Influence of time-restricted feeding schedule on daily rhythm of abcb1a gene expression
and its function in rat intestine

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Abstract

P-glycoprotein (P-gp) is one of the ATP-binding cassette transporters and acts as an efflux pump for cytotoxic substances. P-gp mRNA expression and its transporting activity show the daily rhythm and contribute to the chrono-pharmacokinetic profiles of many drugs. It is reported that daily rhythm of \textit{abcb1a} mRNA is regulated by a circadian clock-controlled output pathway. Time-restricted feeding is well known to shift the peripheral circadian phase of clock gene expression without changing the central clock function. This study was undertaken to examine the influence of time-restricted feeding procedure during the light phase on the daily rhythms of \textit{abcb1a} mRNA expression and P-gp activity. The \textit{abcb1a} mRNA and P-gp activity showed the daily rhythm with a peak at the early in the dark phase in rat intestine under ad lib feeding. Time-restricted feeding during the light phase shifted these rhythms to 12 hr advance. The mRNA expression of clock genes (\textit{DBP} and \textit{HLF}, the transcript activators of \textit{abcb1a}) also showed daily rhythms, and their phases were shifted by time-restricted feeding procedure. The peak time of \textit{DBP} mRNA expression was similar to that of \textit{abcb1a} mRNA expression under ad lib feeding and time-restricted feeding conditions. These results indicate that time-restricted feeding procedure changes \textit{DBP} mRNA expression, which in turn influences \textit{abcb1a} mRNA expression and P-gp activity.
Introduction

In mammals, daily rhythms in behavior and physiology are regulated by the circadian timing system, which is comprised of a master pacemaker in the suprachiasmatic nuclei (SCN) (Ripperger and Schibler, 2001; Reppert and Weaver, 2002). A circadian oscillatory mechanism, which is constituted by "clock gene" products, is based on the self-sustained transcriptional/translational feedback loops (Mitsui et al., 2001; Ueda et al., 2005; Ukai-Tadenuma et al., 2008). This systematic mechanism interconnects the positive and negative limbs of circadian clockwork circuitry and controls the 24-hr variation in output physiology (Jin et al., 1999; Oishi et al., 2003). For example, the circadian-controlled output pathway regulates the expression of many enzymes, which are involved in xenobiotic detoxification such as cytochrome P450 enzymes, carboxyl esterase and xenobiotic transporters (Gachon et al., 2006).

Many studies have shown that the changing of feeding schedule can alter the biological rhythms regardless of lighting condition (Boulos and Terman, 1980). While the SCN clock is mainly entrained by the light/dark cycle, peripheral clock-gene-oscillators are strongly influenced by daily feeding cycles (Damiola et al., 2000; Le Minh et al., 2001; Stokkan et al., 2001). It is also reported that the time-restricted feeding modifies drug toxicity by changing the rhythms of the kinetics and metabolic enzyme activities (Song et al., 1993; Matsunaga et al., 2004). Therefore, food-intake schedule is considered as a dominant Zeitgeber for peripheral circadian oscillators, but not for SCN pacemaker (Damiola et al., 2000; Stokkan et al., 2001).

It is well known that the pharmacokinetics of many drugs is influenced by their dosing-time (Levi, 2002; Hermida and Smolensky, 2004). These are caused by the daily changes in
absorption, distribution, metabolism and elimination of drugs (Ohdo, 2007). P-glycoprotein (P-gp), one of ATP-binding cassette (ABC) transporters, is expressed in epithelial cells of several organs including intestine, liver and kidney (Schinkel et al., 1994) and acts as an energy-dependent efflux pump by expelling cytotoxic substances (Gottesman et al., 2002). We have previously observed that abcb1a mRNA expression in mouse intestine shows a 24-hr rhythmicity (Ando et al., 2005). The daily change in the activity of intestinal P-gp, which followed the daily rhythm in the mRNA expression, was also detected. Recently, Murakami et al. reported that mouse abcb1a gene expression was regulated by a circadian clock-controlled output pathway (Murakami et al., 2008). In addition, they showed that the circadian rhythm of abcb1a expression in mouse intestine was dampened in the clock-mutant mice (Murakami et al., 2008). Based on these observations, it is speculated that an alteration in clock-gene oscillations could modify the daily rhythm in abcb1a expression.

