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HEXOSE TRANSPORTER EXPRESSION AND FUNCTION IN MOUSE SMALL INTESTINE: ROLE OF DIURNAL RHYTHM

Javairiah Fatima, Corey W. Iqbal, Scott G. Houghton, Michael S. Kasperek, Judith A. Duenes, Ye Zheng, and Michael G. Sarr

Department of Surgery and Gastroenterology Research Unit, Mayo Clinic College of Medicine, Rochester, Minnesota, USA

Abstract

BACKGROUND—Expression and function of hexose transporters vary diurnally in rat small intestine; however, this subject remains unexplored in mice.

AIM—Investigate diurnal expression and function of hexose transporters SGLT1, GLUT2, and GLUT5 in mouse small bowel.

METHODS—24 c57bl6 mice maintained in a 12-h light/dark room (6AM–6PM) were sacrificed at 9AM, 3PM, 9PM, and 3AM (n=6 each). In duodenal, jejunal, and ileal mucosa, total cellular mRNA and protein levels were quantitated by real time PCR and semi-quantitative Western blotting, respectively. The everted sleeve technique measured transporter-mediated glucose uptake at 9AM and 9PM.

RESULTS—mRNA expression of SGLT1, GLUT2, and GLUT5 varied diurnally in all 3 intestinal segments (p 0.03). SGLT1, GLUT2, and GLUT5 protein levels varied diurnally in duodenum and jejunum (p<0.05) but not in ileum. Transporter-mediated glucose uptake was greater at 9PM than 9AM (p 0.04) in all three segments. V_{max} was greater in duodenum (10 vs 6 nmol/cm/sec) and jejunum (8 vs 5 nmol/cm/sec) at 9PM compared to 9AM (p=0.01); K_m remained unchanged.

SUMMARY—mRNA levels of intestinal hexose transporters varied diurnally. Protein levels peaked 6–12 hours later during dark cycle when >70% of food intake occurred; glucose transport followed a similar pattern with increased uptake at 9PM.

CONCLUSION—Hexose transporter expression and function vary diurnally with nocturnal feeding patterns of mice.

Keywords

hexose transporters; diurnal rhythm; mice; small intestine; sugar absorption

INTRODUCTION

Sodium glucose co-transporter 1 (SGLT1), glucose transporter 2 (GLUT2), and glucose transporter 5 (GLUT5) are the primary hexose transporters present in rodent small intestine [1,2]. Expression of these transporters is regulated by luminal substrates, hormonal

Address correspondence to Michael G. Sarr, MD, Gastroenterology Research Unit (GU 1001), Department of Surgery, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. Phone: 507-255-5713. Fax: 507-255-6318. Sarr.Michael@mayo.edu with cc to Deborah Frank (frank.deborah@mayo.edu) and Javairiah Fatima, MBBS, (fatima.javairiah@mayo.edu).

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regulation, neural regulation, ontogenic programmed expression, and by diurnal rhythm possibly via clock genes [1,3–8]. Expression of mRNA and protein of these transporters vary diurnally and in a temporally coordinated, segmental manner throughout rat small intestine [1,7–10]. Elucidating the interaction between regulatory mechanisms of these transporters may help develop novel treatments for patients with malabsorptive disorders and diabetes.

Virtually all the previous work on diurnal variation in glucose transporters has been limited to the rat model [1,7,8,10,11]. Uncovering the molecular mechanisms regulating this periodicity would be more feasible in the mouse model where the genome is more readily exploited [11]; however, this diurnal variation has not been characterized in mice.

It is important to understand both the mechanisms regulating the expression of nutrient absorptive proteins as well as the presence or absence of a diurnal variation in their expression and activities at a segmental level to better understand neural and/or hormonal control of this regulation. We hypothesized that the expression and function of the hexose transport proteins SGLT1, GLUT2, and GLUT5 follow a coordinated diurnal rhythm throughout the mouse small intestine.

METHODS

Animal handling and care was conducted in conformity with the NIH guidelines on the humane use and care of laboratory animals after approval from our Institutional Animal Care and Use Committee.

