Capsaicin-sensitive vagal afferents modulate posttranscriptional regulation of the rat Na\(^+\)/glucose cotransporter SGLT1

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**THE APICAL na\(^+\)/glucose cotransporter (SGLT1),** localized on the brush-border membrane of small bowel enterocytes, is responsible for all secondary active absorption of glucose from the small bowel (14, 19). Dysregulation of SGLT1 is increasingly recognized in type 2 diabetes mellitus, with expression increasing up to fourfold in both patients and animal models (4, 7–9). Similarly, knockout of the SGLT1 regulatory subunit RS1 in mice leads to both SGLT1 overexpression and severe obesity (27). Therapy aimed at modulating SGLT1 expression may therefore have clinical benefits in diabetes and obesity through slowing of glucose absorption in a manner similar to amylase inhibitors (5) and through increased ileal glucose delivery (38).

SGLT1 expression is regulated by several inputs, including diet composition (11) and the timing of food intake (28). Despite significant progress in characterizing the physiological signaling mechanisms regulating SGLT1 expression, these are still poorly understood. In particular, SGLT1, in common with several other absorptive proteins, exhibits a profound and rapid anticipatory diurnal rhythm to temporally match transporter capacity to nutrient intake (1, 6, 31, 35). However, the pathways controlling this rhythm are largely unknown.

Anticipatory cuing of SGLT1 rhythmicity implies the presence of an entrainment pathway initiated by sensing the temporal pattern of nutrient delivery and culminating in Sglt1 transcription and subsequent translation. The nature of this nutrient signaling pathway remains unclear: candidates for afferent and/or efferent components include peptide hormones (systemic or paracrine), neuronal communication, and luminal sensors. Studies on the role of the vagus nerve have yielded contradictory results (16, 36).

In this study, we sought to clarify the mechanism and effect of vagotomy on SGLT1 expression. In particular, the role of afferent vagal pathways using capsaicin will allow specific testing of the contribution of these fibers. Capsaicin leads to a permanent degeneration of capsaicin-sensitive unmyelinated type C and poorly myelinated vagal afferent fibers, while having no effect on myelinated vagal efferent fibers (3, 21, 33).

**MATERIALS AND METHODS**

**Animal models.** All experimental animal procedures and protocols were prospectively approved by Harvard Medical Area Standing Committee on Animals. Male Sprague-Dawley rats weighing 200–211 g were acquired from Harlan (Indianapolis, IN) and acclimatized for 5 days under a strict 12-h light-dark cycle (lights on at 7 AM, zeitgeber time ZT0) with constant temperature and humidity and ad libitum access to chow and water. Animals were randomized to one of three surgical models: total subdiaphragmatic vagotomy, selective deafferentation of afferent vagal fibers, or sham procedure. Animals were anesthetized with 50 mg/kg ip pentobarbital sodium (Ovation Pharmaceuticals, Deerfield, IL). A 3-cm upper midline laparotomy was performed and the anterior esophagus and gastro-esophageal junction exposed. The lesser sac was opened to expose the posterior junction exposed. The lesser sac was opened to expose the posterior

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PO4, pH 7.4). Four adjacent 5-cm sections of jejunum were excised, ZT21). Rats were anesthetized and a midline laparotomy performed. Harvest time: 10 AM, 4 PM, 10 PM, or 4 AM (ZT3, ZT9, ZT15, or ZT31). All rats were weighed daily at ZT0. After operation, rats were caged in pairs of the same model. Food was restricted to ad libitum food at night only, with chow placed at 7 PM and retrieved at 7 AM. Buprenorphine (0.05 mg/kg sc; Bedford Laboratories, Bedford, OH) was provided for postoperative analgesia for 48 h.

Food consumption. Excess chow of known mass was provided to each cage (containing two rats) at ZT12 (7 PM), and chow remaining at ZTO (7 AM) was removed and weighed. Consumption per rat in any given cage was calculated by halving the mass consumed by both for that day. The mean consumption per rat for each group was then calculated by averaging the values for all rats in that group. Animals were all weighed daily at ZT0.

