Journal of Surgical Research **156,** 123–128 (2009)
doi:10.1016/j.jss.2009.03.052

Diurnal Expression and Function of Peptide Transporter 1 (PEPT1)[1]

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Submitted for publication January 8, 2009

Background. **Protein is absorbed primarily as di/tripeptides, which are transported into the enterocyte exclusively by H^+/peptide cotransporter 1 (PEPT1). Diurnal changes in expression and function of several other mucosal transporters occur in rat. Diurnal variations in mRNA, protein, and transport function of PEPT1 occur in rat duodenum and jejunum, but not in ileum.**

Methods. **Mucosal levels of mRNA and protein were determined at 9 AM, 3 PM, 9 PM, and 3 AM ($n = 6$ each) by real time RT-PCR and Western blotting, respectively, in rats maintained in a 12-h light/dark room [light 6 AM to 6 PM]; transporter-mediated uptake of dipeptide (Gly-Sar) was also measured by everted sleeve technique.**

Results. **mRNA transcripts of PEPT1 and Gly-Sar uptake varied diurnally in duodenum and jejunum (peak at 3 PM, $P < 0.05$), but not in ileum; maximal uptake was in jejunum. V_{max} (nmol/cm/min) was greater at 3 PM and 9 PM compared with 9 AM (3 PM *versus* 9 AM: 104 *versus* 62 in duodenum, and 185 *versus* 101 in jejunum; $P < 0.03$); K_m was unchanged across time points or locations. Protein levels varied minimally in jejunum and ileum with peaks at 9 PM and 3 AM.**

Conclusion. **Gene expression and transport function of PEPT1 vary diurnally in duodenum and jejunum in temporal association with nocturnal feeding of rats.**

Key Words: **peptide transporter 1; PEPT1; diurnal rhythm; rat; small intestine; protein absorption; short peptides.**

INTRODUCTION

Dietary proteins are digested in the lumen of the small intestine into a mixture of primarily short peptides and some free amino acids. Absorption of protein digestion products occurs predominantly as di- and tripeptides rather than individual amino acids [1–3]. Proton-dependent peptide transporter 1 (PEPT1) is the exclusive peptide transporter expressed in the brush border (apical membrane) of enterocytes, and mediates the uptake of essentially all di- and tripeptides from the lumen [3–5]. PEPT1 plays important roles not only as a nutrient transporter but also as a drug transporter for several peptide-like drugs (e.g., β-lactam antibiotics) [5, 6].

Various factors regulate gene expression and transport function of mucosal transporters, such as luminal substrates, hormones, and ontogeny [7–9]. A diurnal rhythm in gene expression and transport function of several other mucosal transporters (e.g., hexose transporters) occurs in the proximal intestine of rodents (i.e., duodenum and jejunum) in coordination with their nocturnal feeding pattern [10–13]. Our aim was to look for and characterize diurnal variations in expression and function of PEPT1 throughout the rat small intestine. Identifying temporal and segmental variations in expression and transport function of nutrient transporters may allow us to modulate their regulatory mechanisms during various diseases or after surgical intervention. Our hypothesis was that diurnal variations in gene expression (mRNA and protein) and transport function of PEPT1 occur in rat duodenum and jejunum but not in ileum.

METHODS

After approval from our Institutional Animal Care and Use Committee and in accordance with the NIH guidelines for the humane use and care of laboratory animals, 24 male Lewis rats weighing 250–300 g (Harlan, Indianapolis, IN), maintained in a controlled,

[1] Presented in part at the Academic Surgical Congress 4th Annual Meeting, Fort Myers, FL, February 6, 2009, and published in Abstract form in the Journal of Surgical Research.

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0022-4804/09 $36.00

12-h light/dark room (light on from 6 AM to 6 PM) with free access to water and standard rat chow (5001 Rodent Diet, PMI Nutrition International LLC, Brentwood, MO), were acclimated for at least 1 wk. Feeding patterns were determined by measuring food consumption twice daily (at 6 AM and 6 PM) in 12 rats for 1 wk prior to sacrifice. To determine diurnal rhythmicity in expression and function of PEPT1, six rats at each of four time points (9 AM, 3 PM, 9 PM, 3 AM) were killed, and the levels of mRNA, protein, and transport activity for PEPT1 were measured in duodenum, jejunum, and ileum.

Tissue Harvest

Rats were anesthetized using inhaled 2% isoflurane and intraperitoneal pentobarbital (50 mg/kg). After celiotomy, the duodenum was cannulated just distal to pylorus, and the small intestine was flushed with cold (4°C) Ringer's solution. The small intestine was excised and placed immediately in cold (4 °C), oxygenated (95% O_2/5% CO_2) Ringer's solution. The proximal duodenum was used for everted sleeves (see below, Uptake Function), and the distal duodenum was used for mRNA and protein measurements. Similarly, mid-jejunum and mid-ileum were studied. The mucosa was scraped bluntly, using a glass slide, into cold, phosphate-buffered saline (PBS). Samples for mRNA analysis were placed in RNA stabilization buffer (RNALater; Qiagen, Valencia, CA) and stored immediately at –80 °C. The samples for protein analysis were collected separately, placed in cold RIPA buffer containing protease inhibitors (Pierce, Rockford, IL), and stored at –80 °C. For histomorphometry, 0.5 cm portions of each anatomic segment were pinned on a support and fixed in 10% buffered formalin.

mRNA Measurement

We used real-time, reverse transcription polymerase chain reaction (RT-PCR) to quantitate mRNA levels for PEPT1. Mucosal samples stored in RNA stabilization buffer were thawed on ice and homogenized; RNA was isolated using the RNeasy Midi kit (Qiagen, Valencia, CA). RNA was then reverse-transcribed into cDNA using the Super Script III kit (Invitrogen, Carlsbad, CA); cDNA levels of PEPT1 and the stably expressed housekeeping gene glyceraldehyde-6-phosphate dehydrogenase (GAPDH) were then determined using RT-PCR in a 7500 Thermocycler using Taqman chemistries with primers and fluorescently-labeled probes in assay mixes (Applied Biosystems, San Francisco, CA). Standard curves from serial dilutions of known copy numbers were used to calculate copy numbers of cDNA for each sample. All samples were run as duplicates with 2 μL of cDNA added to 23 μL of master mix. RT-PCR was carried out at 95°C for 10 min followed by 40 cycles of 15 s at 95° and 1 min at 60°C, after which fluorescence measurements were made. Transporter copy numbers were normalized to copy numbers of GAPDH from each sample.