To address the issue, the influences of time-restricted feeding procedure on the daily rhythm of abcb1a mRNA expression in intestine and its transporting activity were determined in this study. Because glucocorticoid is one of the major circadian oscillators in peripheral clock (Balsalobre et al., 2000), the influence of endogenous glucocorticoid on the daily change in abcb1a mRNA expression was also evaluated using adrenalectomized rats.
Methods

Animal

Six-week-old male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). They were housed under the 12:12h light/dark cycle (light on at 07:00 [zeitgeber time, ZT 0] and off at 19:00 [ZT 12]) with a temperature of 24 ± 1°C and humidity of 60 ± 10%. All animals were exposed to the light/dark cycle for at least 2 weeks before the experiment. Unless otherwise specified, they were free to cross to food and water (ad lib feeding). The experiment was performed in accordance with the Use and Care of Experimental Animals Committee of Jichi Medical University (Tochigi, Japan).

Time-restricted feeding

After the acclimatization period, the time-restricted feeding was performed for 2 weeks as follows; rats were restricted to take food between ZT 3 to ZT 9, but water was available through a day.

Experiment 1: Intestine sample collection

Rats (n=32) were randomly assigned to ad lib feeding or time-restricted feeding group. Animals in each group were randomly divided into four subgroups (n=4 in each). Proximal segment of jejunum was collected at ZT 0, ZT 6, ZT 12 or ZT 18. Samples for real-time PCR experiment were stored in RNAlater™ RNA stabilization reagent (QIAGEN, Valencia, CA) at -80°C until assay.

Experiment 2: Perfusion study

Rats (n=48) were randomly assigned to ad lib feeding or time-restricted feeding group. They were randomly divided into four subgroups (n=6 in each). A proximal segment of
jejunum (length 3.5 cm) was isolated at ZT 0, ZT 6, ZT 12 or ZT 18. Perfusion study described below was immediately started after the isolation.

Adrenalectomy and corticosterone replacement

Adrenal glands were removed via a dorsal approach under sodium pentobarbital (5-ethyl-5-(1-methylbutyl)-2,4,6-trioxohexahydropyrimidine) anesthesia (50 mg/kg, ip). In a preliminary study, all animals died within two weeks after adrenalectomy alone. Therefore, corticosterone (11β, 21-dihydroxy-4-pregnene-3,20-dione, 10 μg/h) (Makino et al., 1995) was continuously infused in adrenalectomized (Adx) rats by osmotic mini pump (Alzet®, DURECT Co., Cupertino, CA), and drinking water was replaced to 0.9% NaCl solution. The experiment was performed at two weeks after the operation. Sham adrenalectomy was conducted by the same procedure without the removal of adrenal glands.

Experiment 3: Tissue and serum collection

Rats (n=20) were randomly assigned to sham or ADx group. A proximal segment of jejunum for real-time PCR and blood for corticosterone were collected at ZT 0 or ZT 12 (n=5 in each point).

RNA extraction and real-time PCR

Isolation of total RNA was carried out using the RNeasy Mini kit according to the manufacture’s instructions (QIAGEN). Reverse transcription was done by AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies, La Jolla, CA). The real-time PCR was performed with the Stratagene Mx3005P (Agilent Technologies). Specific sets of primers and TaqMan probes (for abcb1a, dbp, hlf, e4bp4 and gapdh, TaqMan Gene Expression Assays) were obtained from Applied Biosystems (Foster City, CA). To control a variation in
the amount of cDNA available for PCR in different samples, mRNA expression levels of the
target sequences were normalized to the expression of an endogenous control,
glyceraldehydes-3-phosphate dehydrogenase (\textit{GAPDH}). Data were analyzed using the
comparative threshold cycle method.

