Diurnal rhythm of H\textsuperscript{+}-peptide cotransporter in rat small intestine

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Pan, Xiaoyue, Tomohiro Terada, Megumi Irie, Hideyuki Saito, and Ken-Ichi Inui. Diurnal rhythm of H\textsuperscript{+}-peptide cotransporter in rat small intestine. Am J Physiol Gastrointest Liver Physiol 283: G57–G64, 2002; 10.1152/ajpgi.00545.2001.—In mammals, most physiological, biochemical, and behavioral processes show a circadian rhythm. In the present study, we examined the diurnal rhythm of the H\textsuperscript{+}-peptide cotransporter (PEPT1), which transports small peptides and peptide-like drugs in the small intestine and kidney, using rats maintained in a 12-h photoperiod with free access to chow. The transport of \textsuperscript{[14]C}glycylsarcosine (Gly-Sar), a typical substrate for PEPT1 by in situ intestinal loop and everted intestine, was greater in the dark phase than the light phase. PEPT1 protein and mRNA levels varied significantly, with a maximum at 2000 and minimum at 0800. Similar functional and expression diurnal varia
tions were observed in the intestinal Na\textsuperscript{+}-glucose cotransporter (SGLT1). In contrast, renal PEPT1 and SGLT1 showed little diurnal rhythmicity in protein and mRNA expression. These findings indicate that the intestinal PEPT1 undergoes diurnal regulation in its activity and expression, and this could affect the intestinal absorption of dietary protein.

kidney; intestinal absorption; brush-border membranes

Dietary protein undergoes a series of degradative steps, resulting in a mixture of free amino acids and small peptides. Numerous studies have shown that absorption of the products of protein digestion in the small intestine occurs primarily in the form of small peptides rather than amino acids (1, 17). Cellular uptake of small peptides (di- and tripeptides) is mediated by H\textsuperscript{+}-coupled peptide transporter (PEPT1) localized at the brush-border membranes of intestinal epithelial cells (17, 20). In the kidney, two isoforms of peptide transporters (PEPT1 and PEPT2) are expressed and play a significant role in conserving peptide-bound amino nitrogen (9, 20). On the basis of the nutritional importance of peptides, enteral and parenteral solutions of short-chain pepti
des or activities were observed in dark phases, and these diurnal changes might be assumed to occur in nocturnal animals feeding mainly at night. However, there have been few reports on the diurnal rhythms for transport and metabolism of small peptides, although the physiological and clinical significance of small peptides has been recognized.

In the present study, we focused on the diurnal rhythms of intestinal absorption of small peptides and its molecular mechanism. To achieve this, diurnal changes of \textsuperscript{[14]C}glycylsarcosine (Gly-Sar) transport in the small intestine were examined. Expressional changes of PEPT1 mRNA and protein in the small intestine were also investigated. Furthermore, we compared the expressional changes of intestinal PEPT1 to those of renal PEPT1, to examine the tissue specificity of circadian rhythms. Diurnal rhythms of the Na\textsuperscript{+}-glucose cotransporter (SGLT1) in the small intestine and kidney were also examined to compare with those of PEPT1, because the intestinal SGLT1 was reported to show a circadian rhythm (30, 42).

MATERIALS AND METHODS

 Animals. Animal experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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University. Male Wistar rats (160–180 g) were housed in an air-conditioned room at 22 ± 0.5°C with a 12-h lighting schedule (800–2000). Animals were kept for at least 1 wk before the initiation of any experiments and were allowed free access to water and standard laboratory chow.

**Materials.** [14C]Gly-Sar (1.78 GBq/mmol) was obtained from Daiichi Pure Chemicals (Ibaraki, Japan). [14C]methyl-α-[U-14C]glucopyranoside (αMG); (9.66 GBq/mmol) was supplied by Moravek Biochemicals (Brea, CA). Gly-Sar was purchased from Sigma (St. Louis, MO). All other chemicals used were of the highest purity available.

**In situ loop technique.** We examined [14C]Gly-Sar transport by the in situ loop technique at 1200 and 2400. A cannula with a polyethylene tube was inserted in the portal artery. A duodenum loop 10 cm in length was prepared, and then [14C]Gly-Sar (40 nmol·ml⁻¹·kg body wt⁻¹) was introduced into the loop with a microsyringe. Blood was withdrawn from the portal artery at designated times. Blood samples were centrifuged for 2 min at 14,000 g, and 50 μl of plasma was solubilized in 0.5 ml of ACS II (Amersham Pharmacia Biotech, Uppsala, Sweden). Radioactivity was determined in 5 ml of ACS II (Amersham Pharmacia Biotech) by liquid scintillation counting.

