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Coordinated, Diurnal Hexose Transporter Expression in Rat Small Bowel: Implications for Small Bowel Resection*

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Abstract

Background—Hexose transporter mRNA and protein levels follow a diurnal rhythm in rat jejunum. Their coordinated expression and resultant function throughout the small bowel is not well understood. We hypothesized that hexose transporter levels, and glucose absorption follow a coordinated, site-specific diurnal rhythm in rat duodenum and jejunum, but not in ileum.

Methods—Sprague-Dawley rats were housed in a strictly maintained, 12-hr, light/dark room [light 6AM–6PM] with free access to water and chow. Mucosa was harvested from duodenum, jejunum, and ileum at 3AM, 9AM, 3PM, and 9PM and full thickness 1-cm segments were harvested at 9AM, and 9PM (n=6 for each segment at each time point). mRNA levels were determined by reverse transcription, real-time PCR (n≥5), protein levels by semi-quantitative Western blotting (n≥5), and transporter-mediated glucose uptake by everted sleeve technique (n=6).

Results—mRNA levels of SGLT1 and GLUT5 followed a temporally coordinated, diurnal rhythm in all three segments ($p<0.01$), while mRNA for GLUT2 and protein levels for SGLT1 and GLUT2 varied diurnally only in duodenum and jejunum ($p<0.05$) but not in ileum ($p>0.10$). SGLT1 and GLUT5 mRNA induction decreased aborally. Baseline SGLT1 and GLUT5 mRNA levels and SLGT1 and GLUT2 protein levels did not vary aborally ($p>0.05$ for all). GLUT2 mRNA baseline levels were decreased in ileum ($p<0.01$). Glucose uptake varied diurnally in duodenum and jejunum with no difference in ileum. Transporter-mediated glucose uptake was greater in duodenum and jejunum compared to ileum.

Conclusion—Regulation of hexose absorption in rat small bowel appears to be site-specific and mediated by multiple mechanisms.

Keywords

duodenum; jejunum; ileum; SGLT1; GLUT5; GLUT2; everted sleeve

Introduction

The active transporter protein sodium/glucose co-transporter 1 (SGLT1), and the two facilitated transporter proteins, glucose transporter 2 (GLUT2), and glucose transporter 5

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(GLUT5), are the three main hexose transporters in rat small bowel.[1] The mRNA and protein levels of these transporters follow a well-described, daily diurnal rhythm in rat jejunum.[1,2] Additionally, mRNA and/or protein levels of SGLT1, GLUT2, and GLUT5, as well as sucrase-isomaltase and H^+/peptide co-transporter 1 (PEPT1), follow a diurnal rhythm in rat duodenum, with increased expression at night.[3–8] These rhythms are entrained by the nocturnal feeding behavior of rats and can be altered by changes in chow composition and changing the times of access to food.[1,9,10] SGLT1, GLUT2, and GLUT5 are expressed in ileum;[3,9,11–13] however, the diurnal variations, if any, of mRNA and protein levels, and the resultant transporter function in ileum are not known. Furthermore, whether these diurnal rhythms are coordinated temporally in a site-specific manner throughout the small bowel is unknown.

Under normal conditions, the majority of ingested sugars are absorbed by the proximal gut and present at low concentrations in ileum. Furthermore, the absorptive capacity of the small bowel for hexoses is not constant throughout its length.[14] Therefore, a coordinated pattern of transporter expression in the presence of decreased luminal substrate concentrations would suggest a role for either humoral factors and/or the enteric nervous system in regulating transporter expression. Knowledge of these mechanisms would be important in understanding the enteric pathophysiology of bowel resection or bowel denervation (e.g. intestinal transplantation) and could be exploited for potential treatment of short gut syndrome and small bowel transplantation. Given the relative absence of ingested sugars in the ileal lumen, we hypothesized that the mRNA and protein levels of SGLT1, GLUT2, and GLUT5 would follow a temporally coordinated, diurnal rhythm in duodenum and jejunum with complimentary changes in transporter-mediated glucose uptake but not in ileum. Our aim was to determine semi-quantitatively the coordinated, diurnal expression patterns of mRNA and protein levels of SGLT1, GLUT2, and GLUT5 in addition to the complimentary transporter-mediated glucose uptake in these three segments of small bowel.

Methods

Tissue Acquisition

Handling of animals and conduct of experiments was carried out after approval by the Institutional Animal Care and Use Committee of the Mayo Clinic and Mayo Foundation. All experiments were carried out in accordance with the NIH guidelines for the humane care and use of laboratory animals.

Thirty-six adult, male, Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250–300g were housed for 7 days in a strictly maintained, 12-hr light/dark room (6AM to 6PM) with free access to water and standard rat chow (5001 Rodent Diet, PMI Nutrition International LLC, Brentwood, MO). At 3AM, 9AM, 3PM, and 9PM, mucosa from duodenum, jejunum, and ileum was harvested from 6 rats (n=6 for each segment at each time point). Transporter-mediated glucose uptake was measured at 9AM and 9PM in a separate group of 12 rats (n=6 for each time point). Glucose uptake was measured in all three intestinal segments using the everted sleeve technique described below. For purposes of this study, duodenum was defined as the segment of bowel between the pylorus and ligament of Treitz, jejunum was defined as the first 15 cm of bowel distal to ligament of Treitz, and ileum was defined as the last 15 cm of small bowel proximal to ileocecal junction. Rat weights were similar at each of the 4 time points ($p=0.60$ by ANOVA).

Rats were anesthetized using inhaled isoflurane followed by 50 mg/kg of pentobarbital intraperitoneally. Intestinal segments for mRNA and protein analysis were placed immediately in ice-cold (4°C), phosphate buffered saline (PBS) and flushed as described previously to remove luminal debris.[2] Samples were harvested by scraping off the mucosa with a glass slide. Half of the mucosa was placed in cold RNA stabilization buffer, (RNAlater, Qiagen, Valencia,

CA), snap frozen in liquid nitrogen, and stored at −80°C. The other half of the harvested mucosa was placed in cold PBS, snap frozen in liquid nitrogen, and stored at −80°C. For uptake studies using everted sleeves, the duodenum was cannulated at the pylorus and flushed in situ with cold Ringer's solution. The entire small intestine was then excised and kept in cold Ringer's bubbled with 95%O_2/5%CO_2. All rats were killed under anesthesia by aortic transection.

RNA Isolation and cDNA Synthesis

Total RNA was isolated using the RNeasy Midi kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and as described previously; mucosa was homogenized by multiple passages through a needle.[2] Isolated total RNA was stored at −80°C in RNase-free water until cDNA synthesis. Isolated total RNA was first subjected to DNase digestion to remove any genomic DNA contamination. For each set of comparisons, equal amounts of RNA (0.5–2.0 μg) were incubated at room temperature for 15 min with 0.5–2.0 U of DNase (Invitrogen, Carlsbad, CA) and DNase buffer. After digestion, DNase was inactivated by incubation in 25 mM EDTA at 65°C for 10 min. DNase reaction products were used immediately for reverse transcription. Reverse transcription was performed using the Super Script III kit (Invitrogen, Carlsbad, CA) with random hexamer priming according to the manufacturers instructions. For studies of diurnal variation, all 24 samples from a segment (duodenum, jejunum, or ileum) were reverse transcribed simultaneously in an attempt to minimize any variability in cDNA synthesis.[15–17] For experiments evaluating mRNA induction, baseline expression, and peak expression across all 3 segments, all 36 samples were reverse transcribed simultaneously. cDNA was stored at −20°C until real-time PCR analysis.

Real-Time PCR

Real-time PCR utilizing TaqMan chemistries (Applied Biosystems, Foster City, CA) was employed to determine the number of cDNA copies present for each transporter in a sample. Real-time PCR reactions were performed using an Applied Biosystems 7500. TaqMan primers and fluorescently labeled probes for SGLT1, GLUT2, and Glyceraldehyde-6-Phosphate Dehydrogenase (GAPDH) were purchased from Applied Biosystems. GLUT5 primers and labeled probe were designed by Applied Biosystems with a forward primer sequence of 5′-CTCATCTTCCCGTTCATTCAAGTG - 3′, reverse primer sequence of 5′-GATGTAGATGGAGGTGAGGAGACA - 3′, and probe sequence of 5′-ACAGCTTCATCATCTTTGC - 3′. Each reaction volume was 25 μL and consisted of 23 μL of a master mix containing 12.5 μL of a 2X TaqMan Universal reaction mix (Applied Biosystems), 1.25 μL of gene-specific, primer-probe assay mix (Applied Biosystems), and 9.25 μL PCR grade water plus 2 μL of a standard or unknown reverse transcription product. Reaction conditions for all four genes consisted of 10 min at 95°C followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Fluorescence was measured during the 60°C cycle.

Serial dilutions of a standard with known copy numbers were analyzed during every real-time PCR run and used to generate a standard curve.[2] Unknown cycle threshold (C_t) values were compared to the standard curve and used to determine the number of cDNA copies of a given gene present in a volume of sample. Every sample, including standards, was run in duplicate during every real-time PCR run for a given gene. Negative controls consisted of 23 μL of reaction mix and 2 μL of water and were run in duplicate or triplicate throughout the plate during each run. Copy numbers for each transporter in a given sample are reported as a ratio to the copy numbers of GAPDH present in that cDNA sample. GAPDH is a stably expressed, housekeeping gene devoid of diurnal variation.[1,18] The reported ratios are completely arbitrary and can only be used to make comparisons between those samples undergoing reverse transcription simultaneously.[17]

Segmental Differences in mRNA Expression

Once mRNA peak and trough times for each segment were identified, we then compared the segmental mRNA induction (fold change from baseline to peak values), baseline expression, and peak expression levels for each transporter. A total of 36 mRNA samples were reverse transcribed simultaneously representing mucosa from each segment at the time points at which peak and trough transporter mRNA levels respectively occurred (duodenum: 3PM and 9AM; jejunum: 3 PM and 9AM; ileum: 9 PM and 9AM (n=12 from each segment, n=6 peak and trough mRNA samples for each segment)). cDNA samples were then compared using real-time PCR. For induction comparisons, peak levels were normalized to 9AM for that segment. For baseline and peak expression studies, mRNA levels of the hexose transporters were normalized to those in duodenum for that transporter.