Tissue harvest. On postoperative day 9, rats were randomized to harvest time: 10 AM, 4 PM, 10 PM, or 4 AM (ZT3, ZT9, ZT15, or ZT21). Rats were anesthetized and a midline laparotomy performed. Jejunum was retrieved from 5 cm distal to the ligament of Trietz and flushed with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, and 10 mM PO4, pH 7.4). Four adjacent 5-cm sections of jejunum were excised, opened longitudinally over ice,blotted to remove debris, and scraped with glass microscope slides to remove the mucosa. Mucosal scrapings were flash frozen in liquid nitrogen and stored at −80°C for later analysis. RNA was extracted from the two proximal sections and protein from the two distal sections.

Confirmation of surgical procedure. Rats were examined clinically and at harvest for gastric distension as evidence of adequate vagotomy. Vagal integrity was assessed in all animals with the use of the neuronal tracer fluorogold (Fluorochrome, Denver, CO) as described by Powley et al. (29). On postoperative day 4, rats were injected with 1 mg fluorogold in 1 ml sterile saline, administered as two 0.5-ml ip injections 1–2 min apart. Following removal of the intestine, the brain was fixed by transaxial perfusion first with 50 ml of ice-cold PBS, then 50 ml of 4% formaldehyde. The brain was removed and placed in formaldehyde for 24 h. A 5-mm block was cut from the brainstem at the level of the dorsal motor nucleus (DMN) of the vagus under a dissecting microscope. The block was embedded in paraffin, and 30-μm sections were cut at 500-μm intervals. Sections were mounted, deparaffinized and coverslipped, and examined with a high-spectrum microscope. Nonspecific fluorogold staining of the area postrema was used as the criterion for adequate tracer injection. Bilateral staining of DMN demonstrated both trunks of the vagus to be intact. Exclusion criteria were absence of the area postrema staining in any animal, any DMN staining for vagotomized animals, and absence of or unilateral DMN staining for the deafferented or sham animals. Assessments were performed by an investigator blinded to the treatment group.

RNA analysis. RNA was extracted from frozen tissue samples with a mirVana mRNA Isolation Kit (Ambion, Austin, TX) and quantified with a microplate reader (Spectramax M5; Molecular Devices, Sunnyvale, CA). Reverse transcription was performed simultaneously on 2.5 μg of RNA from each rat with Superscript III (Invitrogen, Carlsbad, CA).

To facilitate inter- and intragroup comparisons of Sglt1 and Actin expression, quantitative PCR of all cDNA samples were run on a single 384-well plate. RSI mRNA expression was analyzed on a separate plate. The cDNA product was diluted and added to forward and reverse primers (see Table 1), together with SYBR green master mix (Applied Biosystems, Foster City, CA). Quantitative PCR was performed in triplicate with diluted cDNA, primers (Table 1) and SYBR green master mix using an Applied Biosystems ABI 7900HT thermal cycler (2 min, 50°C; 10 min, 95°C; 40 cycles of 15 s, 95°C and 1 min, 60°C). Dissociation curves were obtained to ensure generation of a single, authentic amplicon.

Protein analysis. Whole cell protein extraction was performed on jejunal tissue scrapings. Jejunal mucosa was lysed in 1 ml Trition lysis buffer (Boston Bioproducts, Ashland, MA) containing 10 μl of protease inhibitor mix (Sigma). Samples were homogenized with a polytrom and then sonicated before separation on a centrifuge. Protein was quantified with a bichinonic acid assay (Sigma) with bovine serum albumin as a standard.

