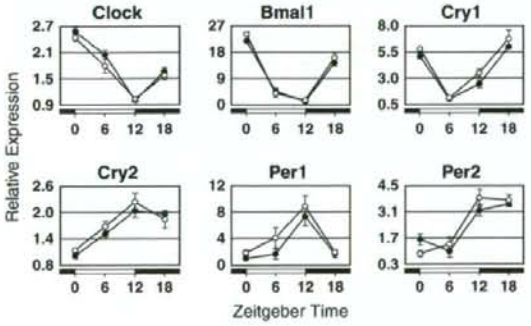

Fig. 1. Daily mRNA expression profiles of clock genes in the livers of mice fed a regular (black circles) or an atherogenic (white circles) diet. Transcript levels of the clock genes were determined by real-time quantitative PCR. Data are means ± SEM of four mice at each time point and are expressed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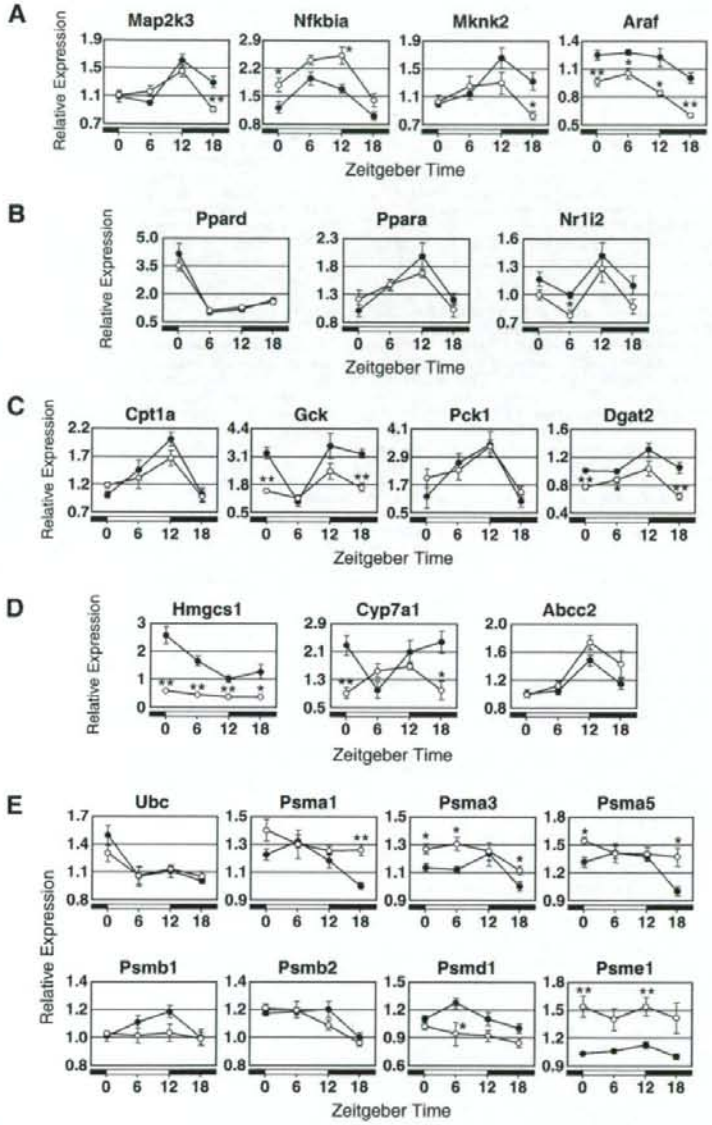

Fig. 2. Daily mRNA expression profiles of the circadianly expressed genes related to the MAPK cascade (A), nuclear receptors (B), energy metabolism (C), cholesterol metabolism (D), and protein degradation (E) in the livers of mice fed a regular (black circles) or an atherogenic (white circles) diet. 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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Forum Minireview

New Topics in Vasopressin Receptors and Approach to Novel Drugs: Vasopressin and Pain Perception

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Abstract. Arginine vasopressin (AVP) activates three vasopressin receptors and it also has an agonistic activity on the oxytocin receptor. For an accurate description of the target receptor subtype(s) responsible for complex AVP and oxytocin actions, a careful evaluation of ligand specificity and receptor activities are required, particularly when these receptors are co-expressed in the central nervous system. Previous studies suggest that AVP plays a regulatory role in nociception through the direct activation of central vasopressin receptors and also through the receptors that reside in the peripheral tissues. Genetically altered rodent models, including the AVP-deficient mutant Brattleboro rat and gene knockout mice lacking an endogenous opioid peptide, advanced the understanding of the interactions between the pain perception process and AVP system. This report reviews previous findings in this important field and reconciles them with the findings of recent gene knockout/knockdown studies.