Protein Isolation

Our method of total protein isolation, as adapted from Zhang et al,[19] has been described previously.[2] In brief, mucosal samples in PBS were thawed and placed in ice-cold (4°C) lysis buffer containing Complete™ protease inhibitors (Roche Applied Science, Indianapolis, IN) for 30 min. A Kontes Pellet Pestle (Fischer Scientific, Pittsburg, PA) was used to homogenize samples which were then centrifuged at 5000 × g for 15 min at 4°C after which the supernatant was collected carefully. Protein concentrations were measured by the bicinchoninic acid method (Pierce, Rockford, IL) using bovine serum albumen as a standard. Aliquots containing 100 μg of protein in XT sample buffer (Bio-Rad, Hercules, CA) were stored at −80°C for use in Western blotting.

Western Blotting

Our technique for Western blotting has been described previously in detail.[2] Briefly, equal amounts of protein (100–150 μg) were resolved on a 10% SDS-PAGE gel (Bio-Rad), transferred electrically to a PVDF membrane (Millipore, Bedford, MA), and blocked in TBS-T containing 5% milk (Bio-Rad). Membranes were cut between SGLT1 or GLUT2 and GAPDH. The bottom half was incubated overnight in a 1:500 dilution of primary antibody against GAPDH (US Biological, Swampscott, MA), and the top half was incubated overnight in primary antibody containing either 1:3000 SGLT1 or 1:500 GLUT2 (both from Chemicon International, Temecula, CA). Membranes were rinsed with TBS-T and incubated for 1 hr in secondary antibody. Dilutions of secondary antibody (peroxidase conjugated, goat anti-rabbit IgG; Sigma-Aldrich, St Louis, MO) were 1:10,000 for SGLT1 and GLUT2 and 1:10,000 (peroxidase conjugated, goat anti-mouse IgG; Sigma-Aldrich) for GAPDH. Membranes were washed and incubated in Opti-4CN™ (Bio-Rad) colorimetric substrate for 10 min to induce a colorimetric reaction. GLUT5 protein levels were not able to be determined accurately because of lack of a consistently reliable antibody despite numerous attempts to develop a reliable antibody.

Membranes were scanned and imported into Scion Image for Windows, based on NIH Image (Frederick, MD), which was used to determine band densitometry. The values reported are the band densitometry values for SGLT1 and GLUT2 normalized to the corresponding values for GAPDH in each individual sample. Each sample was run at least in duplicate, and the ratios to GAPDH for each sample were averaged and used in the final analysis. Samples that did not possess adequate levels of GAPDH were excluded from analysis; 1 of 24 samples in duodenum, 2 of 24 samples in jejunum, and 1 of 24 samples in ileum lacked a sufficient GAPDH band for analysis. Because of the inability to detect GAPDH in some samples by Western blot analysis, n≥5 for each time point studied.

Segmental Differences in Protein Expression

Once protein peak and trough times for each segment were identified, we then compared the segmental protein induction (fold change from baseline to peak values), baseline expression, and peak expression levels for each transporter. For induction comparisons, peak levels were normalized to the trough value for that segment. For baseline and peak expression studies, protein levels of the hexose transporters were normalized to those in duodenum for that transporter.

Everted Sleeve

Transporter-mediated glucose uptake was measured using the everted sleeve technique as described by Karasov and Diamond,[20] After the small bowel was removed and flushed to remove any luminal contents, the entire length of intestine was everted over a steel rod exposing the mucosal surface to the surrounding bath. Approximately one centimeter lengths of the segment being studied were then mounted on steel rods and secured with two 5-0 silk ties placed in pre-formed grooves one centimeter apart. The remaining edges were excised leaving a one centimeter everted segment. These sleeves were kept in cold (4°C) mammalian Ringer's (in mM: NaCl 128, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 20; pH 7.3–7.4 and 290mOsm) bubbled with 95% O_2/5% CO_2. Prior to uptake measurements, tissues were preincubated for 5 min in 8ml of 38°C mammalian Ringer's bubbled with 95% O_2/5%CO_2. After preincubation, segments were placed in an 8ml incubation bath (38°C) consisting of mammalian Ringer's with iso-osmotic replacement of NaCl with either 1mM, 20mM, or 50mM d-glucose, and stirred at 1,200 rpm (to mix the unstirred layer). ^{14}C-D-Glucose was used as a marker for transporter-mediated uptake. ^{3}H-L-glucose, which is not taken up via facilitated transport, was used to correct for passive diffusion and adherent fluid. After following incubation, tissues were rinsed in 30ml of chilled (4°C) mammalian Ringer's stirred at 1,200 rpm to remove adherent glucose and placed in glass scintillation vials. One-half ml of tissue solubilizer (Solvable™, PerkinElmer, Boston) was used to solubilize the segments in a 50°C water bath over 3 h. After complete solubilization, 10ml of opti-flour scintillation counting cocktail (Opti-Flour®, PerkinElmer, Boston) was added and probe counts were determined using liquid scintillation counting.

Counts were performed using dual isotope counting on a Beckman Liquid scintillation counter. A standard quench curve was made and corrections were performed to account for spillover. All counts were then expressed as disintegrations per minute (DPM). Uptake calculations performed using the following equation:

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

Where P =DPM of $^{14}C_{tiss}$, M =DPM of $^{3}H_{inc}$, R = DPM $^{14}C_{inc}$/DPM $^{3}H_{inc}$, H= DPM $14C_{inc}$/nmol $glucose_{inc}$, t= time, m=length.

Statistical Analysis

The Kruskal-Wallace test was used to compare non-parametric data across multiple groups; Wilcoxon rank sum tests were used between individual groups. The Bonferoni method for multiple comparisons was used to correct p values for the Wilcoxon tests. Results for experiments of induction, baseline, and peak expression are reported as fold change, with fold changes of less than 0.5, the discriminatory limit of real-time PCR, considered not significant.[15,16] For continuous data, ANOVA was used to compare multiple groups; single group comparisons were made using a two-tailed Student's *t* test. P-values less than 0.05 were considered significant. Ratios of transporter mRNA and protein levels to GAPDH are reported as the median value and interquartile range unless otherwise noted, with continuous data reported as mean±SEM.

Results

Coordinated Diurnal Variation of SGLT1 mRNA and Protein Levels

mRNA levels of SGLT1 showed a diurnal variation in all three small bowel segments ($p<0.01$ for all) (Figure 1A–C). mRNA levels were greatest at 3PM, 3 hr prior to lights out, in both duodenum and jejunum, while mRNA levels in ileum were greatest at 9PM, about 6 hr later than in the proximal bowel. Protein levels of SGLT1 followed a diurnal variation in duodenum and jejunum ($p<0.02$ for both) with peak levels occurring 18 hr (duodenum) and 12 hr (jejunum) after mRNA peaked in both segments; however, in ileum, no diurnal variation in SGLT1 protein levels was seen ($p>0.15$) (Figure 1A–C).

Coordinated Diurnal Variation of GLUT5 mRNA Levels

mRNA levels of GLUT5 also showed a diurnal variation in all three small bowel segments ($p<0.001$ for all) (Figure 2A–C). As with SGLT1, mRNA levels were greatest in duodenum and jejunum at 3PM, 3 hr prior to lights out. mRNA levels in ileum varied diurnally with a peak level at 9PM, 6 hr after duodenum and jejunum. Protein levels of GLUT5 could not be measured.

Coordinated Diurnal Variation of GLUT2 mRNA and Protein Levels

mRNA levels of GLUT2 also showed a diurnal variation in duodenum and jejunum ($p<0.001$ for both) with greatest levels at 3PM in both segments (Figure 3A–B). Peak levels occurred 3 hr prior to lights out, corresponding to those observed for SGLT1 and GLUT5. In contrast to SGLT1 and GLUT5, mRNA levels of GLUT2 did not vary diurnally in ileum ($p=0.8$) (Figure 3C) and had much larger inter-rat variations. Protein levels of GLUT2 followed a diurnal rhythm in duodenum and jejunum with greatest levels occurring at 9AM ($p<0.005$ for both), but protein levels of GLUT2 did not vary diurnally in ileum ($p>0.3$).

Segmental mRNA Induction, Baseline, and Peak Expression

To determine differences in induction, baseline, and peak levels of hexose transporters between segments, we analyzed RNA samples from peak and trough time points (as determined by diurnal studies) respectively in each segment (duodenum: 3 PM and 9AM; jejunum: 3 PM and 9AM; ileum: 9 PM and 9AM [n=6 peak and trough mRNA samples from each segment]) simultaneously by reverse transcription real-time PCR. mRNA levels of SGLT1 demonstrated a 4.5-fold change in duodenum, 3.5-fold change in jejunum, and 3.1-fold change in ileum ($p<0.004$ for all) (Figure 4A). GLUT5 mRNA levels demonstrated a 6.8-fold change in duodenum, 6.3-fold change in jejunum and 5.2-fold change in ileum ($p<0.004$ for all) (Figure 4B), while mRNA levels of GLUT2 demonstrated a 2.3-fold change in duodenum and jejunum ($p<0.004$ for both), but no induction was seen in ileum ($p= 0.60$) (Figure 4C), consistent with an absence of diurnal variation in GLUT2 levels of mRNA in ileum. When compared to expression of mRNA in duodenum, baseline levels of mRNA for SGLT1 and GLUT5 at 9AM were similar for all three segments ($p>0.05$ or less than 0.5-fold change) (Figure 5A and B, respectively). Baseline GLUT2 mRNA levels were similar in duodenum and jejunum ($p>0.05$) but were much less in ileum ($p<0.01$) (Figure 5C). Peak SGLT1 mRNA levels were similar in duodenum and jejunum ($p>0.05$) but were less in ileum ($p<0.02$) (Figure 6A). Peak GLUT5 mRNA levels were similar in duodenum and jejunum ($p>0.05$) but were less in ileum ($p<0.02$) (Figure 6B). Peak GLUT2 mRNA levels were similar in duodenum and jejunum (less than 0.5-fold change) (Figure 6C), no diurnal variation was noted in ileum.