Design

To determine the presence or absence of diurnal rhythmicity, relative levels of total cellular hexose transporter mRNA and protein levels for mucosal hexose transporters from 6 mice at each of four time points (9AM, 3PM, 9PM, and 3AM) were determined from mucosal scrapings of duodenum, jejunum, and ileum. In addition, transporter-mediated glucose uptake was determined using the everted sleeve technique at 9PM and 9AM (n=6 at each time point; corresponding with presumed peak and trough levels of protein expression, respectively) [12].

Procedure

Adult male, c57bl6 mice (Harlan, Indianapolis, IN) weighing 20–25g were maintained in an alternating 12-h, light-dark cycle (6AM–6PM, respectively) with free access to chow (Lab Diet 5001, Brentwood, MO) and water. Mice were acclimated to this facility at least 1 wk prior to study. Food consumption was monitored every 12 h (6AM–6PM) for 1 wk separately in 6 mice.

Mice were anesthetized using inhaled 2% isoflurane (Abbott Laboratories, North Chicago, IL) for induction and intraperitoneal pentobarbital (40 mg/kg; AmproPharmacy, Arcadia, CA) for maintenance of anesthesia. After a midline celiotomy, the proximal duodenum was cannulated, and the entire small intestine was flushed with cold (4°C), iso-osmotic phosphate buffer solution (PBS) to remove intraluminal content. Care was taken to prevent disruption of blood supply to the bowel during this procedure. Duodenal, jejunal, and ileal segments were placed immediately in PBS at 4°C. Harvested duodenal segments were 4 cm in length, extending from the pylorus to the ligament of Treitz, jejunal specimens were 8 cm in length with the proximal limit as ligament of Treitz, and ileal samples were 10–12 cm lengths extending proximally from the cecum. Mucosa was obtained from each of these segments by scraping the opened segment with a glass slide. Part of the mucosal sample was placed in RNA stabilization buffer (RNALater, Qiagen, Valenica, CA), and the remainder in

cold PBS with protease inhibitor (Complete tablets, Roche Diagnostics, Indianapolis, IN) for protein studies. All samples were snap-frozen in liquid nitrogen immediately after sample acquisition and kept at -80°C until analysis.

RNA

Total RNA was isolated using the RNeasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. RNA concentrations in each sample were then determined using a spectrophotometer (DU 650, Beckman, Fullerton, CA). Then, $2\text{ }\mu\text{g}$ of RNA from each sample was incubated for 15 min with $2\text{ }\mu\text{l}$ of DNase enzyme (amplification grade) in DNase buffer (Invitrogen, Carlsbad, CA) to digest any residual DNA. Next, each sample was reverse transcribed using the SuperScriptIII kit (Invitrogen, Carlsbad, CA) with random hexamer priming. To eliminate variability, all samples from each segment were reverse transcribed simultaneously. cDNA was kept at -20°C until further analysis.

Real-time PCR analysis was performed using Taqman assays (Applied Biosystems, Foster City, CA). Each reaction consisted of $2\text{ }\mu\text{l}$ of standard, control, or unknown sample combined with $23\text{ }\mu\text{l}$ of a standard master mix (Applied Biosystems). Plasmids containing the cDNA transcripts for SGLT1, GLUT2, and GLUT5 were purchased from Open Biosystems (Huntsville, AL). Serial dilutions of plasmids with known copy numbers were used to generate a standard curve during each real-time PCR run. The cycle threshold for unknown samples was then compared to the standard curve to determine the copy numbers present for each transcript in each sample. DNase-free water was used as negative control. Each sample was run in duplicate, and all samples to be compared were run together on the same analysis to avoid variability between runs of real-time PCR analysis. Expression levels of each transporter were expressed as a ratio to glyceraldehyde 6-phosphate dehydrogenase (GAPDH), a housekeeping gene whose mRNA and protein levels are expressed in stable amounts in intestinal mucosal cells.

