Altered Diurnal Rhythm of Intestinal Peptide Transporter by Fasting and Its Effects on the Pharmacokinetics of Ceftibuten

XIAOYUE PAN, TOMOHIRO TERADA, MASAHIRO OKUDA, and KEN-ICHI INUI

Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Kyoto, Japan
Received June 19, 2003; accepted July 30, 2003

ABSTRACT

We previously demonstrated that H+/peptide cotransporter PEPT1 shows a diurnal rhythm in the rat small intestine. In the present study, we examined the effect of food intake on the diurnal rhythm of intestinal PEPT1 using fed and fasted rats and also determined whether such variation affected the pharmacokinetics of peptide-like drugs. In fed rats, PEPT1 protein level was significantly higher at 8:00 PM than at 8:00 AM. However, during fasting for 2 to 4 days, the differences of PEPT1 protein levels between 8:00 AM and 8:00 PM gradually disappeared. Intestinal absorption of an oral antibiotic ceftibuten (CETB), a pharmacological substrate for PEPT1, was also greater at 8:00 PM than at 8:00 AM in fed rats, but not different in 4-day fasted rats. In contrast to PEPT1 protein levels, PEPT1 mRNA levels retained a diurnal rhythm after 4 days of fasting. Pharmacokinetic analyses of CETB after intraintestinal administration demonstrated that both $C_{\text{max}}$ and area under the plasma concentration-time curve from 0 to 3 h were greater at 8:00 PM than at 8:00 AM in fed rats. In contrast, pharmacokinetic parameters showed no significant difference between 8:00 AM and 8:00 PM for intraintestinal administration in 4-day fasted rats and for intravenous administration in fed and 4-day fasted rats. These findings suggested that the diurnal rhythm of intestinal PEPT1 transport activity was disrupted by fasting and that diurnal variation of intestinal PEPT1 functionality could influence the pharmacokinetics of peptide-like drugs such as CETB.

Di- and tripeptides and various peptide-like drugs such as β-lactam antibiotics are taken up into the intestinal and renal epithelial cells by H+-coupled peptide cotransporters. Cloning studies have identified two peptide transporters, PEPT1 and PEPT2, and many functional studies using heterologous expression systems have demonstrated molecular natures in their transport characteristics (Leibach and Ganapathy, 1996; Daniel and Herget, 1997; Inui and Terada, 1999). Furthermore, molecular identification of PEPT1 and PEPT2 provided a novel opportunity to determine the mechanisms of their regulation. For example, it was reported that the intestinal PEPT1 is regulated by various factors, including dietary conditions (Ogihara et al., 1999; Shiraga et al., 1999; Naruhashi et al., 2002), hormones such as insulin, leptin, and thyroid hormone (Buyse et al., 2001; Ashida et al., 2002; Gangopadhyay et al., 2002), epidermal growth factor (Nielsen et al., 2001), development (Shen et al., 2001), and some pharmacological agents (Fujita et al., 1999; Berlioz et al., 2000).

In addition to the above-described regulations, we recently found that PEPT1 in the rat small intestine is under the regulation of diurnal rhythm (Pan et al., 2002). Briefly, the transport of [14C]glycylsarcosine (Gly-Sar), a typical substrate for PEPT1, by in situ intestinal loop and everted intestine was greater in the dark phase rather than the light phase, and PEPT1 protein and mRNA levels varied significantly, with a maximum at 8:00 PM and minimum at 8:00 AM (Pan et al., 2002). In contrast to the intestine, renal PEPT1 and PEPT2 showed little diurnal rhythmicity. Because rodents show nocturnal feeding behavior, the diurnal rhythm of intestinal PEPT1 is reasonable for the preparation of anticipated dietary load.