Segmental Protein Induction, Baseline, and Peak Expression

Measurement of SGLT1 protein induction demonstrated a 1.6-fold change in duodenum and a 1.7-fold change in jejunum ($p<0.03$ for both), while no induction was seen in ileum ($p=0.15$)

(Figure 7A) consistent with absence of diurnal variation in SGLT1 protein levels in ileum. GLUT2 protein levels showed a 1.6-fold change in duodenum and a 2.2-fold change in jejunum ($p<0.01$ for both), again no induction was seen in ileum ($p=0.47$) (Figure 7B) consistent with absence of diurnal variation in GLUT2 protein levels in ileum. When compared to expression of protein in duodenum, baseline levels of SGLT1 and GLUT2 protein were similar in all three segments ($p>0.05$ for both) (Figure 8A–B). Peak SLGT1 and GLUT2 protein levels were similar in duodenum and jejunum ($p>0.05$ for both) (Figure 9A–B), no diurnal rhythm was seen in ileum.

Transporter-Mediated Glucose Uptake

Transporter-mediated glucose uptake was greater at 9PM compared to 9AM in the duodenum at all three glucose concentrations ($p=0.004$) (Figure 10A). Duodenal V_{max} was also greater at 9PM compared to 9AM (6.0±0.5 vs. 3.5±0.2nmol/cm/min, $p=0.002$) (Figure 11A). In the jejunum uptake was greater at 9PM compared to 9AM at the 1mM and 50mM concentrations ($p<0.01$), however, there was no difference at the 20mM concentration (253±25 at 9PM vs 223 ±13nmol/cm/min at 9AM, $p=0.3$) (Figure 10B) nor was there a difference in V_{max} (6±0.9 vs 5±0.3nmol/cm/min, $p=0.2$) (Figure 11A). The ileum demonstrated no difference in uptake at any glucose concentration between 9AM and 9PM (p=NS) (Figure 10C) and the V_{max} remained unchanged (1.7±0.2 vs 2.0±0.5nmol/cm/min, $p=0.7$) (Figure 11A). K_m values remained unchanged from 9AM to 9PM in all three segments (p=NS, data not shown).

Duodenum and jejunum demonstrated greater transporter-mediated glucose uptake compared to ileum at all three glucose concentrations and at both time points ($p\leq0.04$ for all) (Figure 11B). Similarly, V_{max} was greater in both the duodenum and the jejunum compared to the ileum ($p\leq0.01$) at both 9AM and 9PM (Figure 11A).

Discussion

The mechanisms controlling diurnal variations in expression of SGLT1, GLUT2, and GLUT5 mRNA and protein levels as well as overall function in the small bowel are not known. Because ingested hexoses are absorbed predominantly in the proximal gut, there is a relative absence of these hexoses in the lumen of the distal small bowel. Therefore, we hypothesized that the mRNA and protein levels of SGLT1, GLUT2, and GLUT5 would follow a coordinated, diurnal rhythm in duodenum and jejunum, but a similar diurnal rhythm would be absent in ileum with complimentary changes in transporter-mediated glucose uptake. In this study, we demonstrated a temporal, aborally coordinated, site-specific diurnal rhythm of SGLT1 and GLUT5 mRNA levels throughout the length of rat small bowel. In contrast, the protein levels of SGLT1 and mRNA and protein levels of GLUT2 followed a coordinated diurnal rhythm only in duodenum and jejunum but not in ileum. Similarly, transporter-mediated glucose uptake demonstrated a diurnal rhythm in the proximal small bowel but not in ileum, correlating with mRNA levels but not protein levels. Diurnal rhythms in proximal bowel were anticipatory in nature, with peak mRNA levels occurring prior to maximal chow intake (dark cycle) and a corresponding peak in protein levels during or after maximal chow intake. The diurnal rhythms seen in ileum were coordinated temporally, occurring 6 hours after the diurnal pattern in proximal bowel and with a decrease in levels of induction. Glucose uptake was greater at the time point of maximal chow intake with no change in the distal small bowel. Unexpectedly, baseline expression of SGLT1 and GLUT5 mRNA levels were similar in duodenum, jejunum, and ileum. In contrast mRNA and protein levels of GLUT2, which did not have a diurnal rhythm in ileum, had a marked decrease in baseline mRNA levels. Given the relative absence of hexoses in the ileal lumen, these finding suggest a possible role for humoral factors in regulating protein levels of hexose transporters and the corresponding transporter-mediated glucose uptake.

Several authors have reported previously that the mRNA and protein levels of nutrient transporters follow a diurnal rhythm in rat duodenum or jejunum.[1–8,10] In agreement with these reports, we demonstrated that the diurnal rhythms in hexose transporter mRNA and protein levels in proximal bowel appear to be entrained by or are at least associated with the nocturnal feeding behavior of rats. Studies of transporter-mediated glucose uptake studies correlated with the nocturnal feeding behavior of rats showing maximal uptake during the dark cycle; this has been shown previously for SGLT1.[7] We again showed that the diurnal changes are anticipatory, with peak mRNA levels occurring prior to maximal chow intake and total cellular protein levels peaking after maximal intake in proximal bowel, and the coordinated expression (mRNA and protein) of these transporters occur concurrently in duodenum and jejunum.

The presence of diurnal variations in mRNA levels of SGLT1 and GLUT5 in ileum was unexpected. Because of the relative lack of hexoses in the ileal lumen, we hypothesized that there would be no diurnal variations in mRNA and protein levels in ileal mucosa. We also expected baseline expression of these transporters to be decreased in ileum; however, this pattern was present only for GLUT2. Although gene expression of SGLT1, GLUT2, and GLUT5 occurs in ileum, the presence of a diurnal rhythm had not been demonstrated previously.[3,9,11–13] In contrast to mRNA expression, we were not able to demonstrate diurnal variations in glucose uptake and total cellular protein levels of SGLT1 or GLUT2 in ileum. The lack of a diurnal rhythm in ileal protein levels of SGLT1 and GLUT2 could be a consequence of the method used to measure protein levels.[2] We used semi-quantitative Western blotting, which is likely not as sensitive for detecting changes in protein levels as real-time PCR is in detecting changes in levels of mRNA. Nevertheless, the lack of a diurnal rhythm in transporter-mediated glucose uptake in ileum supports the absence of changes in protein levels. The lack of a diurnal rhythm in ileal transporter-mediated glucose uptake and protein levels in the face of diurnal changes in mRNA levels could indicate the presence of post-transcriptional or post-translational mechanisms in regulating the protein levels of hexose transporters. Interestingly, diurnal rhythms in transporter-mediated glucose uptake also correlated with GLUT2 mRNA levels, suggesting that this transporter has an important role in overall glucose uptake.

Diurnal changes in mRNA and protein levels are variable and can be effected by changes in chow intake or operative manipulation.[2,8,9,10] Furthermore, changes in mRNA and protein levels are not always linked and have been shown to be discordant after operative manipulations.[2,21] These observations suggest that more than one mechanism mediates diurnal changes in mRNA and protein levels.[2,8,21,22] These and other findings have led several authors to suggest that post-translational modification plays a major role in expression and function of hexose transporters.[2,21,23] Others have shown that a mutation in a glycosylation site on SGLT1 alters membrane trafficking, resulting in compromised function and severe glucose malabsorption.[22] Similar post-translational (or post-transcriptional in the case of mRNA) modifications that regulate trafficking to and from the membrane could explain the presence of a diurnal rhythm in expression of SGLT1 and GLUT5 mRNA in the absence of SGLT1 protein, GLUT2 mRNA, and GLUT2 protein variation in the ileum. In this study, peak SGLT1 and GLUT2 mRNA levels in the proximal bowel preceded peak protein levels by 6 to 12 hr. Others have shown a simultaneous increase,[6,8] however, most authors report a delay of 6 to 12 hr.[1–5] This discrepancy could be due to minor variations in housing conditions and/or diet and because mRNA and protein levels were not measured continuously but at discrete time points. These variations could also be explained by post-transcriptional or post-translational mechanisms which could also account for the variable time interval reported between peaks in mRNA and protein expression in diurnal studies, even by our own group.[2]

We observed the expected diurnal rhythms in transporter-mediated glucose uptake throughout the length of the small bowel with diurnal rhythms present in proximal bowel but absent in ileum. In addition, overall absorptive capacity was greater in duodenum and jejunum compared to ileum. This observation seems logical given hexose levels are greater in the lumen of proximal bowel and virtually absent in ileum. Interestingly, peak protein levels did not correlate with transporter-mediated glucose uptake, however if a segment demonstrated a diurnal rhythm in protein levels the segment also demonstrated a diurnal rhythm in transporter-mediated glucose uptake suggesting some correlation. This correlation is further supported by the use of different animals for protein and uptake studies. For technical reasons we were not able to use the same animals for all 3 experiments which could potentially alter the time of maximal levels. One possible explanation for differences in protein and uptake peaks was our use of total cellular protein for Western blotting. Using this method, we were not able to differentiate between functional and non-functional protein. This would allow us potentially to measure partially degraded, non-functional, or compartmentalized protein not available to the brush boarder and therefore not able to participate in glucose uptake but still present on Western blotting.