Protein

The intestinal mucosal samples in PBS buffer containing protease inhibitors were thawed on ice and homogenized in RIPA lysis buffer, also containing the protease inhibitors, using a Kontes Pellet Pestle (Fischer Scientific, Pittsburg, PA) [6]. Cellular debris was removed by centrifugation at 10,000 rpm for 15 min at 4°C , and total protein was collected in the supernatant. Protein concentrations were then determined using the bichinchonink acid method (Pierce, Rockford, IL) and protein plate reader (Microplate Manager III, BioRad, Hercules, CA) at a wavelength of 570 nm. Next, $200\text{ }\mu\text{g}$ of protein from each sample was resolved on a 10% polyacrylamide gel (BioRad, Hercules, CA). Precision Plus protein standards (BioRad) were used for identification of molecular weights. Proteins were then transferred onto a PVDF membrane (Millipore, Bedford, MA) using a semi-dry technique and blocked with 5%, non-fat milk (BioRad) in Tris buffered saline in Tween (TBS-T). Membranes were then incubated overnight in primary antibody against SGLT1 (1:3000 dilution, Chemicon International, Temecula, CA), GLUT2 (1:500 dilution, Chemicon International), GLUT5 (1:25 dilution, CoCalico Biologicals, Reamstown, PA), or GAPDH (1:500 dilution, US Biological, Swampscott, MA). Subsequently, membranes were washed 3X in TBS-T and blocked in 5% milk for 10 min prior to incubation in the secondary antibodies (1:10,000 dilution, anti-rabbit IgG for SGLT1, GLUT2, and GLUT5, and 1:10,000 dilution of anti-mouse IgG for GAPDH; Sigma-Aldrich, St. Louis, MO) at room temperature for 1 h. Membranes were washed 3X in TBS-T and once in TBS alone. Colorimetric reaction was then performed using Opti-4CN (Bio-Rad) substrate for 10 min. Amplified Opti-4CN substrate kit (Bio-Rad) was used to enhance the SGLT1 band. Immunoreactive bands produced by this method were identified at about 70 kDa for SGLT1, 65 kDa for GLUT2, 55 kDa for GLUT5, and 35 kDa for GAPDH. Membranes were

scanned, and densitometric analysis of the bands was performed using Scion Image (Scion Corporation, Frederick, MD). All samples were run in duplicate; values were expressed as a ratio of the transporter to GAPDH levels in each sample.

GLUT5 primary antibody production—Due to the unavailability of commercially prepared GLUT5 primary antibody, we developed an antibody using a GLUT5 epitope (the last 15 amino acids at the C-terminal of GLUT5 protein according to the NCBI sequence—EEKELNDLPPATREQ). The epitope was used by CoCalico Biologicals (Reamstown, PA) to induce production of antibody to the GLUT5 epitope in rabbits. After several boosts, serum from the production bleed was obtained. Western blot was performed using this unpurified antibody, and a band was identified as expected at 55 kDa. To validate that the antibody was indeed against GLUT5, we repeated our experiment by incubating GLUT5 primary antibody with the epitope peptide for 1 h; the band at 55 kDa disappeared, supporting the specificity of this antibody.

Transporter-Mediated Glucose Uptake

Transporter-mediated glucose uptake was measured using the everted sleeve technique [12], whereby the harvested intestine was everted to expose the mucosal surface externally. One-cm, everted segments of duodenum, jejunum, and ileum were then mounted on steel rods and secured with 5-0 silk ties in pre-formed grooves, leaving the mucosal surface exposed to the bath solutions. These everted sleeves were kept in chilled (4°C) mammalian Ringers solution (in mM: 128 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 20 NaHCO₃; pH 7.3–7.4; 290 mOsm) bubbled with 95% O₂/5% CO₂ until studied. Prior to transport studies, the everted sleeves were then preincubated in 8 ml of 38°C mammalian Ringers solution bubbled with 95% O₂/5% CO₂ for 5 min. After preincubation, the segments were immersed in an 8-ml incubation bath maintained at 38°C containing Ringers solution with iso-osmotic replacement of NaCl with either 1 mM, 20 mM, or 50 mM d-glucose and stirred at 1200 rpm. ¹⁴C-D-Glucose was used as the marker of transporter-mediated uptake, while ³H-L-glucose (which is not absorbed via carrier-mediated facilitated transport) was used to correct for passive diffusion and adherent fluid. After a 1-min incubation, tissues were rinsed in 30 ml of chilled Ringers solution stirred at 1,200 rpm for 20 s and placed in glass scintillation vials. One-half ml of tissue solubilizer (Solvable™, PerkinElmer, Boston, MA) was used to solubilize the segments over a 3-h period in a 50°C water bath. After complete solubilization, 10 ml of scintillation counting cocktail (Opti-Fluor®, PerkinElmer) was added, and disintegrations per minute (DPM) were obtained using liquid scintillation counting. Radioactivity was measured using dual-isotope counting on a Beckman liquid scintillation counter. A standard quench curve was constructed and corrections performed to account for spillover. All counts were then expressed as DPM. Uptake was calculated using the following equation [11]:

$$J = (P - R \cdot M) / H \cdot t \cdot m$$

where P = DPM of ¹⁴C_{tiss}, M = DPM of ³H_{inc}, R = DPM ¹⁴C_{inc}/DPM ³H_{inc}, H = DPM ¹⁴C_{inc}/nmol glucose_{inc}, t = time, m = length; tiss = tissue, inc = incubation fluid.

Lineweaver Burke plots were constructed to calculate V_{max} and K_m values.

Statistical Analysis

Ratio of total cellular mRNA and protein expression for each transporter is expressed as a ratio of the transporter to the housekeeping gene GAPDH and is reported as the median value with interquartile ranges (IQR). Kruskal-Wallis and Wilcoxon rank sum tests were

used to analyze the variation between the four time points and within each group at different anatomic regions (duodenum, jejunum, ileum). Fold changes in mRNA and protein were calculated as the maximal levels divided by minimum levels. Glucose uptake values are reported as the mean and standard error of the mean ($\bar{x} \pm \text{SEM}$). ANOVA and Student's *t*-tests were used to compare transport data across segments and within each segment, respectively. *P* value of <0.05 after Bonferroni corrections was considered significant; *n* values are number of mice.

RESULTS

Feeding Pattern

Mice followed a nocturnal-based feeding pattern. Greater than 70% of chow intake occurred between 6PM and 6AM (data not shown).

Segmental Diurnal Variation in Expression of mRNA and Protein

All three intestinal segments showed a diurnal variation in expression of hexose transporter mRNA; total cellular protein levels showed a similar diurnal variation in duodenum and jejunum but with a delay of 12–18 h in time.

Duodenum—mRNA levels of the three hexose transporters SGLT1, GLUT2, and GLUT5 peaked at 3PM in duodenum ($p < 0.05$ in all; Figure 1). The relative fold changes (peak over minimum level) were 2-fold for SGLT1, 3-fold for GLUT2, and 9-fold for GLUT5. Protein levels peaked at 9AM, 18 h after mRNA levels for all three transporters ($p < 0.02$ each for all three transporters (Figure 1a). The relative fold-change was 2-fold for all the three transporters.

In about half of the duodenal samples, we were unable to measure any protein for the hexose transporters or for GAPDH. This inability to identify and thus quantify protein tended to occur at 9PM and 3AM, requiring us to harvest tissues from additional mice at these time points to obtain an *n* of at least 6 mice at each time point.

Jejunum—SGLT1 mRNA levels peaked at 9PM ($p = 0.04$; Figure 1b). GLUT2 and GLUT5 mRNA levels peaked at 3PM ($p = 0.03$ each). Relative fold-changes were 3-fold for SGLT1 and GLUT2, and 24-fold for GLUT5. Peaks in protein expression followed with a delay of 6–12 h for all three transporters; protein levels of SGLT1, GLUT2, and GLUT5 were greatest at 3AM ($p < 0.05$ each; Figure 1b). GLUT2 levels remained high at 9AM, while the other two transporter levels declined. Relative fold-changes were 2-fold for SGLT1 and 1.5-fold for GLUT2 and GLUT5. Representative Western blots for jejunal protein levels are shown in Figure 2.

Ileum—mRNA levels of all three hexose transporters peaked at 9PM ($p < 0.03$ for all; Figure 1c). Relative fold changes were 3-fold for SGLT1, 10-fold for GLUT2, and 8-fold for GLUT5. In contrast to duodenum and jejunum, protein levels of all three hexose transporters did not vary diurnally in ileum ($p = 0.5$ each).