In our previous study, we used rats kept with free access to water and laboratory chow to clarify the diurnal rhythmicity under standard environmental conditions. However, because feeding conditions greatly affected the expression and function of intestinal PEPT1 (Ogihara et al., 1999; Shiraga et al., 1999; Naruhashi et al., 2002), there is a possibility that feeding also influences the diurnal rhythm of this transporter. Based on these hypotheses, in the present study, we examined the effect of food intake on the diurnal rhythm of intestinal PEPT1 using fed and fasted rats. In addition, using an oral β-lactam antibiotic ceftibuten (CETB), a good pharmacological substrate of PEPT1, we examined whether the regulation of intestinal PEPT1 in the diurnal rhythm and fasting could affect the intestinal absorption and pharmacokinetics of peptide-like drugs such as CETB.

ABBREVIATIONS: PEPT, peptide transporter; CETB, ceftibuten; HPLC, high-performance liquid chromatography; AUC, area under the plasma concentration-time curve.
kinetics of this drug. The expressionional changes of renal PEPT1 and PEPT2 were also examined to clarify the tissue specificity of the diurnal rhythm and fasting effect.

### Materials and Methods

**Materials.** CETB was supplied by Shionogi Co. (Osaka, Japan). All other chemicals used were of the highest purity available.

**Animals.** Male Wistar rats (160–180 g) were housed in an air-conditioned room at 22 ± 0.5°C with a 12-h lighting schedule (8:00 AM–8:00 PM). Animals were fed ad libitum on the light/dark schedule for 1 week before they were divided into fed and fasted groups. Two different groups of rats were used in this study: 1) control group fed normal chow ad libitum (F0); and 2) fasted for 1 to 4 days (F1, F2, F3, and F4). Water was available ad libitum to all groups throughout the experiments. Five or six rats were used in each group. Daily changes in body and mucosal weights were examined over the 4 days of fasting (27 and 35% decreased in body and mucosal weights after 4 days of fasting, respectively, compared with those in fed rats). The animal experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University.

**Western Blot Analysis.** Under anesthesia, the duodenum and kidney were removed at 8:00 AM and 8:00 PM of 1 day. The duodenum was flushed with ice-cold phosphate-buffered saline, and the mucosa was scraped. The kidney was decapsulated, and slices of the renal cortex were prepared with a Stadie-Riggs microtome. A portion of the mucosa and renal slices were rapidly frozen in liquid nitrogen for later preparation of brush-border membranes and total RNA. Brush-border membranes from rat small intestine and kidney cortex were prepared as described previously (Inui et al., 1984; Okano et al., 1986). The membrane fractions (small intestine, 10 μg/lane; kidney cortex, 50 μg/lane) were separated by 8.5% SDS-polyacrylamide gel electrophoresis, and analyzed by Western blot analyses as reported previously (Saito et al., 1995 and 1996). The relative densities of the bands in each reaction were determined using NIH Image 1.61 (National Institutes of Health, Bethesda, MD).

**Northern Blot Analysis.** Total RNA was isolated from small intestinal mucosa using TRIzol reagents (1 ml/100 mg of tissue) (Invitrogen Japan KK, Tokyo, Japan) per the manufacturer’s protocols. Total RNA (20 μg) was electrophoresed in 1% denaturing agarose gel containing formaldehyde, and Northern blot analyses were performed as reported previously (Saito et al., 1995 and 1996). The relative densities of the bands in each reaction were determined using NIH Image 1.61 (National Institutes of Health, Bethesda, MD).

**HPLC Analysis.** Plasma concentration-time profiles of CETB were measured with an HPLC LC-6A (Shimadzu, Kyoto, Japan) equipped with an UV spectrophotometric detector SPD-10A (Shimadzu). The condition was as follows: column, Zorbax ODS 4.6-mm inside diameter × 150 mm (Agilent, Palo Alto, CA); mobile phase, 50 mM ammonium acetate–methanol (80:20); flow rate, 1.0 ml/min; wavelength, 262 nm; injection volume, 50 μl, and column temperature, 45°C. Peak area size was measured with a Chromatopac C-R6A (Shimadzu).