Several potential limitations of the current study deserve mention. First is the normalization of all values in this study (and most other related studies) to GAPDH, a housekeeping gene which is presumably expressed stably throughout the light/dark cycle.[1,18] Although a full discussion on the use of housekeeping genes for mRNA and protein quantitation, particularly with real-time PCR, is not warranted, it is important to remember that there is no perfect housekeeping gene, and any normalization introduces a potential for error.[15,24,25] A second limitation of the current study is the different sensitivities of Western blotting and real-time PCR. Real-time PCR is very sensitive and consistent with a dynamic range spanning 8 orders of magnitude.[15,16,25] Western blotting, however, has a lower sensitivity and dynamic range, and possesses large variations between antibody lots and epitopes being probed. The use of densitometry for quantitative estimation is also less precise. Moreover, a third limitation is that immunoreactivity for measuring protein levels is not necessarily indicative of transporter activity *in vivo*. In this study we measured total cellular protein levels in an attempt to avoid any alterations that would be incurred by measuring protein levels only in brush boarder membrane vesicles (BBMV). Measuring protein levels in BBMV may have accentuated any variations in protein levels present, but this method would have excluded large amounts of protein within the cytoplasm that potentially could contribute to transport but may not be constitutively present on the brush boarder. For this reason we feel measuring BBMV protein levels would provide an inaccurate and possibly confounding measurement of protein levels.

We found mRNA levels of GLUT2 to be decreased dramatically and without diurnal variation in ileum compared to proximal bowel. This observation, although not unexpected, is noteworthy in the context of the diurnal variation of mRNA levels of the other two hexose transporters mentioned previously, SGLT1 and GLUT5 in ileum and their equivalent baseline gene expression throughout the length of the small bowel. GLUT2 mRNA levels were 21-fold lower in ileum than in duodenum, compared to less than a 2-fold decrease for both SGLT1 and GLUT5. Several possible explanations may account for this discordance. First, the GLUT2 transcript could contain an as yet unknown, tissue-specific, alternate splice site which removes all or a portion of the sequence (amplicon) detected by our real-time PCR assay in a majority of ileal GLUT2 transcripts, resulting in a GLUT2 mRNA that is not detected by our assay. Most GLUT2 cDNA clones, including the one used as a standard in the present study, were isolated from pancreas or foregut and not ileum.[13] If the GLUT2 gene were to contain such an alternate splice site utilized more commonly in ileum than proximal bowel, we would not have detected it, and our results would be altered. A second possible explanation is the regulation of GLUT2 expression is separate from that of SGLT1 and GLUT5. Moreover, strong evidence suggests that GLUT2 is responsible for the high volume, diffusive component of

hexose absorption that occurs in proximal small bowel in response to a large nutrient load and that SGLT1 activity (either directly or indirectly) is necessary for the rapid trafficking of GLUT2 to the brush border membrane.[26–28] If SGLT1 activation in the presence of large amounts of glucose is necessary not only for rapid trafficking of GLUT2 to the brush boarder membrane, but also for enhanced GLUT2 expression, then the relatively low levels of hexoses in the ileal lumen may not be sufficient to activate SGLT1 and result in lower GLUT2 levels. This situation would, theoretically, result in ileal mucosal cells expressing GLUT2 levels needed for basolateral hexose transport but not those needed for high volume, apical absorption and thus removing the stimulus for diurnal variation. Moreover, transporter-mediated glucose uptake capacity most closely mirrored GLUT2 mRNA levels with both being decreased dramatically in ileum, while SGLT1 and GLUT5 mRNA levels were decreased modestly. This observation again suggests an important role for GLUT2 in hexose absorption.

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Abbreviations

SGLT1
: Sodium/glucose co-transporter 1

GLUT2
: Glucose transporter 2

GLUT5
: Glucose transporter 5

GAPDH
: Glyceraldehyde-6-phosphate dehydrogenase

PCR
: Polymerase chain reaction

mRNA
: Messenger RNA

PBS
: Phosphate buffered saline

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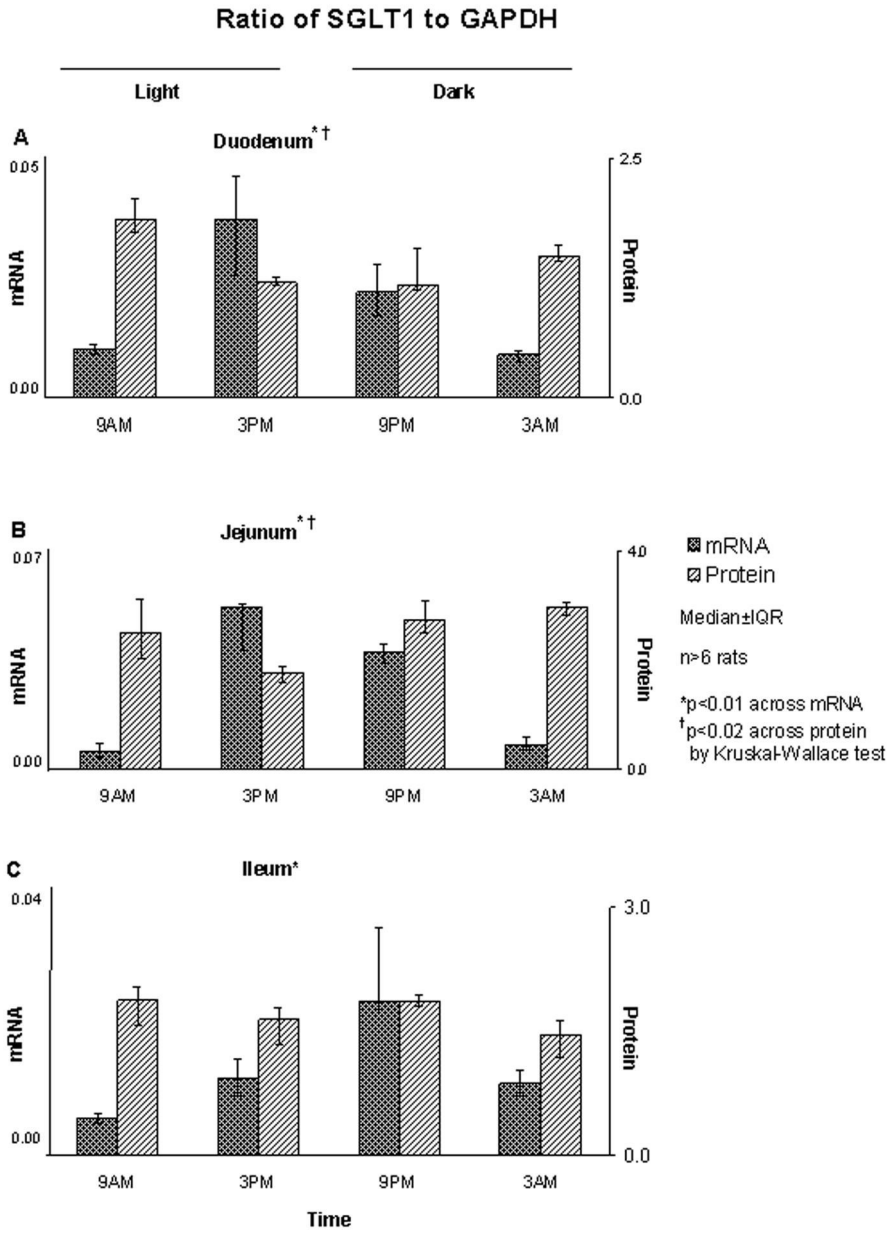


Figure 1.

Diurnal variation of SGLT1 mRNA and protein levels in small bowel mucosa of adult rats expressed as ratios of levels of SGLT1 to the housekeeping gene GAPDH in arbitrary units. Diurnal rhythm analyzed by Kruskal-Wallace test for non-parametric data. A and B) duodenum and jejunum respectively; SGLT1 mRNA levels peak at 3PM with corresponding levels of protein peaking at 3AM and 9AM. C) ileum; SGLT1 mRNA levels peak at 9PM; no variation was seen in protein levels.

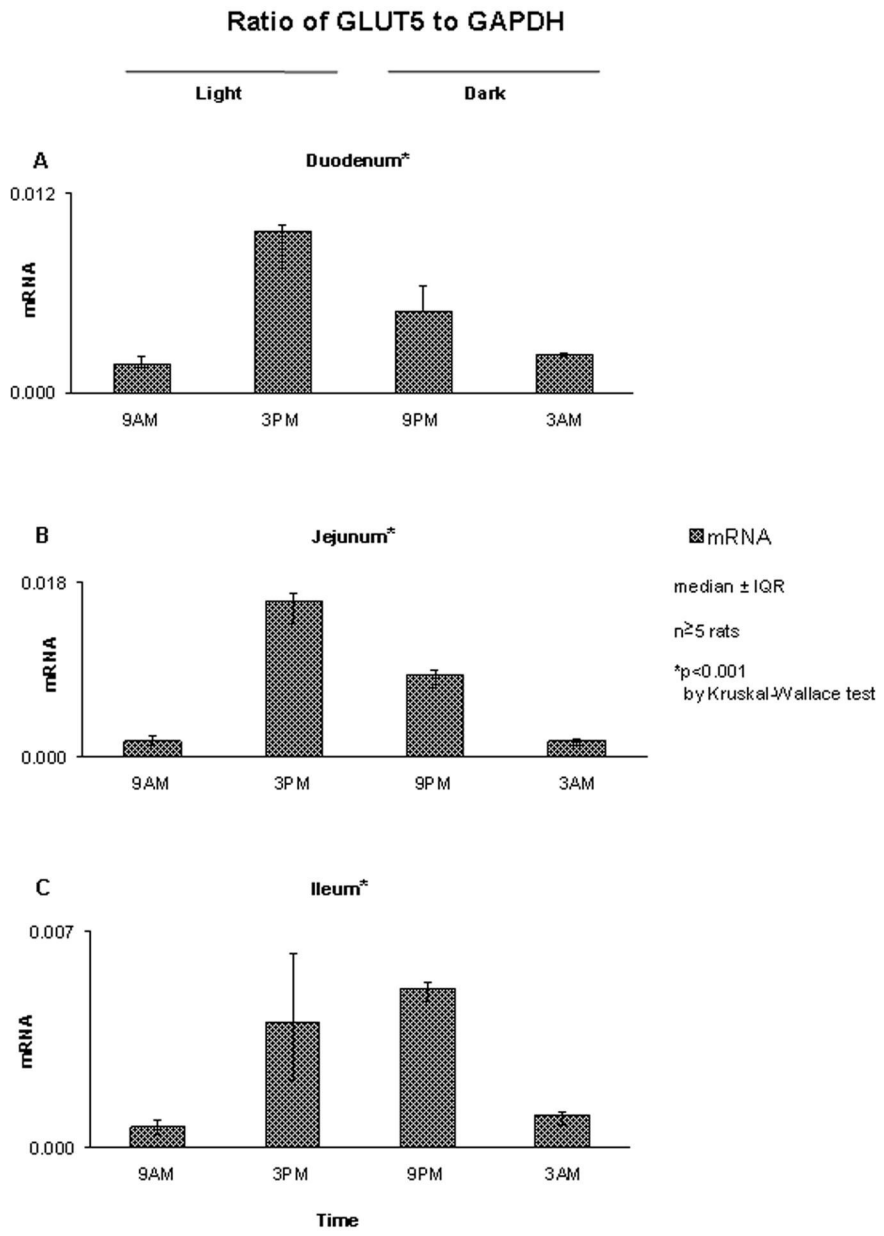


Figure 2.