Transporter-Mediated Glucose Uptake

Uptake in all three segments demonstrated saturation kinetics consistent with transporter-mediated uptake. Glucose uptake in duodenum was greater at 9PM compared to 9AM for all three glucose concentrations (Figure 3a). A similar pattern of increased uptake at 9PM compared to 9AM was also seen in jejunum and ileum, but only at 1 mM and 20 mM glucose concentrations (Figure 3b and 3c). Uptake in ileum was much less than in duodenum and jejunum. Calculated V_{max} increased at 9PM compared to 9AM in duodenum

and jejunum, but remained unchanged in ileum (Figure 4a). K_m did not differ amongst the segments at 9AM vs the 9PM group (Figure 4b).

DISCUSSION

Many groups have studied the regulatory mechanisms of expression of hexose transporter mRNA and protein in the rat small intestine, all demonstrating a diurnal rhythm in the expression of SGLT1, GLUT2 and GLUT5 [1,2,7,9]. Peak levels of mRNA occur in an anticipatory fashion just before or just after the onset of the dark cycle before the majority of feeding takes place, while total cellular protein levels peak 6–12 h later, coinciding with maximal chow intake [10,13]. All these investigations have been conducted in rats, and because of the relative inability to genetically alter the rat genome, the ability to further define the molecular-based mechanisms of underlying signaling pathways and other cellular mechanisms that might contribute to these regulatory changes is complicated. We believed it would be important to establish the existence of a similar, diurnal rhythm in the mouse to broaden genetic investigation into the regulation of hexose transport. To the best of our knowledge, there have been no such comprehensive reports of a diurnal expression of hexose transporters in mouse small intestine nor a correlation of the expression of these transporters with their actual absorptive function.

We demonstrated that when mice had free access to food and water, like rats, most of their food intake (>70%) occurred during the dark cycle between 6PM and 6AM. This diurnal feeding cycle correlated with the total cellular expression of mRNA and protein of the hexose transporters SGLT1, GLUT2, and GLUT5, as well as the timing of maximal transporter function, as measured by the everted sleeve technique. The levels of mRNA of the transporters in the duodenum and jejunum peaked in an anticipatory fashion during the late hours of the light cycle and just before the onset of the dark cycle when feedings occurred; this increased transcription appeared to occur in preparation for translation into the transport proteins to coincide with maximal feeding during the dark cycle. Indeed, the protein levels for SGLT1, GLUT2, and GLUT5 displayed a diurnally varying pattern of expression in the jejunum with greatest expression at 3AM. This finding was supported by the demonstration of maximal transporter-mediated uptake by the small intestine during the dark cycle when food intake was greatest. Also associated with this increased uptake was a 1.6-fold increase in V_{max} (a function of the number of transporters) from 9AM to 9PM, while K_m , a function of the receptor's transporter affinity for its substrate, remained unchanged in the jejunum, reinforcing the concept that the increased protein expression is not a result of a change in type of transporter or conformation rather an increase in the number of transporters expressed.

In the duodenum, transporter function (transport of glucose) showed a similar trend as in the jejunum, with a 1.8-fold increase in the V_{max} from 9AM to 9PM and no change in K_m ; protein expression also had a diurnal trend; however, the peak expression occurred at 9AM (during the early part of light cycle), at least as measured by our technique of isolation and semi-quantitative Western blot. This relative peak in protein expression in mouse duodenum did not correlate with the timing of mRNA expression, transport activity as measured by the everted sleeve technique, maximal food intake, or the peak expression of protein in the rat duodenum [6,10]. We suggest that this apparent discrepancy, as measured by our technique of harvest and Western blot assay, may be an artifact related to the high activity of proteases expected to be present in the duodenal lumen at 9PM and 3AM during feeding, which may have lead to degradation of these membrane transport proteins during tissue harvest. Western blot analysis requires cell lysis to allow the protein transporters to be recognized by the antibodies. Because the proteins are in solution, should any active proteases be present, degradation may occur. Despite the addition of protease inhibitors to the media, it is possible

that high activities of proteases in the duodenal lumen during the nocturnal eating would affect the apparent levels of proteins (both transporters and the housekeeping gene GAPDH), making the measurements less reliable. Evidence for the latter is that in about 50% of mice, we were unable to measure any protein expression of the transporters or even the housekeeping gene GAPDH, suggesting a non-selective degradation of protein in the duodenal samples. These findings question the reliability of the measurements by Western blots of duodenal protein by this technique. The functional uptake studies of membrane-bound hexose transporters may not be as susceptible to these changes, explaining the increase in uptake at 9PM.