\textbf{Everted intestinal perfusion study for assessment of P-gp function}

P-gp function was assessed by serosal (donor) to mucosal (receptor) efflux of digoxin, one
of P-gp substrates, using everted intestinal perfusion system. Each segment was everted and
stainless tube was cannulated at both ends of the intestine, which was tied by 5-0 silk. The
excised jejunum was mounted in a chamber containing 45 mL of DMEM/F-12 culture
medium (GIBCO, Grand Island, NY) and was pre-incubated at 37 °C. After incubation for
15 min, serosal perfusate (DMEM/F-12; 5mL) containing 10 \( \mu \)g/mL digoxin
(12\( \beta \)-hydroxydigitoxin, Digoxin injection\textsuperscript{\textregistered}; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan)
was circulated at a flow rate of 0.5 mL/min during the study. Subsequently, 0.2 mL of
sample was withdrawn from the mucosal side every 30 min for 2 hr and replaced with the
same volume of blank medium. Digoxin concentration was measured by an automatic
fluorescence polarization immunoassay analyzer (TDx\textsuperscript{\textregistered}, Abbot Japan Co., Tokyo, Japan).
Throughout the study period, the mucosal side solution was continuously aerated with 95%
O\(_2\) and 5% CO\(_2\) and warmed to 37 °C in a water bath. As for the performance of assay,
range of reliable response was 0.2-5.0 ng/ml, accuracy (calculate by observed/expected
concentration of standards) was 94-104%, and precision was 0.5-1.9% (intra-day) and
1.9-6.2% (inter-day).

\textbf{Corticosterone assay}
Serum corticosterone concentration was measured using a commercially available radioimmunoassay kit (RPA548, GE Healthcare). Assay was performed according to the instruction manual. Sample was twice assayed. As for the performance of assay, range of reliable response was 0.78-200 ng/ml, accuracy (calculate by observed/expected concentration of standards) was 93-109%, and precision was 0.9-3.2% (intra-day) and 1.5-7.0% (inter-day).

Statistical analysis

Groups were compared by one-way ANOVA, and the difference between two groups was determined using Bonferroni-Dunn test. In the analysis of digoxin concentration profile in intestine perfusion study, groups were compared by the repeated measure ANOVA. The $P<0.05$ was considered to be significant.
Results

**Daily rhythm in rat intestinal abcb1a mRNA expression level**

Rat intestinal abcb1a mRNA expression level showed a significant daily rhythm with a peak at the early dark phase in the ad lib feeding group ($P < 0.01$, Fig. 1A). In contrast, the parameter in the time-restricted feeding group had a significant daily rhythm with a trough in the early dark phase ($P < 0.01$, Fig. 1B).

**Daily rhythm in the P-gp transporting function in the reversed intestinal perfusion system**

Digoxin concentration in the serosal fluid time-dependently increased in both ad lib feeding and time-restricted feeding groups (Fig. 2 and 3). In the ad lib feeding group, the increase of digoxin concentration was significantly larger at ZT 18 than at ZT 0 and ZT 6 ($P < 0.05$). In addition, the AUC value in the concentration-time curve was also significantly higher at ZT 18 than at ZT 0 and ZT 6 ($P < 0.05$ and $P < 0.01$, respectively). On the other hand, the increase of digoxin concentration was significantly larger at ZT 6 than at ZT 12 and ZT 18 ($P < 0.05$) in the time-restricted feeding group. The AUC value was also significantly higher at ZT 6 than at ZT 12 and ZT 18 ($P < 0.01$).

**Daily rhythm in the DBP, HLF and E4BP4 mRNA expression**

DBP, HLF and E4BP4 mRNA expressions in rat intestine showed the significant daily rhythms in both ad lib feeding and time-restricted feeding groups. DBP mRNA peaked at ZT 12 in the ad lib feeding group and ZT 0 in the time-restricted feeding group (Fig. 4A). HLF mRNA peaked at ZT 12 in the ad lib feeding group and ZT 6 in the time-restricted
feeding group (Fig. 4B). \(E4BP4\) mRNA showed a similar daily rhythm with a peak at ZT 18 in the ad lib feeding and time-restricted feeding groups (Fig. 4C).

**Serum corticosterone concentration and intestinal gene expression in the ADx rats**

Serum corticosterone concentration in the sham group was significantly higher at ZT 12 than at ZT 0 (\(P < 0.01\), Fig. 5A). Lower level of the hormone was detected in the ADx group.