**Preparation of intestinal segments and uptake experiments.** Rats were killed at different times during a 24-h period (400, 800, 1200, 1600, 2000, and 2400), and the intestinal segments for the uptake experiments were quickly prepared according to a previous report (24) with some modifications. Isolated duodenum was everted, divided into small segments 5–10 mm in length, and fixed over polyethylene tubes with an outer diameter of 4 mm. Everted intestinal segments were preincubated with incubation medium under an atmosphere of 100% oxygen. The composition of the incubation medium was as follows (in mM): 129 NaCl, 5.1 KCl, 1.4 CaCl₂, 1.3 Na₂HPO₄, and 1.3 NaH₂PO₄ (pH 6.0). After preincubation, each intestinal segment was placed in 1 ml of incubation medium containing [14C]Gly-Sar (20 μM) or αMG (100 μM). The uptake experiments were carried out at 37°C an atmosphere of 100% oxygen. After incubation for 3 min, each segment was rapidly washed with ice-cold incubation medium, blotted on filter paper, weighed, and solubilized in 0.5 ml of ACS II. Radioactivity was then determined in 5 ml of ACS II by liquid scintillation counting.

**Antibodies and Western blot analysis.** Rabbit anti-PEPT1 antibody was raised against the 15 COOH-terminal amino acids of rat PEPT1 (31). Rabbit anti-PEPT2 antibody was raised against synthetic peptides corresponding to amino acids 687–710 of rat PEPT2 (39). Rabbit anti-SGLT1 antibody (a gift of M. Kasahara) was raised against synthetic peptides corresponding to amino acids 564–575 of rabbit intestinal SGLT1 (40). Goat antivillin polyclonal IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). While the animals were under anesthesia, the duodenum and kidney were removed at specified times. The duodenum was flushed with cold PBS, and the mucosa was scraped. The kidney was decapsulated, and slices of 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 (16, 29). The membrane fractions were separated by SDS-PAGE and analyzed by Western blotting with each antibody as reported (23, 26, 31, 39). Relative amounts of band in each reaction were determined densitometrically using Image 1.61 (National Institutes of Health, Bethesda, MD).
specific uptake of $[^{14}C]$Gly-Sar tended to be lower in the light phase than the dark phase. For example, the $[^{14}C]$Gly-Sar specific uptake value was significantly greater at 2400 h ($6.1 \pm 0.6 \text{ nmol/g tissue}$) than at 1200 h ($5.0 \pm 0.5 \text{ nmol/g tissue}$) ($n = 15$ segments from 5 rats; $P < 0.05$). A similar result was obtained in the $[^{14}C]$αMG uptake experiments (Fig. 2D).

Diurnal variation of PEPT1 and SGLT1 proteins in the duodenum. To determine whether the rhythmicity of $[^{14}C]$Gly-Sar uptake was linked to expressional changes of PEPT1 protein, we performed Western blot analysis using intestinal brush-border membranes. As shown in Fig. 3A, the intestinal PEPT1 protein level was highest at 2000 and lowest at 800. A similar pattern of expression was observed for SGLT1 protein (Fig. 3B), and this result was consistent with the previous reports (30, 42). In contrast to both proteins, villin, which is a cytoskeletal marker protein, did not change in expressional level throughout the day (Fig. 3C).

We then analyzed the rat PEPT1 protein expression at different times by immunofluorescence microscopy using a cooled-CCD camera. Immunofluorescence images were captured under the completely same conditions, as previously reported (27). As shown in Fig. 4, localization of PEPT1 is confined to the brush-border membranes of absorptive epithelial cells, and labeling intensity was much stronger at 1600–2400 than at other times.