Diurnal variation of GLUT5 mRNA levels in adult rat small bowel mucosa expressed as ratios of levels of GLUT5 to GAPDH expressed in arbitrary units. Diurnal rhythm analyzed by Kruskal-Wallace test for non-parametric data. A and B) duodenum and jejunum respectively; GLUT5 mRNA levels peak at 3PM. C) ileum; GLUT5 mRNA levels in the ileum peak at 9PM.

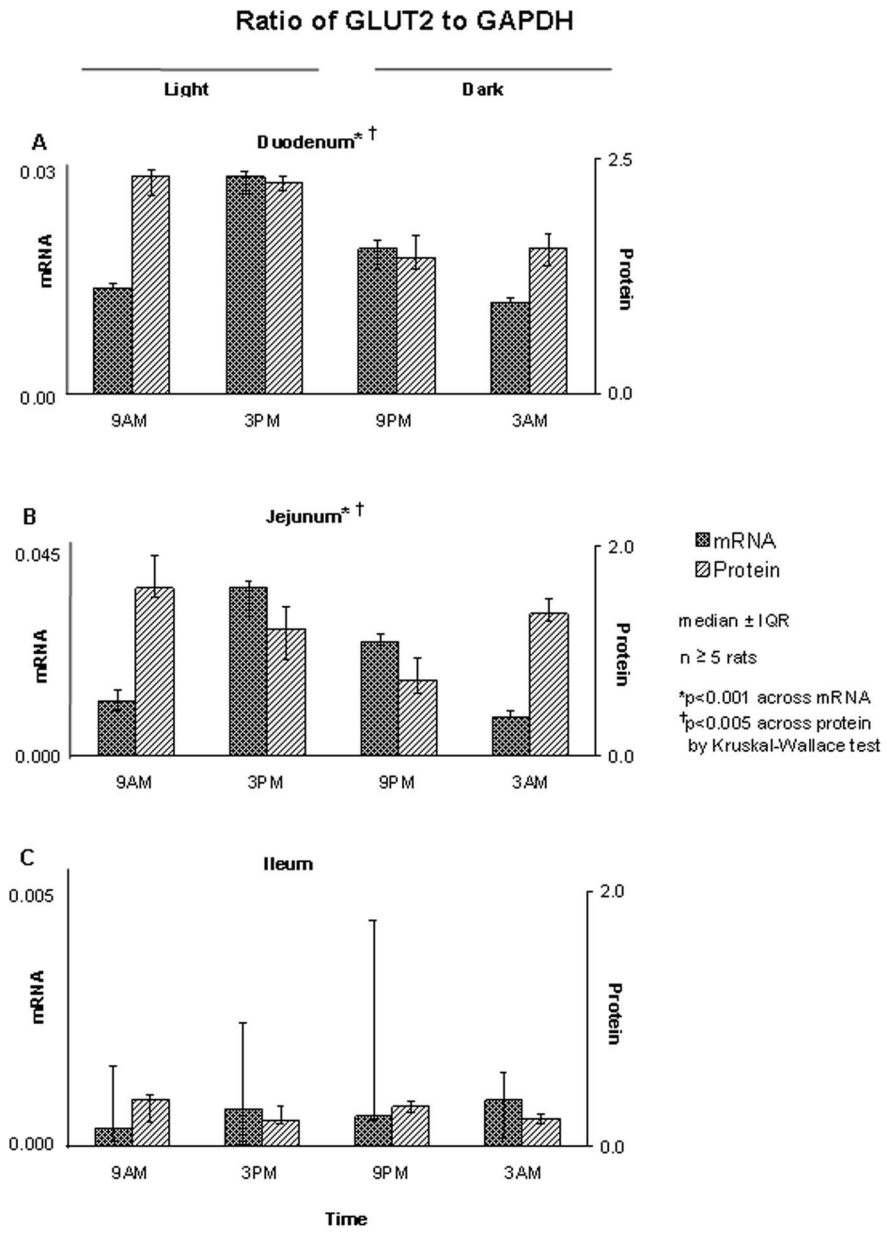


Figure 3.

Diurnal variation of GLUT2 mRNA and protein levels in small bowel mucosa of adult rats expressed as ratios of levels of GLUT2 to GAPDH in arbitrary units. Diurnal rhythm analyzed by Kruskal-Wallace test for non-parametric data. A and B) duodenum and jejunum respectively; GLUT2 mRNA levels peak at 3PM with corresponding levels of protein peaking at 9AM. C) ileum; no variation was seen in GLUT2 mRNA and protein levels.

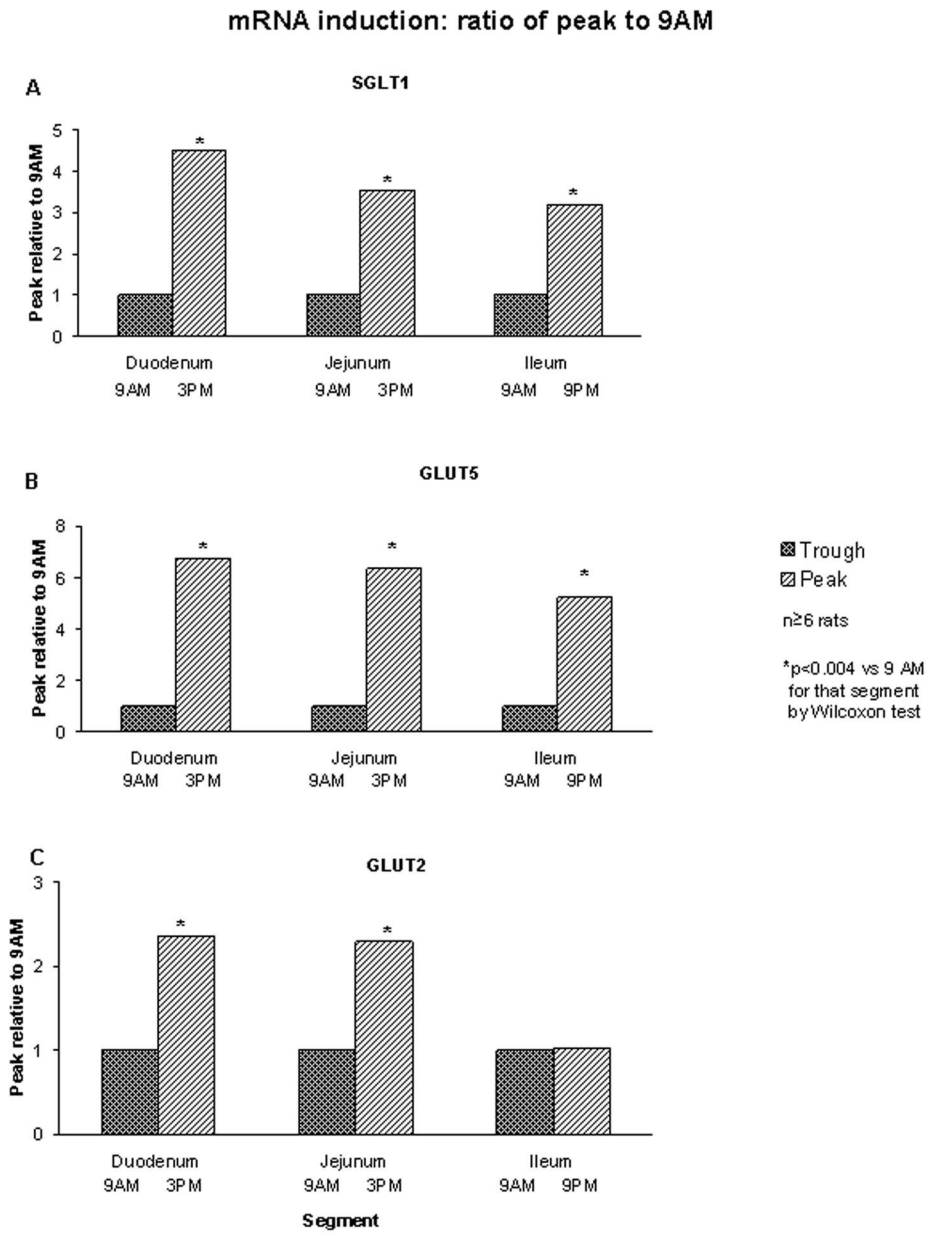


Figure 4.

Hexose transporter mRNA induction in duodenal, jejunal, and ileal mucosa of adult rats. All 36 peak and trough samples were analyzed by reverse transcription real-time PCR simultaneously; trough levels at 9AM are normalized to a value of one. Values are ratios of peak to trough mRNA levels for that segment expressed as fold changes in mRNA levels. A) SGLT1 mRNA induction demonstrating a moderate decrease in aboral induction from 4.5-fold in duodenum to 3.2-fold in ileum. B) GLUT5 mRNA induction demonstrating a decrease in aboral induction from 6.8-fold in duodenum to 5.2-fold in ileum. C) GLUT2 mRNA induction demonstrating equivalent induction in duodenum and jejunum (2.3-fold) but absence of induction in ileum.