Ileal mRNA levels of all three transporters in mice peaked during the early part of the dark cycle with a delay of 6 h from the peak times in the duodenum and jejunum, consistent with findings in the rat [10]. This temporal delay may be a function of the time needed for ingested food to reach the ileum, yet remaining consistent with the anticipation of food for all three transporters. Unlike in the duodenum and jejunum, however, the total cellular protein levels measured by Western blot analyses showed no variation with the time of the day. In large animals, most carbohydrate absorption occurs during transit through the duodenum and jejunum, with minimal intraluminal carbohydrate remaining for absorption in the ileum. Whether this occurs in the mouse is unknown, but this observation might explain the lack of a change in total cellular protein levels or number of transporters as depicted by function (V_{max}) in the ileal segment from 9AM to 9PM. This absence of a diurnal rhythm of total cellular protein expression and hexose transport in the ileum of mice also occurs in the rat [10].

In contrast to total cellular levels of protein expression in the ileum, glucose uptake measured by the everted sleeve technique was increased at 9PM compared to 9AM with a similar trend as in duodenum and jejunum. The explanation of this increase in hexose transporter function cannot be further delineated by our study; however, several potential explanations are possible. Our techniques of measuring total cellular mRNA and protein levels do not necessarily reflect membrane expression or function. Indeed, considerable evidence suggests that there are cytoplasmic pools of preformed SGLT1 and GLUT2 protein that can be translocated rapidly to the apical membrane to increase functional uptake of luminal hexoses [14,15]. Evidence is strongest for GLUT2 translocation [16–19]. Such translocation of preformed cytoplasmic transporter protein(s) to the apical membrane would not be recognized by our techniques for measuring total cellular protein levels but would explain our findings. Further experiments will be necessary to elucidate the mechanisms of this diurnal variation in function of hexose transporters.

An interesting finding from our experiment was an apparent difference in the measured K_m of hexose transport in the ileum compared to the duodenum and jejunum. This difference in K_m was small and of questionable importance but does raise the question of whether there may be a different type or conformational change of the transporter present in the ileum, with a higher affinity for these sugars to allow more efficient absorption of any nutrients that remained in the lumen. This difference could be attributed to differences in the ratio of SGLT1 and GLUT2 expressed at the apical membrane as a result of lesser quantities of glucose in the ileal content, secondary to a change in conformation of the transporter(s) within the membrane, or possibly due to expression of a splice variant of transporters not recognized by our real-time PCR and Western blot assays. Further experiments will be required to differentiate these possibilities.

In summary, our study demonstrates that the mouse, similar to the rat, ingests food primarily at night during the dark cycle, and the expression and function of the hexose transport proteins SGLT1, GLUT2, and GLUT5 follow a diurnal pattern. Expression of mRNA is

anticipatory to feeding, while protein levels, at least in the jejunum, are increased during the time of maximal feeding. The peaks in both protein expression and transport function increase by 1.5- to 2-fold compared to the trough levels. Demonstration of this diurnal variation should facilitate investigation of molecular mechanisms regulating the expression of the transport proteins.

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Figure 1a

Figure 1b

Figure 1c

FIGURE 1.

Variations in mRNA and protein expression levels of SGLT1, GLUT2, and GLUT5 transporters at 4 time-points during the day in **a)** duodenum, **b)** jejunum, and **c)** ileum; mRNA expression relative to expression of the housekeeper GAPDH demonstrated diurnal variation in all three segments of the small intestine. Total cellular protein levels varied diurnally in duodenum and jejunum only.

FIGURE 2.

Representative Western blots for SGLT2, GLUT2, and GLUT5 in the jejunum; note the calculations are based on expression of the transporter protein band compared to the band for GAPDH.

FIGURE 3. Difference in transporter-mediated glucose uptake in **a)** duodenum, **b)** jejunum, and **c)** ileum at 9AM vs 9PM. Glucose uptake was greater at 9PM than at 9AM in all three intestinal segments.

FIGURE 4.

Variation in the **a)** $V_{\max}$ and **b)** K_m for each intestinal segment from 9AM to 9PM. In duodenum and jejunum, the mean $V_{\max}$ was greater at 9PM than at 9AM; K_m remained unchanged.