The intestinal \(abcb1a\) mRNA expression level was significantly higher at ZT 12 than at ZT 0 in both the sham and ADx groups (\(P < 0.01\), sham; \(P < 0.05\), ADx; Fig. 5B). Compared to the sham group, the expression level was significantly (\(P < 0.05\)) higher at ZT 0 and tended to be higher at ZT 12 in the ADx group. On the other hand, \(DBP\), \(HLF\) and \(E4BP4\) mRNA expression levels in the intestine did not significantly differ between the two groups at any observation points (Fig. 5C-5E).
Discussion

In this study, rat intestinal \textit{abcb1a} mRNA expression and P-gp activity showed the daily rhythm with a peak in the dark phase in the ad lib feeding group, which is similar to our previous finding in mice (Ando et al., 2005). Time-restricted feeding procedure during the light period (ZT3-ZT9) shifted the circadian phase of \textit{abcb1a} mRNA expression to 12 hr advance. In addition, the peak of P-gp activity also shifted to the light phase and synchronized with the daily rhythm of \textit{abcb1a} mRNA expression in the time-restricted feeding group. Therefore, the changing of feeding schedule could modify the daily rhythm of rat intestinal \textit{abcb1a} mRNA expression and its activity. Such the effects of time-restricted feeding on the daily rhythms in other functions were already reported (Le Minh et al., 2001; Matsunaga et al., 2004; Koyanagi et al., 2006). In the previous report, P-gp transporting activity was decreased, and subsequently its circadian rhythmicity was blunted in the \textit{clock}-mutant mice (Murakami et al., 2008). In this study, although the daily rhythm of \textit{abcb1a} mRNA expression in rat intestine was altered under time-restricted feeding condition, the P-gp activity in the time-restricted feeding group was similar to that in the ad lib feeding group. These observations support the idea that oscillatory expressions in clock genes are important for composing a circadian expression in \textit{abcb1a} mRNA expression and its activity.

Transcriptional regulation of clock genes via D-box is reported to be involved in the regulation of circadian expression of \textit{abcb1a} mRNA in mouse intestine (Murakami et al., 2008). The consensus sequence of D-box (RTTAYGTAAY) is existed in the 5'-flanking regain of rat \textit{abcb1a} gene. \textit{DBP} and \textit{HLF} are the transcript activators of D-box, and the peak times of their mRNA were identical to that of \textit{abcb1a} mRNA in the ad lib feeding group in this study. In addition, the peak time of mRNA expression of \textit{E4BP4}, which is a transcript
repressor of D-box, was ZT18, and at 6 hr later (ZT0), abcb1a mRNA expression showed the trough level in the ad lib feeding group. These results suggest that the daily expression of intestinal abcb1a mRNA in these nocturnal rodents is at least in part modulated by the transcriptional regulation of clock genes via D-box.

Time-restricted feeding is well known to alter the phase of gene expression rhythm in peripheral tissues (Damiola et al., 2000; Le Minh et al., 2001; Stokkan et al., 2001; Koyanagi et al., 2006). In this study, time-restricted feeding procedure shifted the circadian phases of expressions in not only abcb1a mRNA, but clock genes. The circadian phases of DBP and HLF mRNA expressions were advanced by 12 hr and 6 hr, respectively, while the peak time of E4BP4 mRNA expression was not altered during time-restricted feeding procedure under the present condition. Therefore, the influence of time-restricted feeding procedure on clock genes might be diverse. Murakami et al. reported that HLF, which is one of PAR bZIP proteins, plays a role in the circadian regulation of abcb1a mRNA expression in mouse intestine (Murakami et al., 2008). In this study, the peak time of HLF mRNA expression in the time-restricted feeding group was 6 hr backward compared to that of abcb1a mRNA expression. However, the peak time (ZT 0) of DBP mRNA expression was similar to that of abcb1a mRNA expression in the time-restricted feeding group. Therefore, it is probable that the role of DBP is greater than that of HLF in the circadian regulation of rat intestinal abcb1a gene.

Glucocorticoid is one of the circadian oscillators in peripheral clock (Balsalobre et al., 2000). In addition, it is well known that the manipulation of feeding schedule has also influenced the rhythm of endogenous glucocorticoid secretion. The corticosterone concentration showed a significant daily rhythm peaked at ZT12 in the ad lib feeding group. However, its rhythm was dampened in the time-restricted feeding group (Supplemental Fig.
1). Based on these data, endogenous glucocorticoids did not contribute to modulate the rhythm of abcb1a expression by time-restricted feeding procedure.