Protein samples were prepared with LDS loading buffer and denatured before loading 60 μg of protein on a 15-well gel (10% bis-Tris). A sample from one animal from each time point and each model was run on any individual gel to allow inter- and intragroup comparison. A single reference sample was also loaded on each gel to permit comparisons between gels. Gels were run in MES buffer before transfer to activated PVDF membranes (all above reagents from Invitrogen). After being washed in PBS with 0.05% Tween-20, the membrane was blocked in 10% casein (Vector Laboratories, Burlingame, CA). The membrane was incubated with 1:4,000 rabbit anti-SGLT1 antibody (Chemicon International, Temecula, CA) before being incubated in 1:5,000 dilution of anti-rabbit secondary antibody conjugated to horseradish peroxidase (Vector Laboratories). Antibodies were detected with a standard ECL chemiluminescence kit (Amersham Biosciences, Buckinghamshire, UK) and exposed to Scientific Blue Imaging Film (Eastman Kodak, Rochester, NY). The membrane was then stripped with stripping buffer for 30 min and washed with blocking buffer (Alpha Diagnostics International, San Antonio, TX). The membrane was incubated with 1:500 dilution of mouse antibody to pan-actin (Lab Vision Products, Fremont, CA), followed by 1:500 horseradish peroxidase-labeled anti-mouse antibody (Vector Laboratories). Antibody detection was as above.

Images were digitally acquired from film originals with the use of a Canoscan 4200F scanner (Canon, Lake Success, NY) and ArcSoft Photostudio V5.5 software (ArcSoft, Fremont, CA). Densitometry was performed with Image J software (National Institutes of Health, Bethesda, MD) with band densities obtained for SGLT1 and actin as a loading control. SGLT1 protein expression for each animal was expressed relative to actin and then indexed relative to the SGLT1 expression for the reference sample.

Statistical analysis. Parametric data (weight and intake data, as well as mRNA signal) were analyzed using post hoc ANOVA. These data are reported as means ± SE. We used the Kruskal-Wallis test for analysis of the nonparametric protein data. Protein diurnal rhythm assessment was further confirmed by using a freely available Cosinor Periodogram analysis program (V2.3) to assess circadian periodicity, assuming a circadian period of 24 h and treating individual harvests as different time points.

<table>
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<th>Table 1. Primer sequences</th>
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<tr>
<td>SGLT1 Sense</td>
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<td>SGLT1 Antisense</td>
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<td>RSI Sense</td>
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<td>RSI Antisense</td>
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Primer sequences used for qPCR. SGLT1, sodium-glucose cotransporter 1; RSI, regulatory subunit 1.
a cross-sectional study (30). Statistical analysis was performed using Statistica (StatSoft, Tulsa, OK) or SPSS 16 (SPSS, Chicago, IL) for nonparametric analysis.

RESULTS

Validation of surgical model. Eighty rats survived the postoperative period (29 vagotomized, 25 capsaicin-treated, and 26 sham). One vagotomized rat was euthanized on postoperative day 8 for persistent weight loss.

Fluorogold labeling analysis confirmed adequacy of the surgical techniques (Fig. 1). Nonspecific staining of the area postrema was seen in all animals. Four vagotomized rats had evidence of incomplete vagotomy with unilateral or bilateral staining of the DMN of the vagus. Two rats, one each from the sham and capsaicin-treated groups, had no staining of the DMN. Both rats exhibited gross gastric distension, perhaps due to disruption of the vagus during retrieval of the gauze swab. After exclusion of these six rats, 24–25 rats remained in each group, for 6–7 rats per harvest time.

Vagotomy and deafferentation affects weight gain and food intake. Each group had a similar mean weight at surgery (245 ± 1 g). Sham animals lost ~4.1% weight over the first 3 postoperative days but subsequently recovered to 271 ± 3 g (11% increase). Vagotomized rats lost slightly more weight, dropping to 211 ± 5 g (14% decrease) on postoperative day 4 and only achieved a 1% net gain by harvest. Capsaicin-treated animals gained 2.0 ± 0.9 g (1%) on the first postoperative day. However, they had a net decrease of 3% by postoperative day 3 and then reached 274 ± 2 g by harvest (11% increase). After the initial 24-h excitatory period, capsaicin-treated rats gained less weight than shams (26 ± 2.0 g vs. 33 ± 2.3 g, P = 0.04) between postoperative days 1 and 9 (Fig. 2).