Diurnal variation of PEPT1, PEPT2, and SGLT1 proteins in the kidney. The diurnal variation in the expression of PEPT1 and SGLT1 protein in the kidney was tested further (Fig. 5, A and C). In contrast to their expressional rhythmicity in the duodenum, levels of PEPT1 and SGLT1 protein expression in the kidney did not vary appreciably during the 24-h period. The protein level of PEPT2, which is another H$^+$-peptide transporter predominantly expressed in the kidney, also showed little diurnal variation (Fig. 5B).
Diurnal variation of PEPT1 and SGLT1 mRNAs in the duodenum. To assess whether diurnal variation in PEPT1 expression occurred at the transcriptional level, Northern blot analysis was carried out. As shown in Fig. 6A, significantly more PEPT1 mRNA was present in the small intestine from 1600 to 2400 than at other times. Similar expressional changes were observed for the SGLT1 mRNA (Fig. 6B). On the other hand, the GAPDH mRNA level exhibited no significant diurnal variations (Fig. 6C).

Diurnal variation of PEPT1, PEPT2, and SGLT1 mRNAs in the kidney. We further investigated the diurnal rhythm of PEPT1, PEPT2, and SGLT1 mRNA expression in rat kidney. As shown in Fig. 7, mRNAs of all the transporters exhibited no apparent diurnal variation.

Fig. 3. Diurnal variation of PEPT1 (A), SGLT1 (B), and villin (C) protein expression in rat duodenum. Rats were killed at the indicated time, and brush-border membranes of the rat duodenum were prepared. Membrane proteins (5 μg/lane) were probed with each antibody as described in MATERIALS AND METHODS. Film intensity signals were subjected to scanning densitometry, and protein abundances were expressed as a percentage of the value at 400. Open bars and closed bars show light and dark phases, respectively. Each point represents the mean ± SE of 5 preparations. *P < 0.05, significantly different from the lowest value (800).

Fig. 4. Immunohistochemical analysis of the diurnal variation of PEPT1 protein expression in rat duodenum. Tissue sections obtained from rats killed at the indicated times were processed as described in MATERIALS AND METHODS. Pictures were taken under the same conditions to allow comparison of labeling intensities. Typical pictures from 3 separate experiments are shown.
DISCUSSION

Intestinal PEPT1 is physiologically regulated by various factors including dietary conditions (10, 15, 27, 34), hormones (6, 43), growth factors (25), and development (33). Dietary regulation of intestinal PEPT1 has been extensively investigated (10, 15, 27, 34). For example, we previously demonstrated that short-term starvation markedly increased the amount of PEPT1 protein, whereas dietary administration of amino acids reduced the amount (27). Taking these findings into consideration, it is expected that food content and feeding schedule affect the diurnal rhythmicity of intestinal PEPT1. The aim of the present study is to clarify whether rat PEPT1 shows a diurnal rhythmicity under standard environmental conditions. Therefore, we performed each experiment using rats main-

Fig. 5. Diurnal variation of PEPT1 (A), PEPT2 (B), and SGLT1 (C) protein expression in rat kidney cortex. Rats were killed at the indicated times, and brush-border membranes of the rat kidney cortex were prepared. Membrane proteins (30 μg/lane) were probed with each antibody as described in MATERIALS AND METHODS. Film intensity signals were subjected to scanning densitometry, and protein abundances were expressed as a percentage of the value at 4:00. Open bars and closed bars show light and dark phases, respectively. Each point represents the mean ± SE of 5 preparations.

Fig. 6. Diurnal variation of PEPT1 (A), SGLT1 (B), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (C) mRNA expression in rat duodenum. Northern blot analyses with RNA extracted from the duodenum of rats killed at the times indicated were performed as described in MATERIALS AND METHODS. PEPT1 and SGLT1 message levels were quantified by scanning densitometry, corrected for loading using GAPDH, and expressed as a percentage of the value at 4:00. Open bars and closed bars show light and dark phases, respectively. Each point represents the mean ± SE of 5 preparations. *P < 0.05, significantly different from the lowest value (8:00).
tained in a 12-h photoperiod with free access to water and standard laboratory chow.

The present study has demonstrated that transport activity and expression level (both mRNA and protein) of intestinal PEPT1 and SGLT1 showed diurnal rhythm. Transport activity and expression levels of both transporters appeared to be higher in the dark phase than the light phase. A similar diurnal rhythm has been observed for various intestinal digestive and absorptive activities, such as sucrase-isomaltase (37), lactase (38), γ-glutamyltransferase (38), alkaline phosphatase (38), and glucose and L-histidine transport (12). In addition to the above functional rhythmicity, recent studies have clarified that the expressional rhythms of SGLT1 and facilitative glucose transporters (GLUT2 and GLUT5) are related to these functional rhythms (8, 30, 42). Because rodents show a nocturnal feeding behavior, all of these diurnal rhythms, including the PEPT1 rhythmicity, could seem reasonable for the preparation of nocturnal dietary load.