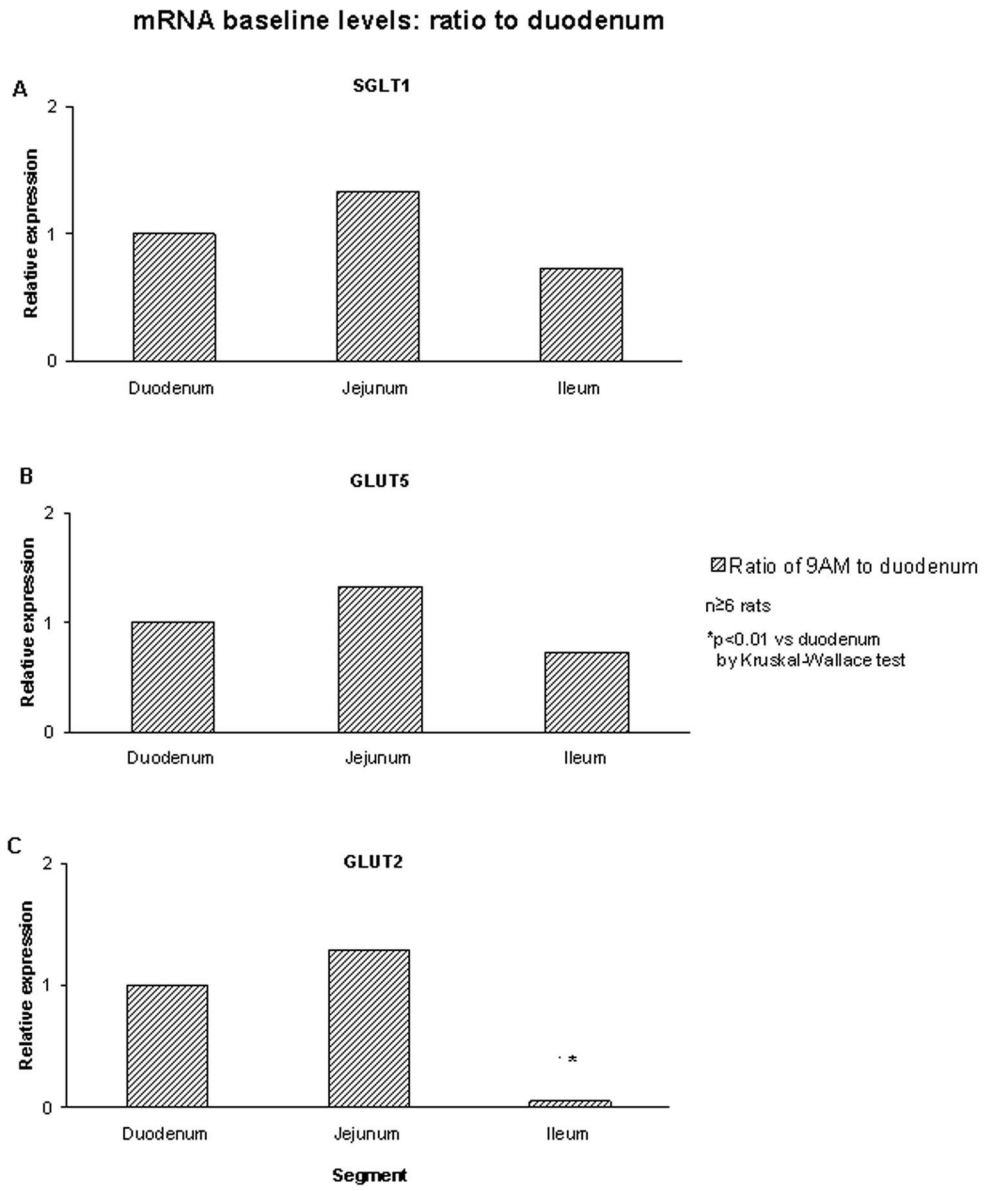


Figure 5.

Baseline mRNA levels of SGLT1, GLUT5, and GLUT2 in small bowel mucosa of adult rats. All 18 baseline samples from trough time points (9AM) underwent reverse transcription real-time PCR analysis simultaneously. Baseline duodenal levels for each transporter are normalized to a value of one and ratios of jejunal and ileal mRNA levels to duodenal levels are expressed as fold changes. A and B) SGLT1 and GLUT5 mRNA levels respectively were similar in all three studied segments. C) GLUT2 mRNA levels were decreased markedly in ileum vs. duodenum and jejunum (21-fold and 28-fold respectively).

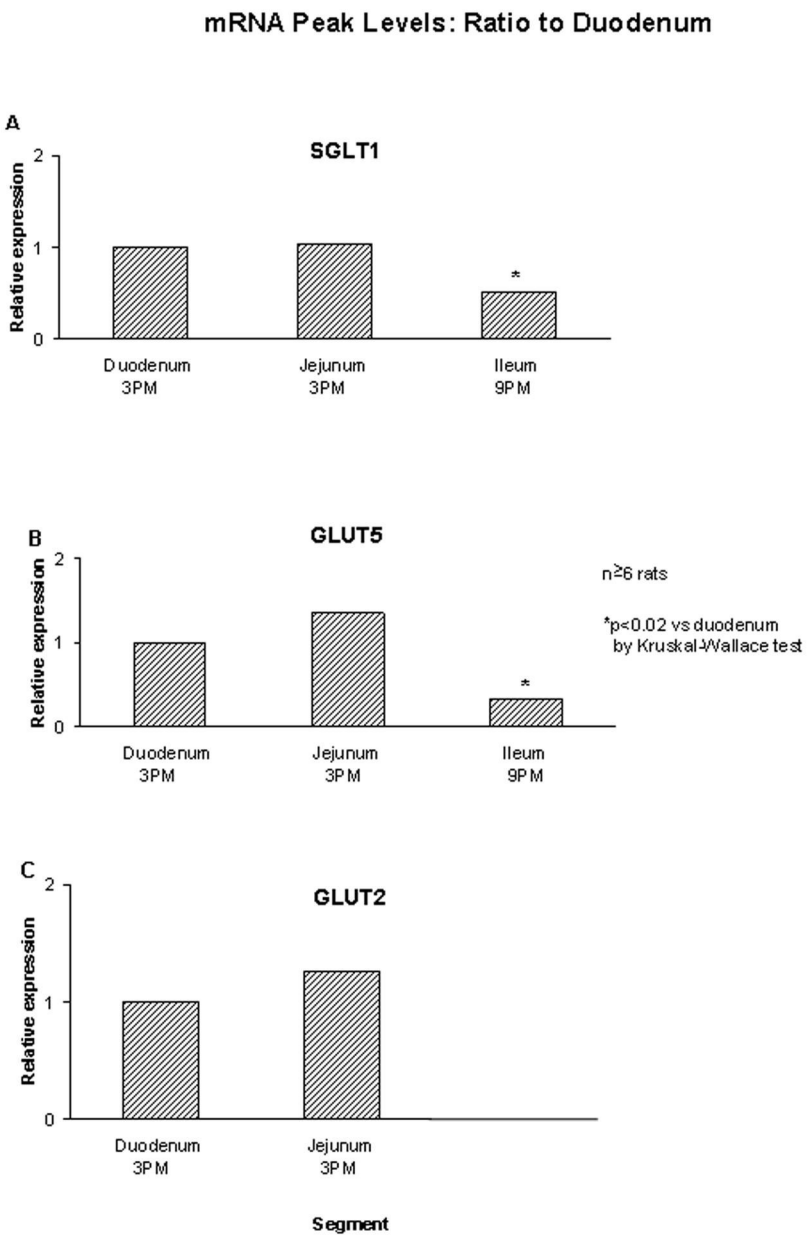


Figure 6.

Peak mRNA levels of SGLT1, GLUT5, and GLUT2 in small intestinal mucosa of adult rats. All 18 samples from peak time points (3PM in duodenum and jejunum, 9PM for ileum) underwent reverse transcription real-time PCR analysis simultaneously. Peak duodenal levels for each transporter are normalized to one, and ratios of jejunal and ileal mRNA levels to duodenal levels for that transporter are expressed as fold changes in mRNA levels. A) Peak SGLT1 mRNA levels are decreased moderately (2-fold) in ileum vs. jejunum. B) Peak GLUT5 levels are decreased in ileum vs. duodenum (3-fold) and jejunum (4.1-fold). C) Peak GLUT2 mRNA levels are similar in duodenum and jejunum, ileal levels were not determined because no diurnal variation was detected.