Mean nightly food intake gradually increased in all groups with an apparent plateau the day before harvest (Fig. 3). During the first night postoperatively, capsaicin-treated animals ate significantly more than sham or vagotomized animals. After this, sham and capsaicin-treated animals ate similar quantities, despite lesser weight gain from the capsaicin-treated animals. Sglt1 transcription is independent of vagal innervation. Quantitative PCR analysis for Sglt1, relative to Actin, confirmed the presence of an anticipatory peak in Sglt1 signal at ZT9 for sham animals. This was a 5.3-fold increase from the nadir at ZT21 (Fig. 4; P < 0.0002). This peak in expression was observed in both capsaicin-treated and vagotomized animals (4.6- and 3.8-fold changes, P < 0.002 compared with ZT21). No significant differences were detected between cohorts at any time point (P = 0.09–0.99).

SGLT1 protein translation is dependent on afferent vagal innervation. Immunoblotting demonstrated peak SGLT1 expression at ZT15 in sham animals (Fig. 5). In contrast, no clear diurnal rhythm in SGLT1 protein expression was discernable for either vagotomized animals or deafferented models.

Semiquantitative densitometry was performed and expression of SGLT1 calculated relative to actin (Fig. 6). Expression in each individual animal was indexed to a standard reference sample to obtain an arbitrary scale. This confirmed peak expression of SGLT1 for shams at ZT15, a 2.3-fold peak compared with the nadir at ZT3 and ZT21 (P = 0.041). In contrast, no significant peak at ZT15 was seen for vagotomized or capsaicin-treated animals (P = 0.19 and 0.58, respectively). Rhythm assessment with cosinor periodogram analysis confirmed a significant rhythm in sham animals (P = 0.036) but excluded a significant rhythm with both capsaicin and vagotomized animals (P > 0.1). SGLT1 in vagotomized and capsaicin-treated animals was significantly different from shams at ZT15 only (1.7-fold higher in shams compared with vagotomized animals, P = 0.046). There was no significant difference between vagotomized and capsaicin-treated animals at this time point (P = 0.92) or any other time.

Vagal influences on protein translation are independent of regulatory subunit RS1 transcription. As regulatory subunit RS1 protein has been shown to influence SGLT1 protein transcription and translation, we examined RS1 mRNA signal across diurnal times for each model (Table 2). At least at mRNA level, RS1 expression was constitutive without a detectable diurnal rhythm. This result suggests that RS1 is not diurnally regulated. Furthermore, RS1 mRNA levels did not differ significantly among the three treatment groups (P = 0.22–0.99).

DISCUSSION

SGLT1 expression is regulated by multiple inputs, including diet composition and the timing of consumption (11, 28). To ascertain the role of vagal innervation on the diurnal rhythmicity of SGLT1 expression, we examined the effects of selective deafferentation vs. total vagotomy, both validated by the flu-
orogold tracer technique. Elimination of SGLT1 protein rhythmicity by both total vagal disruption and afferent ablation suggests that the afferent pathway is specifically required for protein rhythmicity and demonstrates that changes in SGLT1 protein expression after vagotomy are independent of any changes in gastric emptying or intestinal motility. Moreover, we have used whole cell protein extracts, showing that total SGLT1 protein synthesis was affected, not just a single cell compartment such as the brush-border membrane. Although functional glucose uptake was not examined in this study, a direct association between SGLT1 protein expression and function has repeatedly been shown, using both everted sleeves (1, 15) and electrophysiological methods (35).