Protein Measurement

Western blotting was used to measure semiquantitatively the levels of total cellular protein for PEPT1. Tissue samples stored in RIPA buffer containing protease inhibitors were thawed on ice and placed in RIPA lysis buffer containing protease inhibitors in attempt to minimize protein degradation. Samples were homogenized using a Kontes pellet pestle (Fischer Scientific, Pittsburg, PA). The protein-containing supernatant was separated by centrifugation at $5000 \times g$ for 15 min. Protein concentrations were measured by the bicinchoninic acid method (Pierce, Rockford, IL); 200 μg of protein was resolved on a 10% SDS-PAGE gel (Bio-Rad, Hercules, CA), and transferred electrically to a PVDF membrane (Millipore, Bedford, MA). Membranes were blocked using 5% milk in Tris-buffered saline with Tween (TBS-T). GAPDH was used as a stably expressed "housekeeping" protein. Membranes were incubated overnight at 4 °C with primary antibody for PEPT1 (Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH antibody (US Biological, Swampscott, MA). After incubation with primary antibody, membranes were rinsed three times with TBS-T and incubated with secondary antibody in TBS-T containing 5% milk using horseradish peroxidase-conjugated, goat anti-rabbit IgG for PEPT1, and anti-mouse IgG for GAPDH (Sigma, St. Louis, MO). Protein bands visualized with a colorimetric reaction using Opti-4CN Substrate kits (Bio-Rad) were scanned, and Scion Image (Scion Corp., Frederick, MA) was used for semiquantitative measurements based on band densitometry. Protein measurements were normalized to GAPDH to estimate the amount of protein per enterocyte.

Uptake Function

We measured transporter-mediated uptake of the dipeptide glycylsarcosine (Gly-Sar), a nonhydrolysable substrate for PEPT1 [14], using a modified everted sleeve technique described by Karasov and Diamond [15]. Intestinal segments (1 cm) were everted over a pregrooved steel rod and secured with silk ties, thereby exposing the mucosal surface externally. Sleeves were kept in chilled (4°C) Ringer's solution bubbled with 95% O_2/5% CO_2. The sleeves were transferred into 8 mL of warmed (38°C) Gly-Sar-free incubation medium (in mM: 129 NaCl, 5.1 KCL, 1.4 $CaCl_2$, 1.3 NaH_2PO_4, and 1.3 Na_2HPO_4; pH 6.0) [14] for 5 min bubbled with 95% O_2/5% CO_2, and then placed in 8 mL of 38 °C incubation medium with iso-osmotic replacement of NaCl using 0.02, 1, 5, 20, or 40 mM Gly-Sar and stirred at 1200 rpm. One μCi of ^{14}C—Gly-Sar was included in the test solution to measure total uptake of Gly-Sar, from which the transporter-mediated uptake by PEPT1 was calculated (see below). After 1-min incubation, tissues were removed, rinsed in 30 mL of ice-cold (Gly-Sar-free) incubation medium, stirred at 1200 rpm for 20 s, placed in glass scintillation vials containing 1 mL of tissue solubilizer (Perkin-Elmer, Boston, MA), and kept in a 50 °C water bath for 3 h. After complete solubilization, 15 mL of scintillation counting cocktail (Opti-Flour; Perkin-Elmer, Waltham, MA) was added, and DPMs of ^{14}C were determined using liquid scintillation techniques.

Transporter- *versus* Nontransporter-Mediated Uptake

To calculate transporter-mediated uptake of Gly-Sar, total uptake needed to be corrected for passive diffusion and adherent Gly-Sar to the mucosal surface (nontransporter-mediated "uptake"). Based on methods of Matthews et al. [16], nontransporter-mediated uptake at lesser concentrations can be best estimated from observed uptake at greater concentrations. As the substrate concentration increases, nontransporter-mediated passive uptake increases linearly before and after the transporter is saturated; thus, the linear increase in total uptake after the transporter is saturated is attributed "only" to nontransporter mediated "uptake", i.e., passive diffusion and mucosal adherence. We used 20 and 40 mM concentrations of Gly-Sar (at which a linear increase in total uptake was observed) to estimate nontransporter-mediated "uptake" at lesser concentrations (0.02, 1, 5 mM). Subtraction of estimated, nontransporter-mediated uptake from observed total uptake allowed estimation of the transporter-mediated uptake.

Villous Height

Formalin-fixed tissues were embedded in paraffin, sectioned parallel to the villous axis, and stained with hematoxylin and eosin. Maximum villous height was measured from above the crypt to the tip of the villous at 10× magnification using an optical reticule with a micrometer. Measurements from each specimen were made on at least six slides with at least three measurements per slide.

Statistical Analysis

All levels of mRNA and protein expression were expressed as the ratio of PEPT1 to the housekeeping gene (GAPDH). Transporter-mediated uptake of Gly-Sar was measured in nmol/cm/min with