**Data Analysis.** Values are expressed as means ± S.E. Analysis of variance and Fisher’s test were used for the statistical significance of CETB concentration and CETB pharmacokinetic parameters. The statistical significance of differences between mean values of other data was analyzed using the nonpaired t test or one-way analysis of variance followed by Fisher’s test when multiple comparisons were needed. Differences were considered significant at p < 0.05.

### Results

In our previous study, we found that PEPT1 protein and mRNA expressions were minimum at 8:00 AM (beginning of the light phase) and maximum at 8:00 PM (beginning of the dark phase), respectively (Pan et al., 2002), and thus we typically examined PEPT1 expression levels at these times. Furthermore, in preliminary experiments, the body weight and small intestinal mucosal mass of rats fasted for 1 to 4 days were significantly lower than those of fed rats. There was no significant difference in the loss of body weight or intestinal mucosal mass between at 8:00 AM and at 8:00 PM of each day. This result served to confirm the change of the intestinal mucosa during the fasting process.

**Effect of Fasting on Diurnal Variation of Intestinal PEPT1 Protein and mRNA Levels in Fasted Rats.** It has been demonstrated that starvation markedly increased the amount of intestinal PEPT1 mRNA (Naruhashi et al., 2002) and protein (Ogihara et al., 1999). In addition, in the present experiments, PEPT1 mRNA and protein levels in 4-day fasted rats were significantly increased compared with fed rats, and this effect was observed at both 8:00 AM and 8:00 PM (Fig. 1).

Next, we assessed diurnal regulation of intestinal PEPT1 protein using 1- to 4-day fasted rats. As shown in Fig. 2, in fed (F0) and the 1-day fasted rats (F1), PEPT1 protein level was significantly higher at 8:00 PM than at 8:00 AM. However, after fasting for 2 to 4 days (F2–F4), the differences of PEPT1 protein levels between 8:00 AM and 8:00 PM gradually disappeared. To exclude the possibility of the phase shift for intestinal PEPT1 biorhythm, we examined PEPT1 protein levels at 4-h intervals in 4-day fasted rats. As shown in Fig. 3, the intestinal PEPT1 protein of 4-day fasted rats showed no significant diurnal rhythm throughout 1 day.

To assess whether abolished diurnal variation of intestinal PEPT1 protein in 4-day fasted rats were transcriptionally regulated, Northern blot analysis was performed. As shown in Fig. 4, PEPT1 mRNA levels at 8:00 PM were significantly higher in 4-day fasted rats as well as in fed rats. These findings suggested that diurnal regulation of PEPT1 mRNA expression was maintained in the fasting state.
Effect of Fasting on Diurnal Variation of Renal PEPT1 and PEPT2 Protein Levels in Fasted Rats. We next examined the effect of fasting on renal PEPT1 and PEPT2 protein levels at 8:00 AM and 8:00 PM in fed and 4-day fasted rats. As shown in Fig. 5A, PEPT1 protein levels remained mostly constant between fed and 4-day fasted rats both at 8:00 AM and 8:00 PM. PEPT2 protein levels showed a similar pattern as PEPT1, but a modest decrease was observed at 8:00 AM (Fig. 5B).
Renal PEPT1 and PEPT2 did not show diurnal variation in the fed rats (Pan et al., 2002). This result was confirmed in the present study, as shown in F0 of Fig. 6, A and B. However, as the fasting period was prolonged, renal PEPT1 and PEPT2 protein levels showed a slight diurnal variation, with higher expression levels at 8:00 PM than at 8:00 AM in 4-day fasted rats (Fig. 6, A and B), suggesting distinct regulation of renal peptide transporters from that of the intestine.