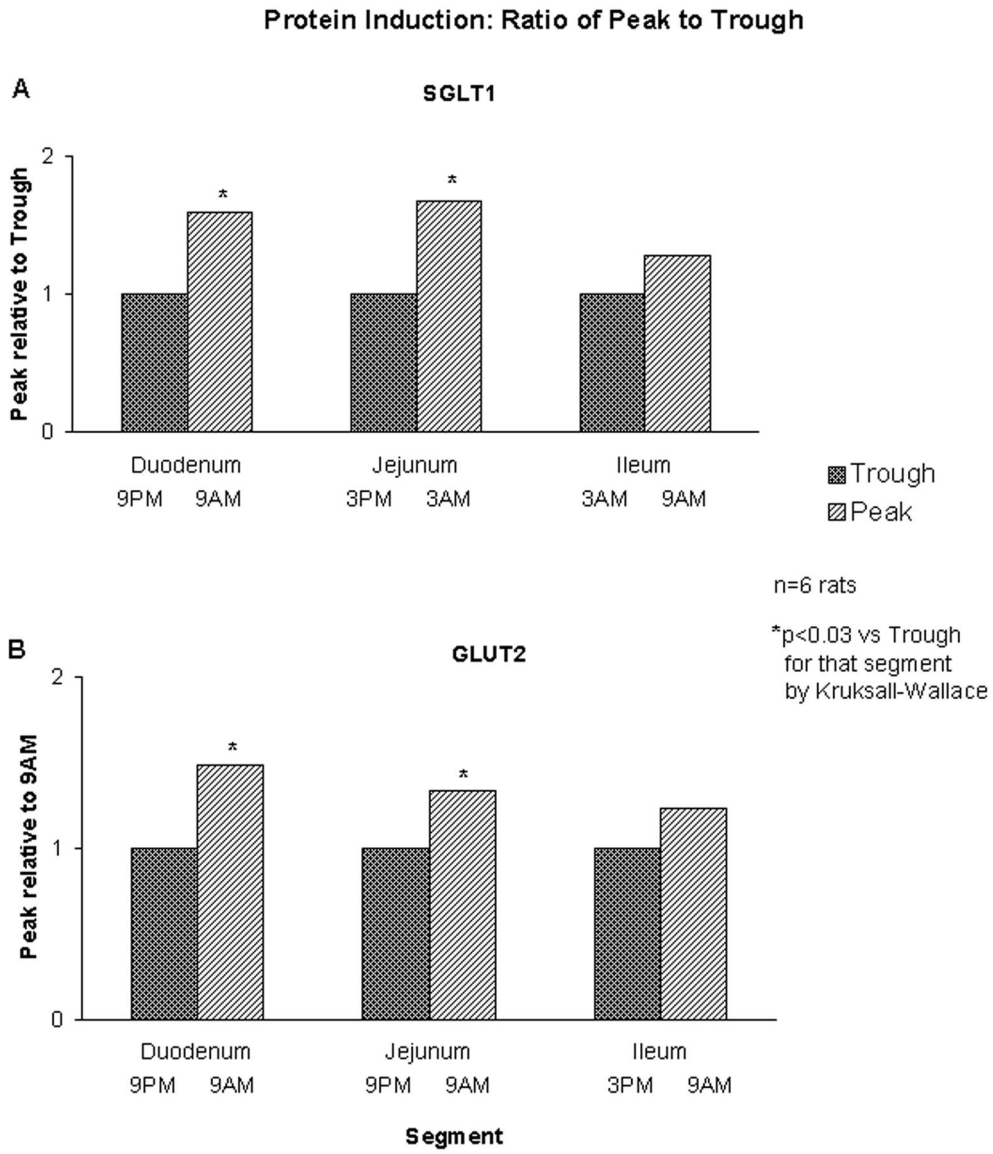


Figure 7.

SGLT1 and GLUT2 protein induction in duodenal, jejunal, and ileal mucosa of adult rats. Peak and trough samples, as determined by diurnal studies, were analyzed by semi-quantitative Western blotting; trough levels are normalized to a value of one. Values are ratios of peak to trough protein levels for that segment expressed as fold changes. A) SGLT1 protein induction demonstrating similar induction in duodenum (1.6-fold) and jejunum (1.7-fold) with absence of induction in ileum. B) GLUT2 protein induction demonstrating 1.6-fold induction in duodenum, 2.2-fold induction in jejunum but absence of induction in ileum.

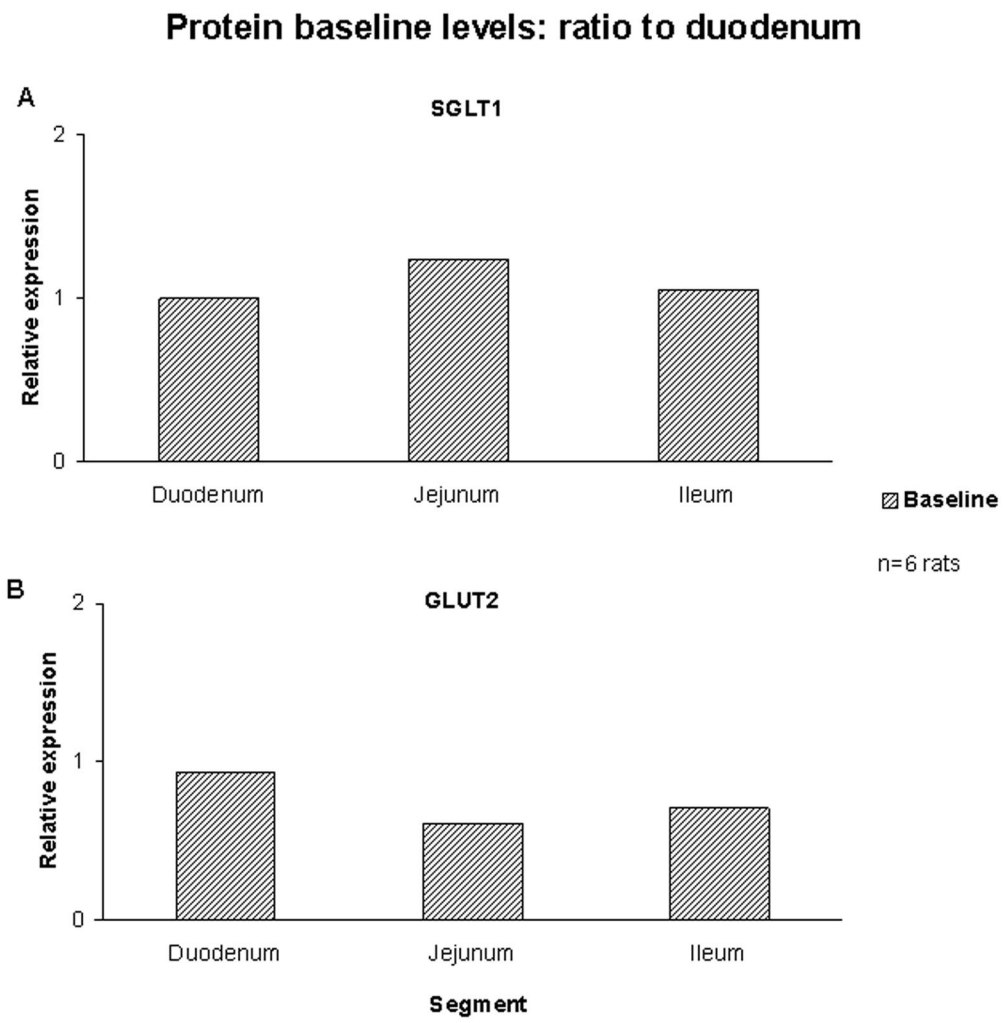


Figure 8.

Baseline protein levels of SGLT1 and GLUT2 in small bowel mucosa of adult rats. Baseline samples from trough time points were analyzed by semi-quantitative Western blotting. Baseline duodenal levels for each transporter are normalized to a value of one and ratios of jejunal and ileal protein levels to duodenal levels are expressed as fold changes. A and B) SGLT1 and GLUT2 protein levels respectively were similar in all three studied segments.

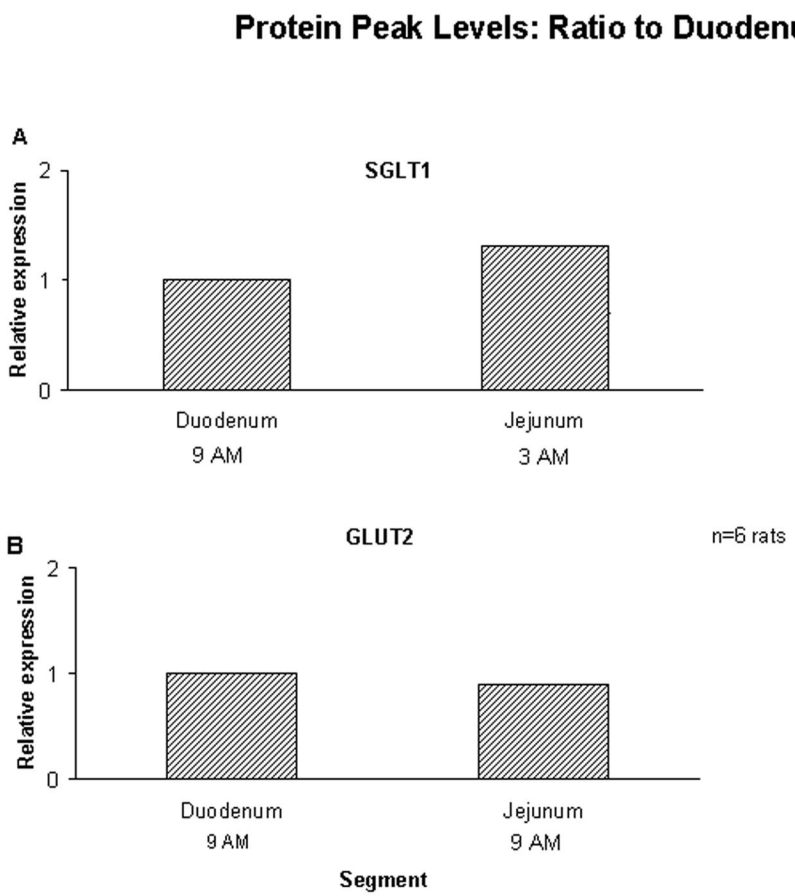


Figure 9.

Peak protein levels of SGLT1 and GLUT2 in small intestinal mucosa of adult rats. Peak time points, as determined by diurnal studies, for duodenum and jejunum were analyzed by semi-quantitative Western blotting. No diurnal rhythm was observed by either transporter in ileum. Peak duodenal levels for each transporter are normalized to one, and ratios of jejunal protein levels to duodenal levels for that transporter are expressed as fold changes in protein levels. A) Peak SGLT1 protein levels are similar in duodenum and jejunum, ileal levels were not determined because no diurnal variation was detected. B) Peak GLUT2 protein levels are similar in duodenum and jejunum, ileal levels were not determined because no diurnal variation was detected.