Keywords: arginine vasopressin, oxytocin, pain, nociception, V1a receptor, V1b receptor

Introduction

Neurohypophyseal hormones, such as arginine vasopressin (AVP) and oxytocin (OT), have a wide range of effects on the central nervous system (CNS), including nociception, learning and memory process, social recognition, central regulation of the cardiovascular system, and stress response (1 – 13). These CNS functions of AVP are not directly related to their peripheral roles as circulating hormones (14). Indeed, neurons containing AVP peptides extend to extrahypothalamic structures, which are important for pain perception (15 – 19). OT, on the other hand, is present in several thalamic nuclei, the mesencephalic central gray nucleus, the substantia nigra, the locus coeruleus, the raphe nucleus, the nucleus of the solitary tract, and the spinal cord (20, 21).

Because AVP is a principal agonist to all vasopressin receptors and also has agonistic activity on OT receptors, it has been difficult to define the target receptor responsible for the complex AVP actions in the CNS. There are three types of G-protein–coupled vasopressin receptors, termed V1a, V1b, and V2 receptors (22); and a single OT-receptor gene has been identified (20). V1a, V1b, and OT receptors activate Gq heterotrimeric GTP binding protein and V2 stimulates Gs protein (22). The main receptors for AVP in the brain could be presumably of the V1 type (23); no V2-specific ligand binding or V2 mRNA were detected in previous reports (24, 25), while other studies suggest that there is a V2-like receptor in the CNS (3, 26). A relatively good correlation was reported between the autoradiographic distribution of mRNA transcripts for the V1a and autoradiographic distribution reported for V1-specific binding sites (25, 27). The V1b receptor transcript, on the other hand, is localized in several brain regions, including the olfactory bulb, CA2 pyramidal neurons in the hippocampus, supraoptic, suprachiasmatic and dorsomedial hypothalamic nuclei, piriform and entorhinal cortices, substantia nigra, and dorsal motor nucleus of the vagus (28 – 30). The functional interactions between AVP /OT peptides and their receptor system have been shown to provide diverse opportunities to modulate the efficiency of sensory transmission (31). This review will

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discuss the roles of endogenous and external AVP in nociception. Owing to limitations of space, this article is not a comprehensive overview of the subject. For discussions concerning the variety of neurophysiological roles of central AVP and OT, other previous reviews should also be consulted (3, 14, 20, 21, 32 – 35).

Effect of AVP in pain perception

The pain perception process can be altered by a variety of peptide and non-peptide neurotransmitters (33). Using rodent withdrawal reflex tests, such as the tail flick test, or more complex behavioral tests, such as the hot plate test, in which supraspinal motivational processing is involved, AVP and OT were shown to modify the nociception threshold induced by these noxious heat stimuli (33). In early reports, the antinociceptive activity was observed after either the intraventricular or subcutaneous administration of lysine vasopressin (LVP) in the rat or after intraperitoneal injection of AVP in mice (1, 36, 37). Des-glycinamide-LVP, a vasopressin analog with no apparent pressor or antidiuretic action, or des-amino-AVP, a vasopressin analog with minimal pressor activity but greatly enhanced antidiuretic activity, was also relatively ineffective (36, 37). Later, intracerebroventricular injections of AVP or OT were shown to lead to antinociceptive effects in rats and in human cancer patients (38 – 40).

In addition to the capacity of administered AVP to show antinociception, studies on nociceptive condition of Brattleboro rats, which are deficient in mature vasopressin, are informative concerning the role of AVP in pain sensitivity. When examined using the flinch-jump threshold test, Brattleboro rats were in a hyperalgesic state and stress analgesia was impaired in comparison to controls (41). These analgesic deficits observed in Brattleboro rats was vasopressin-dependent; LVP introduced into the lateral cerebral ventricle, or subcutaneously at a high dose, induced an antinociceptive effect (42). One of other methods to inhibit AVP action in vivo is the administration of antiserum against AVP directly into the cerebral ventricle or brain nucleus. Administration of antiserum to the cerebral ventricle resulted in a small reduction of tail-flick latency from 3.9 s in the vehicle adminstration to 3.3 s at a high radiant heat level (50°C), but it prolonged the latency at moderate (46°C) heat (43).