As expected from our previous study examining total vagotomy (36), rhythmicity of Sglt1 mRNA persisted following selective vagal afferent ablation. The present study provides further support for a nonvagal pathway, e.g., hormonal or luminal, linking Sglt1 transcriptional patterns to food intake rhythms. However, vagal deafferentation (as well as total vagotomy) prevented the normal diurnal increase in SGLT1 protein. This induced discordance between SGLT1 transcription and translation suggest that these processes are regulated independently to some degree and that SGLT1 expression can be regulated at multiple levels. This discordance may be due either to reduced translation or a higher turnover of protein. Future work using in vivo labeling of SGLT1 may permit distinction between these mechanisms. Of interest, the “basal” or fasting level of SGLT1 protein is maintained; only “peak” or feeding levels of SGLT1 are affected by deafferentation or vagotomy.

The role of the vagus on glucose homeostasis is predominantly through vagal afferent mediation of stimuli. Deafferentation has been shown to improve oral glucose tolerance tests in diabetic rats (13), though surprisingly has little influence on intravenous glucose challenges (10). This supports the effect of deafferentation on glucose metabolism being mediated at an intestinal or portal level.

At a molecular level, vagal regulation of jejunal transporters remains poorly understood. Our present study confirms a previous report suggesting that vagotomy reduces peak diurnal SGLT1 protein expression (as well as facilitated glucose transporter GLUT2 and GLUT5 protein expression) through post-translational mechanisms (16). This differs from a previous report from our laboratory showing no change on SGLT1 expression after vagotomy (36), although notably in this latter

Fig. 2. Weight changes after surgery. Capsaicin-treated animals weighed significantly more than shams on the first and second postoperative day (P < 0.001 and 0.039, respectively). Thereafter, this significant difference between shams and deafferented animals was lost (P = 0.1–0.64). Vagotomized animals weighed significantly less than shams from the second postoperative day onwards (P < 0.001).

Fig. 3. Mean daily food intake per animal over the postoperative time course (postoperative night one is the night immediately after the operation). There was no statistically significant difference between capsaicin-treated and sham animals after the first postoperative night (P = 0.10–0.89). Vagotomized animals ate less than sham animals throughout the postoperative course (P < 0.001). Data for postoperative night 10 are not shown because harvests were at differing times.

Fig. 4. Quantitative PCR data demonstrates a persisting diurnal rhythmicity in Sglt1 signal for all 3 animal cohorts. The anticipatory peak in signal occurs at zeitgeber time (ZT9 (4 PM) with a 5.3-fold peak compared with ZT21 (P < 0.001 compared with ZT3 and ZT21).

Fig. 5. Representative Western immunoblot images showing clear diurnal rhythmicity in sham animals, with a peak at ZT15 (10 PM). No clear peak is seen for vagotomized (VAG) or deafferented animals (CAP). SGLT1, sodium-glucose cotransporter.
study we only examined daylight time points and did not confirm successful vagotomy in our animals by the fluorogold tracer technique. The role of vagal afferents in the regulation of intestinal transporter expression is even less well studied. Studies have shown that intraluminal capsaicin infusion (which is acutely excitatory on vagal afferents) in the short term suppresses alanine transport and expression of the PepT1 peptide transporter (2, 18). In contrast, vagal deafferentation leads to upregulation of alanine transport (25). Functional studies (using brush-border membrane vesicle uptake of glucose) have demonstrated that vagal deafferentation prevents upregulation of glucose transport after switching to a high-carbohydrate diet in guinea pigs (3). It seems, therefore, that vagal afferent signaling may lead to the preferential uptake of glucose at the expense of peptide transport.

We hypothesize that vagal afferents may be detecting nutrient delivery, for example, through activation of SGLT1 or SGLT3 (12, 17), intestinal sweet taste receptors such as T1R3 (23), or portal/hepatic glucose receptors (26, 32). An output pathway then leads to upregulation of SGLT protein synthesis. This may provide an explanation for the rapid resolution in diabetes observed in obese patients after Roux-en-Y gastric bypass (RYGBP), as vagal afferent nutrient signaling is likely to be impeded by duodenal and proximal jejunal bypass. The consequent reduction in SGLT1 activity would reduce portal ent delivery, for example, through activation of SGLT1 or GLUT2 transporter protein expression, whereas vagotomy appears to dissociate GLUT2 protein expression from mRNA signal in a similar manner to SGLT1 (16), again suggestive that the effects of vagotomy and deafferentation are independent of RS1.