In the ADx experiment, abcb1a mRNA expression level of ADx group was significantly higher than that of sham group at ZT0 and ZT12. In addition, ADx did not change the expression level and the time-dependent change of DBP, HLF or E4BP4 mRNA. Therefore, it is speculated that the transcription of abcb1a gene in rat intestine is partially suppressed by endogenous corticosterone via DBP-, HLF- or E4BP4-unrelated mechanism. Several in vitro studies have shown that the effect of glucocorticoid on P-gp expression differs among the cell types (Fardel et al., 1993; Zhao et al., 1993; Schuetz et al., 1995; Seree et al., 1998). For example, dexamethasone, a potent glucocorticoid, decreased (kidney), increased (liver) or did not affect (testis, heart, muscle, spleen and stomach) P-gp expression in rat (Demeule et al., 1999). In addition, the possibility is remained that the bindings of DBP and HLF to D-box in abcb1a gene promoter are enhanced by adrenalectomized. Thus, the influence of glucocorticoid on the P-gp expression is quite complex and a mechanism responsible for the elevation of rat intestinal abcb1a mRNA expression by ADx observed in this study remains to be determined.

In this study, we showed the daily rhythms in abcb1a mRNA expression and P-gp transporting activity in rat intestine in ad lib feeding group, which were shifted by time-restricted feeding procedure. These results indicate that time-restricted feeding might alter pharmacokinetic profiles of drugs, which are substrates of P-gp, and subsequently lead to the intra-individual variety in pharmacokinetics. Further study is needed to evaluate the influence of irregular meal timing on the pharmacokinetics and pharmacodynamics of drugs and clinical outcome of patient.
References


Footnote

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Legends for Figures

**Fig. 1** Daily changes in rat intestinal *abcbla* mRNA expression level in the ad lib feeding (A) and time-restricted feeding (B) groups.

Mean value at highest observation point was set to 1.0. Mean±SE, N=4, * P<0.05, ** P<0.01

**Fig. 2** Time course of digoxin concentration in mucosal fluid in the everted intestinal perfusion system (A) and AUC of time-digoxin concentration-time (B) in the ad lib feeding group.

△ =ZT0  ○ =ZT6  ▲ =ZT12  ● =ZT18,  Mean±SE, N=6, * P<0.05, ** P<0.01

**Fig. 3** Time course of digoxin concentration in mucosal fluid in the everted intestinal perfusion system (A) and AUC of time-digoxin concentration (B) in the time-restricted feeding group.

△ =ZT0  ○ =ZT6  ▲ =ZT12  ● =ZT18,  Mean±SE, N=6, * P<0.05

**Fig. 4** Daily changes in the mRNA expressions of *DBP* (A), *HLF* (B) and *E4BP4* (C).

Mean value at highest observation point was set to 1.0 in each group.

○ =ad lib feeding group  ● =time-restricted feeding group,  Mean±SE, N=4

**Fig. 5** Serum corticosterone concentration (A) and intestinal genes mRNA expression (B~E) in the ADx rats.

Mean mRNA expression value of the sham group at ZT12 was set to 1.0.
☐ = ad lib feeding group  ■ = time-restricted feeding group, Mean±SE, N=5,  * P<0.05,
** P<0.01
Fig. 1
Fig. 2

(A) Digoxin concentration in mucosal fluid (ng/mL) vs. Time after perfusion (min).

(B) AUC (ng/hr/mL) at different ZT (ZT = 0, 6, 12, 18).

* indicates significance at the 0.05 level.

** indicates significance at the 0.01 level.
Fig. 3

(A) Digoxin concentration in mucosal fluid (ng/mL)

(Time after perfusion (min))

(B) AUC (ng hr/mL)

(ZT 0 6 12 18)
Fig. 4
Fig. S1 Daily changes in serum corticosterone concentration in the ad lib feeding (A) and time-restricted feeding (B) groups

Corticosterone concentration showed a significant daily rhythm in the ad lib feeding group (P<0.01), but not in the time-restricted feeding group (P=0.09). Mean ± SE, N=6, ** P<0.01
Supplemental method

Rats were randomly assigned to ad lib feeding or time-restricted feeding group (n=6 in each). Blood was collected at five different times (ZT 0, ZT 6, ZT 12 and ZT 18) from each rat. Serum was separated by centrifugation, and stored at -80 °C until assay.