**CETB Pharmacokinetics at 8:00 AM and 8:00 PM in Fed and 4-Day Fasted Rats.** It has been demonstrated that several drugs vary in potency and/or toxicity based on the rhythmicity of biochemical, physiological, and behavioral processes (Lemmer and Labrecque, 1987; Labrecque and Belanger, 1991; Lemmer, 1999). There is a possibility that the diurnal regulation of intestinal PEPT1 may affect the intestinal absorption of peptide-like drugs, which in turn may influence the pharmacokinetic parameters of such drugs. Thus, we assessed this hypothesis using a representative pharmacological PEPT1 substrate CETB. Fasting effects on the intestinal absorption and the pharmacokinetic parameters of CETB were also investigated. As shown in Fig. 7, in the fed state, the initial absorption rate of CETB was significantly higher at 8:00 PM than at 8:00 AM. On the other hand, in the 4-day fasted state, the initial absorption rate of CETB was not significantly different between 8:00 AM and 8:00 PM. Comparing the initial absorption rate of CETB between fed and fasted rats, the latter showed higher absorption rate.

We next measured plasma concentrations of CETB after intraintestinal and intravenous administration at 8:00 AM and 8:00 PM in fed and 4-day fasted rats. Pharmacokinetic parameters of CETB after intraintestinal and intravenous administration are summarized in Tables 1 and 2, respectively. In the fed condition, rats administered at 8:00 AM showed greater $C_{\text{max}}$ and AUC$_{0-3\text{h}}$ and faster $T_{\text{max}}$ rather than those administered at 8:00 AM (Table 1; Fig. 8A). However, there were no significant differences of pharmacokinetic parameters of CETB between 8:00 AM and 8:00 PM in 4-day fasted rats (Table 1; Fig. 8B). Comparison of pharmacokinetic parameters between fed and 4-day fasted rats, all parameters exhibited higher absorption of CETB in 4-day fasted rats compared with fed rats irrespective of administration time (Table 1). In the intravenous administration, there were no differences in $K_{\text{e,C}}$, CL$_{\text{tot}}$, and $t_{1/2}$ between 8:00 AM and 8:00 PM in the fed and 4-day fasted rats (Table 2; Fig. 8, C and D), although there were significant differences between fed and 4-day fasted rats at both administration times (Table 2).

**Discussion**

A daily periodicity in the intestinal transport activity and several other digestive proteins were documented before the responsible genes were identified. Nevertheless, the observed activity changes were clearly ascribed to the feeding pattern (ad libitum and scheduled) rather than to an inherent circadian signal (Fisher and Gardner, 1976; Stevenson and Fi-
The intestinal PEPT1 protein showed significant diurnal rhythm in feeding and 1-day fasted conditions. However, this diurnal rhythm disappeared after 2 days of fasting. In accordance with the diurnal rhythm of PEPT1 protein expression in feeding and fasting conditions, the CETB absorption rate assessed by the in situ loop technique was higher at 8:00 PM rather than at 8:00 AM in the feeding state, but not in the 4-day fasted condition. Interestingly, the diurnal regulation of the PEPT1 mRNA level was maintained even in the 4-day fasted state. This finding suggested that the transcription of intestinal PEPT1 gene in the diurnal rhythm was mediated by factors other than food intake. Currently, there is no information regarding transcription factors involved in the constitutive and regulatory expression of intestinal PEPT1 mRNA. Further studies about promoter analysis of rat PEPT1 may clarify the molecular mechanisms for the diurnal rhythm of intestinal PEPT1 mRNA expression. For Na\(^+\)/glucose cotransporter 1, it was demonstrated that periodicity in transcription factor hepatocyte nuclear factor-1 contributes to circadian changes in glucose transport activity in the intestine (Rhoads et al., 1998). Because there is a potential site for HNF-1 in the rat PEPT1 promoter region (Shiraga et al., 1999), this factor can be a candidate for diurnal transcriptional regulation of the intestinal PEPT1 expression.