In the CNS, AVP is released synaptically in the lateral septum, hippocampus, amygdala, habenula, and several other brain structures (15). Therefore, the brain region(s) where AVP acts as antinociceptive neurotransmitter is of large concern. The amygdala, an important region regulating emotional responses, such as anxiety or fear, has a critical role in conditional pain perception. When AVP was injected into the central nucleus of the amygdala and the nociceptive jaw opening reflex monitored in freely moving rats, AVP showed an analgesic effect in diagnostic electromyograms, which was inhibited by a V1, but not V2, receptor antagonist (44). Other studies examined the effect of AVP in nociception by injecting it into the periaqueductal gray and observed an increase in pain threshold (26). AVP increased the endorphin and enkephalin concentration in liquid samples perfused through the periaqueductal gray (45). Not only intracranial injection, but also intrathecal administration of AVP showed an antinociceptive effect (46, 47). However, another study reported that AVP failed to influence nociceptive thresholds or to modify the antinociceptive action of morphine (48). Intrathecal AVP also produced scratching bouts and suppression of hindbody motor function (47).

Opioid-dependency of AVP-induced antinociception

So far, evidence has been accumulating that AVP administered into the intracranial and, in some reports, intrathecal spaces causes an increase in the pain threshold. The next question to be answered is whether the pain-inhibiting effect of AVP could be mediated by intrinsic opioid peptide–receptor systems. To clarify this, a selective μ-opioid–receptor antagonist, naloxone, or several opioid agonists, such as morphine and other peptide agonists, were used together with AVP. The series of results so far have been equivocal. Naloxone inhibited AVP-induced analgesia in several studies (44, 49, 50), but in other studies, the antinociceptive actions of AVP were not mediated by opioids (1, 37, 38, 46).

Apart from the intracranial regions, anterior and posterior pituitary functions are closely related to AVP and OT. Since AVP is secreted under stress conditions and several reports describe evidence of AVP-secreting stimulation also causing secretion of opioid peptides. In the posterior pituitary where AVP and OT neurons terminate, the hypophysial nerve terminals contain enkephalin peptides together with AVP or OT in rat pituitary (51). Dynorphin immunoreactivity was also localized in AVP and OT neurons (52). These reports suggested that AVP and opioid peptides could be secreted from the posterior pituitary and in the CNS. On the other hand, AVP acts as stimulator of adrenocorticotropin secretion in the anterior pituitary. The adrenocorticotropin peptide is processed from the pro-opiomelanocortin (POMC) gene. Although the POMC gene can also produce β-endorphin in the CNS of the human and rat pituitary, a lack of an appreciable amount of active β-endorphin has been reported in basal condi-

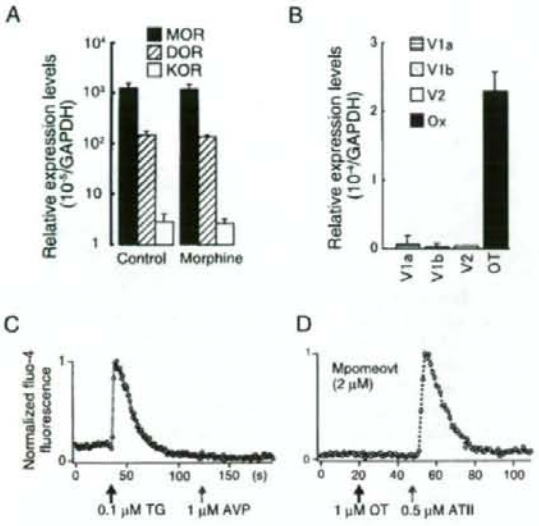

Fig. 1. The co-expression of MOR1 and DOR, but not κ (KOR), opioid receptors and OT oxytocin receptor transcripts in a human neuroblastoma cell line. Real time PCR analysis detected MOR1 and DOR (A) and OT receptor (B) transcripts as the predominant opioid receptors and vasopressin/OT receptor, respectively, in human neuroblastoma cells. C: The specific OT-receptor agonist TG stimulated $[Ca^{2+}]_i$ mobilization and desensitized the corresponding receptor, resulting in no Ca^{2+} response upon subsequent AVP (1 μM) application. The TG-induced $[Ca^{2+}]_i$ mobilization was inhibited by pretreatment of the cells with 2 μM Mpomeovt, a selective OT-receptor antagonist (D), but Mpomeovt failed to inhibit the angiotensin II–stimulated Ca^{2+} response. Mpomeovt: 1-deamino-2-*O*-methyl-tyrosyl-8-ornithine-1-(β-mercapto-(β,β-cyclopentamethylene)propionic acid)oxytocin; TG, [Thr^4,Gly^7] oxytocin.