There are three aspects of the experimental design that merit comment. Importantly, we imposed a feeding schedule on the animals. Vagotomy leads to a loss in the normal nocturnal feeding patterns of rats, with animals consuming food with a nearly continuous pattern (22, 34). We therefore imposed a feeding rhythm to ensure comparability between arms. This study therefore supports the similar findings by Houghton et al., showing that the loss of SGLT1 protein rhythm after vagotomy was not due to changes in feeding patterns. Furthermore, we used an objective method to confirm adequate vagotomy. As our study demonstrates, despite best efforts, in 14% of attempts at vagotomy, there was incomplete surgical division of the nerve. In particular, we found the posterior trunk of the vagus can be difficult to identify intraoperatively. Fluorogold tracer was identified in the DMN to a similar extent in both capsaicin- and sham-treated animals, supporting other reports showing that capsaicin at the dose or higher is selective for the vagal afferents (3). Further support for this is seen by the lack of gastric distension in capsaicin-treated animals. We ensured selectivity of capsaicin for vagal afferents by anatomical isolation with parafilm to prevent deafferentation of the spinal afferents; however, spinal afferent disruption cannot be excluded as contributing to the results observed. Last, disruption due to topical application of capsaicin implies the pathway is due to ascending signals through the long vagal afferents. However, many capsaicin-sensitive vagal afferents coursing through the vagal trunks will be affected and subsequently degenerate (20), including specialized terminal processes such as intramuscular arrays. It is possible, therefore, that the effects observed are occurring at an intramural level through branches off the vagal afferents, without passing through a central level.

Summary. In summary, this study has shown that anticipatory transcriptional control of Slgt1 is independent of the vagus. However, the temporal matching of SGLT1 protein to diurnal patterns in nutrient intake is dependent on the vagus nerve, specifically requiring the presence of an afferent component. This is likely independent of the regulatory subunit RS1 pathway. Disruption of afferent signals may contribute to the improvement in diabetes after proximal small bowel bypass in RYGBP surgery.

Table 2. Relative mRNA signal for RS1

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<tr>
<th></th>
<th>ZT3</th>
<th>ZT9</th>
<th>ZT15</th>
<th>ZT21</th>
<th>P (ANOVA)</th>
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<tr>
<td>Sham</td>
<td>2.3±0.5</td>
<td>1.9±0.8</td>
<td>2.2±0.4</td>
<td>2.3±0.6</td>
<td>0.46–1.00</td>
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<td>Vagotomy</td>
<td>1.6±0.2</td>
<td>1.7±0.3</td>
<td>2.5±1.1</td>
<td>2.6±0.3</td>
<td>0.43–1.00</td>
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<tr>
<td>Capsaicin</td>
<td>2.5±0.3</td>
<td>2.0±0.4</td>
<td>2.4±0.4</td>
<td>1.9±0.4</td>
<td>0.73–1.00</td>
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Values are means ± SE. RS1 mRNA expression relative to Actin, showing no significant change either on a diurnal basis or as a consequence of vagotomy or deafferentation. The range of P values presented are for ANOVA comparison between time points. ZT, zeitgeber time.

Fig. 6. Semiquantitative densitometry on Western immunoblotting images showing expression of SGLT1 relative to actin and indexed to a single control animal. Data points show means ± SE. Sham animals show a peak in protein level at ZT15 (2.3-fold change compared with ZT3 and ZT21, *P < 0.05). This peak is absent for both vagotomized and capsaicin-treated animals.
VAGAL AFFERENTS REGULATE EXPRESSION OF INTESTINAL SGLT1

GRANTS
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REFERENCES