The diurnal variation of intestinal PEPT1 protein expression was abolished in 4-day fasted rats, although the diurnal variation in the mRNA expression was retained. There are some possible explanations for this finding. First, the rates of the translation process and degradation of PEPT1 protein at 8:00 AM and 8:00 PM may be different between the fed and fasted conditions. Second, the alteration in the cellular distribution of PEPT1 protein (cytoplasmic pool and apical membranes) seems to be involved in the disappearance of diurnal variation. For example, the increased translocation of the cytoplasmic pool of PEPT1 to the apical membrane was demonstrated by hormone treatment such as insulin (Gangopadhyay et al., 2002; Thamotharan et al., 1999) and leptin (Buyse et al., 2001). Insulin and leptin showed a diurnal rhythm with a nocturnal peak, but this rhythm of both hormones was completely abolished by the fasting state (Ahrén, 2000), which seemed to correspond to the alteration of PEPT1 membrane expression between the fed and fasted conditions.

Several clinical studies, performed in a crossover design, have provided evidence that the pharmacokinetics of many lipophilic drugs can be circadian phase-dependent in C\(_{\text{max}}\) and \(T_{\text{max}}\) (Lemmer, 1999). Because most drugs tested are absorbed by passive diffusion, the circadian variation in gastric emptying time (Goo et al., 1987) and the perfusion of the gastrointestinal tract (Lemmer and Nold, 1991) have been considered for this reason. In the present study, we found that pharmacokinetic parameters of CETB such as C\(_{\text{max}}\) and \(T_{\text{max}}\) showed a significant diurnal variation after intraintestinal administration in fed rats. The initial absorption rate of CETB assessed by the in situ loop technique was significantly higher at 8:00 PM than at 8:00 AM, and pharmacokinetic parameters of intravenous administration of CETB did not show diurnal variation. These findings suggested that pharmacokinetic variation of CETB between 8:00 AM and 8:00 PM was caused by the intestinal absorption process and that the diurnal rhythm of the intestinal PEPT1 expression plays a pivotal role in this variation. The diminished diurnal
variation of the pharmacokinetic parameters for CETB administered intraintestinally in 4-day fasted rats corresponding to the disappearance of the diurnal rhythm of intestinal PEPT1 also supported this idea. As far as we know, this is the first demonstration that the intestinal transporter is involved in the diurnal variation of pharmacokinetics of drugs. In the intravenous administration, there were no significant differences in pharmacokinetic parameters of CETB.
between 8:00 AM and 8:00 PM both in fed and 4-day fasted rats, although the expressions of renal PEPT1 and PEPT2 were increased in 4-day fasted rats. Although we did not assess the renal handling of CETB in detail, it is plausible that renal PEPT1 and PEPT2 may little affect the CETB pharmacokinetics.

CETB pharmacokinetic parameters such as $C_{\text{max}}$ and AUC$_{2-\infty}$ obtained by the intestinal administration were significantly larger in 4-day fasted rats compared with the fed rats. In addition, the CETB absorption rates assessed by the in situ loop technique in 4-day fasted rats were significantly increased compared with the fed rats both at 8:00 AM and at 8:00 PM. All these findings seemed to be accountable by increased intestinal PEPT1 protein expression. Recently, Naruhashi et al. (2002) reported that PEPT1 mRNA expression was induced by starvation and its level correlated with absorptive transport of cefadroxil, an oral $\beta$-lactam antibiotic, in the rat intestine. Furthermore, it was reported that the expression level of PEPT1 assessed by Western blot analysis in the rat jejunum was significantly correlated with intestinal peptide transporter PEPT1 transport activity. In conclusion, we have demonstrated that the diurnal rhythm of intestinal PEPT1 transport activity is altered by fasting. The diurnal rhythm of intestinal PEPT1 would be responsible for the diurnal variation for pharmacokinetics of peptide-like drugs such as CETB, which was also abolished by fasting.

References


Pan et al.


Address correspondence to: Professor Ken-ichi Inui, Department of Pharmacy, Kyoto University Hospital, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: inui@kuhp.kyoto-u.ac.jp