In the present study, we found that the diurnal rhythm of the expression of intestinal PEPT1 protein is linked with the periodicity of the transcription of the PEPT1 mRNA. But it remains unclear what factors regulate the transcription of intestinal PEPT1. In the case of SGLT1, Tavakkolizadeh et al. (42) suggested two distinct and separate pathways regulating the expression and function in intestinal epithelial cells. One pathway is the utilization of gut luminal signals (presumably food intake) to induce the diurnal variation. This mechanism may be involved in the regulation of PEPT1 rhythmicity, because the dietary regulation of intestinal PEPT1 was reported (10, 15, 27, 34). The second is a daily anticipatory mechanism preparing the intestine for an expected increase in nutrients before exposure to the luminal contents. This mechanism may also contribute to the diurnal rhythm of intestinal PEPT1, because the PEPT1 mRNA level in the small intestine has begun to increase at 1600 before the onset of feeding. It was reported that the bulk of food ingestion occurs in the first 4–6 h of the dark phase when rodents are fed ad libitum (44). It is, therefore, hypothesized that these two factors have complexly influenced the diurnal variation of the intestinal PEPT1 expression.

Rhoads et al. (30) demonstrated that the periodicity in the activity of hepatocyte nuclear factor 1 (HNF-1) contributed to the circadian rhythm of SGLT1 transcription. Because it was reported that there is a potential site for HNF-1 in the rat PEPT1 promoter region (34), this factor may be involved in the daily anticipatory mechanism of intestinal PEPT1 expression. A neuroendocrine mechanism, such as insulin circadian variation, was also proposed to affect the glucose absorption in rats (5, 14). Likewise, insulin was demonstrated to regulate the PEPT1 function by increasing the population of PEPT1 protein in membranes (43). These findings suggest that insulin circadian variation may affect the PEPT1 expression and function, although this mechanism is not involved in the transcriptional regulation. More recently, Buyse et al. (6) reported that PEPT1-mediated epithelial transport of dipeptides and cephalexin is enhanced by gastric leptin, the ob gene product, on the luminal side of the small intestine. Although the diurnal rhythm of gastric leptin secretion into the lumen has not been investigated, there are various reports on the circadian rhythm of the plasma leptin levels (2, 3, 22). Thus the luminal leptin secreted by the stomach could be involved in the diurnal rhythm of intestinal PEPT1 expression.

Fig. 7. Diurnal variation of PEPT1 (A), PEPT2 (B), and SGLT1 (C) mRNA expression in rat kidney cortex. Northern blot analyses and quantification of signals were carried out as described in Fig. 6. Open bars and closed bars show light and dark phases, respectively. Each point represents the mean ± SE of 5 preparations.
In contrast to the intestinal PEPT1 and SGLT1 expression, the mRNA expression of both transporters in the kidney showed little diurnal rhythmicity. Crypt-villus turnover in the intestine has been demonstrated to show a circadian rhythm; i.e., enterocyte differentiation is increased and peaks at 300 in a 12:12-h light-dark cycle beginning at 600 (35). In addition, the length of the villus and the number of mature enterocytes peak before the onset of feeding (36). However, to our knowledge, there are few reports on the circadian rhythms of cell differentiation and number in renal tubular cells. It is, therefore, assumed that some distinct features of cell dynamics between the intestinal and renal epithelial cells contribute to the different diurnal rhythmicities of the intestinal and renal PEPT1 and SGLT1 expressions. Alternatively, transcription factors, such as HNF-1 may have different expressions. PEPT1 and SGLT1 expressions. Alternatively, tran-rhythms of cell differentiation and number in renal and renal epithelial cells contribute to the different diurnal rhythmicities of the intestinal and renal PEPT1 and SGLT1 expressions. The diurnal rhythm of intestinal and renal PEPT1 show a diurnal rhythm as well as SGLT1. The diurnal rhythm of intestinal PEPT1 expression occurred in the transcription.

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