tions and under corticotropin-releasing stimuli, such as AVP administration or insulin-induced hypoglycemia (53). Therefore, in mice the pituitary gland is one of the major sources of peripherally circulating β-endorphin, but its role in analgesia remains uncertain (54). Previous studies have shown that hypophysectomy may in fact enhance most types of opioid analgesia (55). Furthermore, the lack of stress-induced analgesia documented in β-endorphin–knockout mice may be due to the loss of central β-endorphin rather than loss of pituitary-derived β-endorphin (54).

OT and analgesia

Physiological stimulations that induce a large increase in OT concentrations in the blood, such as parturition and vaginal dilation, are known to raise the pain threshold (21). The analgesic effect of this type of OT action is not a morphine-sensitive process and peripheral injections of OT have no analgesic effect (21). OT, however, attenuates the development of tolerance to the analgesic action of morphine (56). Lesions of the hypothalamic paraventricular nucleus deplete endogenous immunoreactive AVP and OT from the rat spinal cord, but fail to modify the nociceptive thresholds (48). Therefore, complex interactions are suggested between the central OT systems and opioid analgesia.

Using a human neuroblastoma cell line the relationship of opioid- and OT-dependent cellular signaling was recently examined in our laboratory to determine the molecular mechanism for the enhanced nociceptive effect produced by OT. The neuroblastoma cells express both the μ-opioid receptor (MOR1) and δ-opioid receptor (DOR), in addition to the OT receptor (Fig. 1). OT receptor–Gq coupling and Ca^{2+} responses are evoked by application of a specific OT-receptor agonist, which

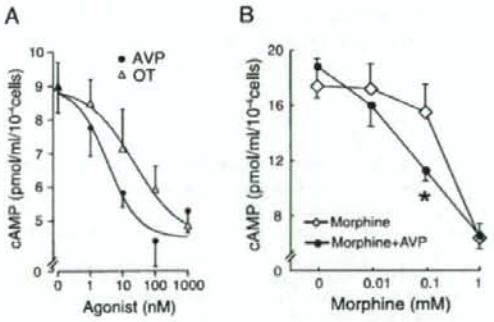

Fig. 2. The inhibition of cyclicAMP production by OT, AVP, and co-application of AVP and morphine. A: The inhibitory effects of OT and AVP against forskolin-induced cyclic AMP production. The cells were stimulated at the indicated concentrations of OT or AVP for 5 min at ambient temperature and reactions were terminated by heating cells at 100°C for 5 min. Cyclic AMP concentrations of the cellular extracts were examined by an enzyme-linked immunosorbent assay. B: The co-applications of morphine and AVP enhanced inhibition of adenylate cyclase activities. In the presence of 1 μM AVP, the inhibition of forskolin-stimulated adenylate cyclase by morphine was significantly enhanced. *P<0.05.

are inhibited by an antagonist, Mpomeovt (Fig. 1: C and D). Interestingly, OT inhibits forskolin-induced cyclic AMP production in a concentration-dependent manner (Fig. 2A). The co-application of 1 μM AVP and morphine enhances adenylate cyclase inhibition, when the effect is compared with morphine alone (Fig. 2B). These previously unpublished results from our investigations suggested that when expressed in the same cell and stimulated simultaneously, MOR1, DOR, and OT receptors cooperatively enhance the Gi-signaling pathway. These results might be one of the possible mechanisms for the analgesic effect of central OT-receptor activation.

Concluding remarks

A majority of the studies performed in vivo on the roles of central AVP and OT receptors suggested that these intrinsic peptides show analgesic effects through an undetermined mechanism. The complexities in delineating the AVP- and OT-receptor functions could partly originate from mutual receptor–ligand interactions. In addition, direct interactions between receptor molecules, resulting in homomer and heteromer receptor complexes, as well as indirect intracellular signaling cross talks, have also been suggested (57, 58). To obtain a more detailed picture, both genetically modified animal models and specific pharmacological tools continue to be useful by perturbing one signal domain so that the other remaining one could be more clearly demonstrated. In this regard, mice lacking the V1a- or V1b-receptor gene are important animal models for unequivocally identifying receptor subtype(s) responsible for AVP actions in the CNS [see the following review article in this issue by K Honda and Y Takano (ref. 59)].

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