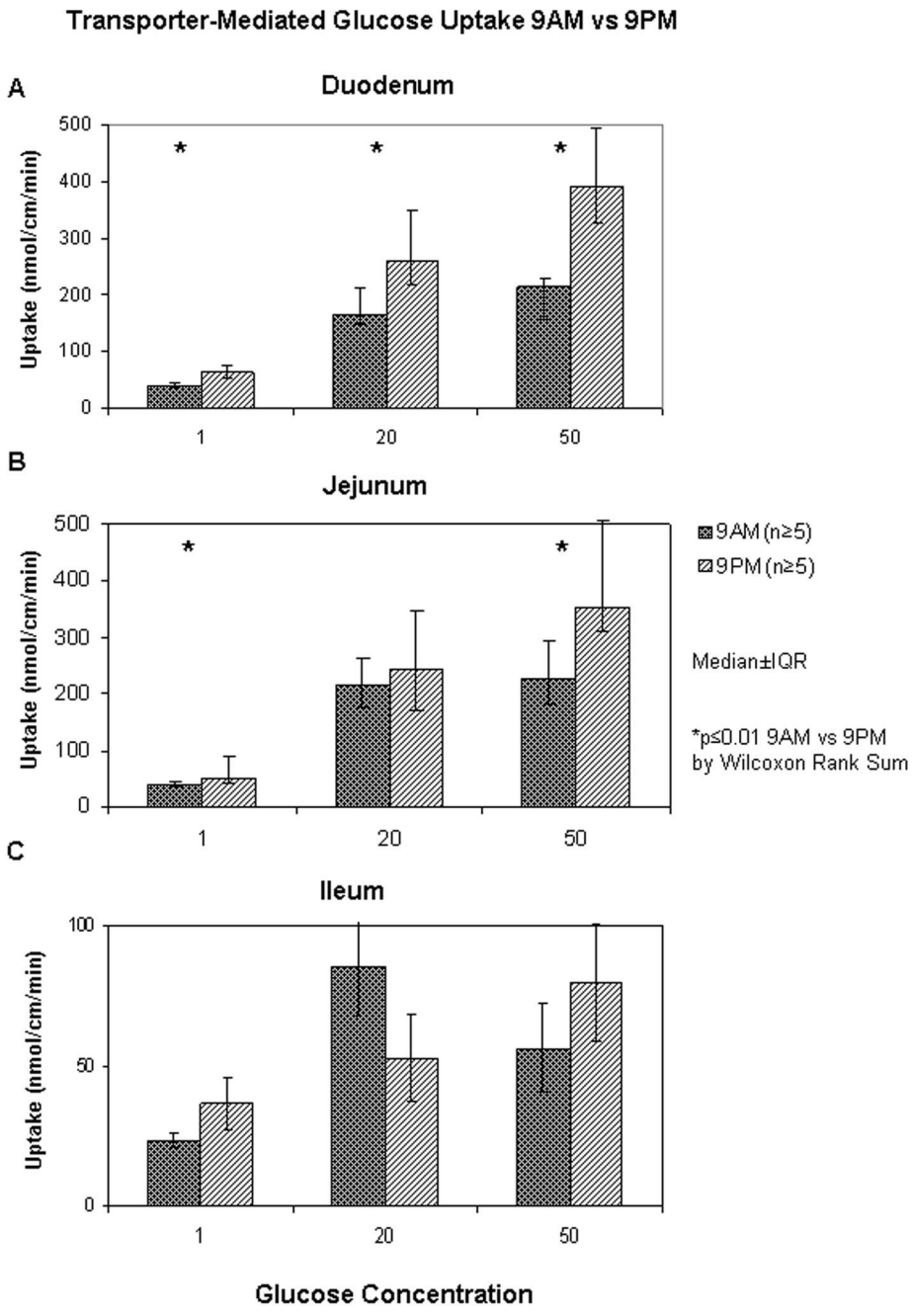
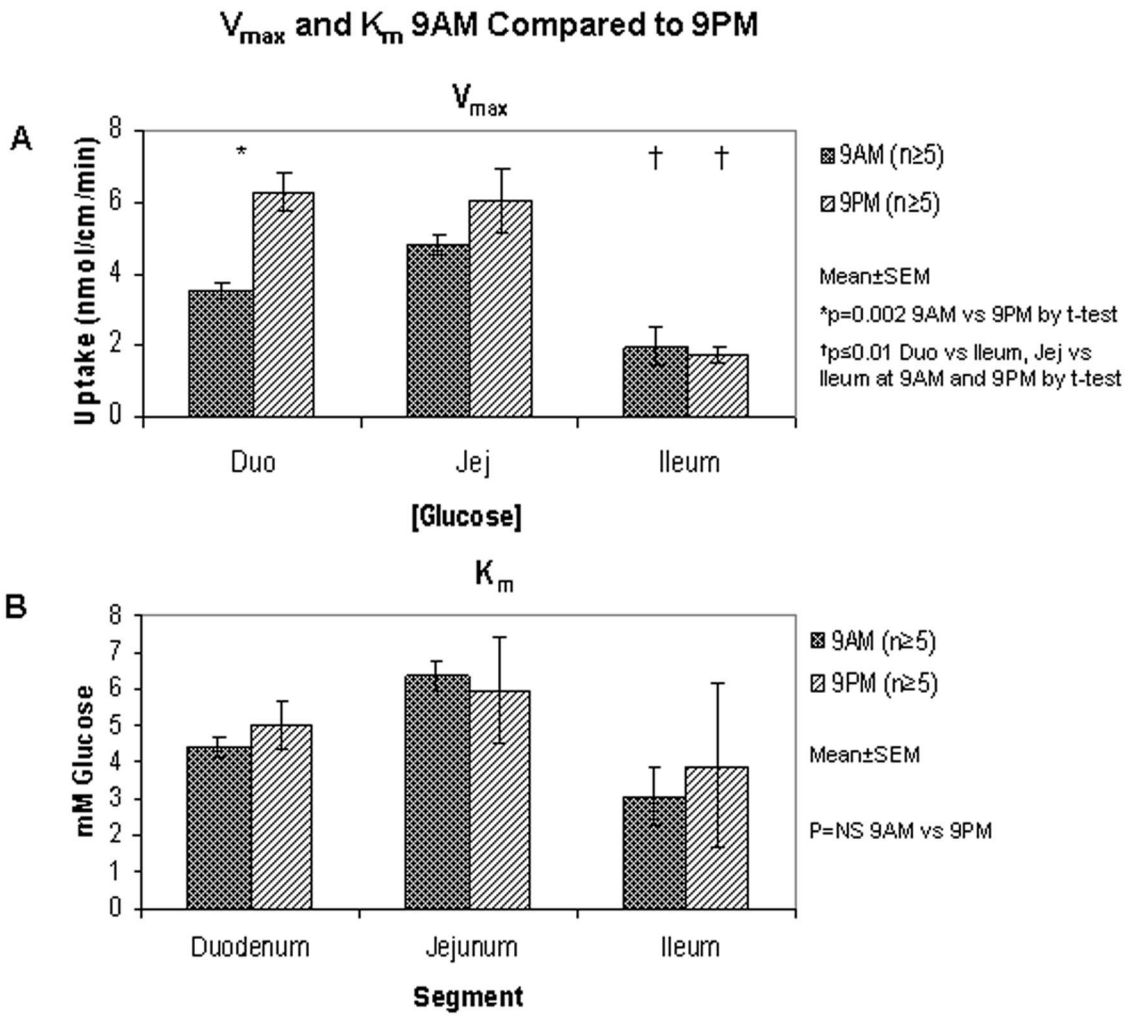


Figure 10.

Transporter-mediated glucose uptake at 9AM and 9PM in small bowel of adult rats. Glucose uptake was measured by the everted sleeve technique in A) duodenum, B) jejunum, and C) ileum. Transporter was greater at 9AM vs 9PM in duodenum and jejunum; no difference was noted in ileum.



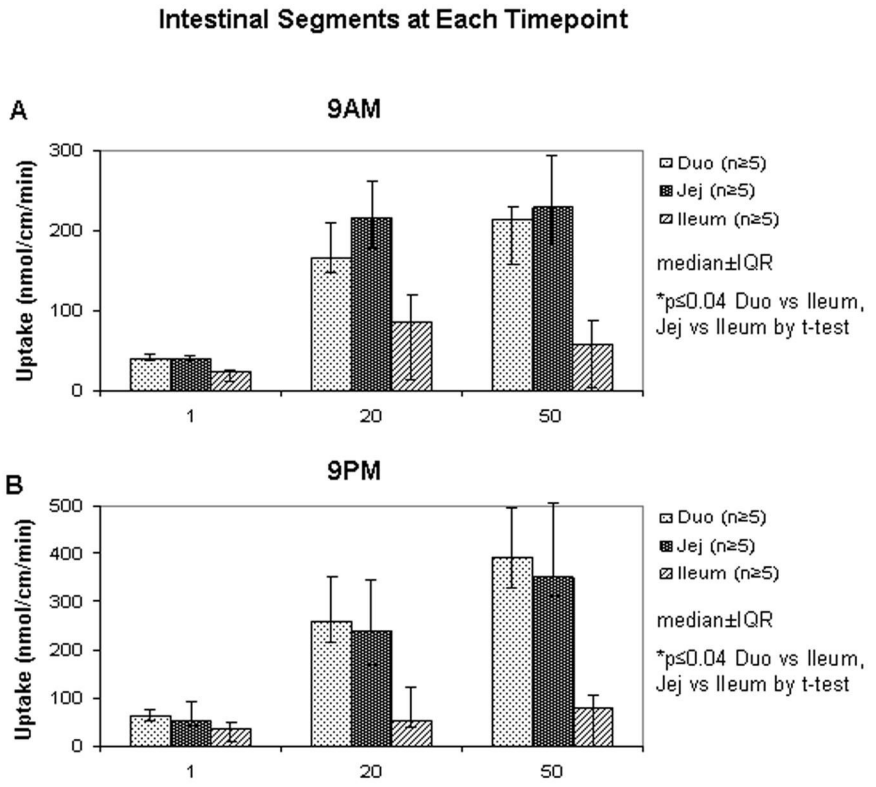


Figure 11.

V_{MAX} and K_M of transporter-mediated glucose uptake in duodenum, jejunum, and ileum from everted sleeve technique using Michealis-Menten Kinetics. A) V_{MAX} levels were greater in duodenum and jejunum than in ileum. B) K_M values did not differ across anatomic segments.