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Absence of Evidence of Translocation of GLUT2 to the Apical Membrane of Enterocytes in Everted Intestinal Sleeves

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Abstract

INTRODUCTION—Traditional models of intestinal glucose absorption confine GLUT2 to the basolateral membrane. Evidence suggests that GLUT2 is translocated to the apical membrane when the enterocyte is exposed to high luminal glucose concentrations.

HYPOTHESIS—GLUT2 translocates to the apical membrane by a PKC signaling mechanism dependent on activity of SGLT1 and the cellular cytostructure.

METHODS—Transporter-mediated glucose uptake was studied in rat jejunum using everted sleeves under 7 conditions: Control, SGLT1 inhibition (phlorizin), GLUT2 inhibition (phloretin), both SGLT1 and GLUT2 inhibition, PKC inhibition (calphostin C or chelerythrine), and disruption of cellular cytostructure (nocodazole). Each condition was tested in iso-osmotic solutions of 1, 20, or 50 mM glucose for 1 or 5 min incubations (n=6 rats each).

RESULTS—Control rats exhibited a saturable pattern of uptake at both durations of incubation. Phlorizin ($p \leq 0.006$ each) inhibited markedly and phloretin ($p \leq 0.01$ each) inhibited partially glucose uptake in all concentrations and time. Phloretin and phlorizin together completely inhibited uptake ($p = 0.004$ each). Calphostin C, chelerythrine, and nocodazole had little effect on glucose uptake at either 1 or 5 min.

SUMMARY—Inhibition of SGLT1 led to near complete cessation of transporter-mediated glucose uptake, while GLUT2 inhibition led to partial inhibition, suggesting some constitutive expression of GLUT2 in the apical membrane. Disruption of PKC signaling or cytoskeletal integrity partially inhibited transporter-mediated glucose uptake only in 1 mM glucose, suggesting a non-specific effect.

CONCLUSIONS—Under these conditions, it does not appear that GLUT2 is translocated to the apical membrane on the cellular cytostructure in response to PKC signaling.

Keywords

SGLT1; GLUT2; hexose transport; glucose uptake; intestinal absorption; protein kinase C

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INTRODUCTION

The traditional model of glucose absorption from the brush border of the intestine has attributed the majority, if not all, of transporter-mediated glucose uptake, from the lumen into the enterocyte, to the secondary active glucose transporter, sodium glucose co-transporter 1 (SGLT1) (1–4). In this model, glucose transport into the enterocyte is driven by a basolateral, sodium-potassium ATPase pump which maintains a sodium gradient; this gradient allows SGLT1 to transport glucose actively along with sodium into the cell, after which glucose is either metabolized or exits the cell into the portal venous system via the facilitated transporter, glucose transporter 2 (GLUT2) which has been thought to be present only in the basolateral membrane of the enterocyte. *In vivo* rat studies, however, have demonstrated a very rapid, progressive, increase in glucose uptake at substrate concentrations far exceeding concentrations of glucose that fully saturate the SGLT1 transport system; thus, the idea that SGLT1 is responsible for all glucose uptake does not fit with experimental findings (5–8).

Two prominent theories have been proposed to explain this accessory pathway for glucose uptake (9–15). The first proposal (1983) to explain this phenomenon in intestinal glucose uptake has been termed “solvent drag” (9,12–15). Investigators supporting this theory have demonstrated what appears to be opening of tight junctions between enterocytes mediated either by activation of SGLT1 or by the increases in luminal solute (glucose) concentrations, which, coupled with large water and solute shifts, leads to an augmented influx of glucose into the paracellular space where it is transported into the cell along a concentration gradient by a facilitated transporter, GLUT2 (14).

The second theory, proposed more recently by Kellett and others (7,16–19), suggests that GLUT2, a facilitated transporter, is translocated rapidly to the apical membrane from pre-formed, intracellular stores of GLUT2 via a signaling mechanism initiated by activation of SGLT1 through protein kinase C (PKC); the increase in the brush border membrane of a transporter (GLUT2) with a lesser affinity but a greater transport capacity would explain this rapid increase in uptake of glucose at luminal concentrations of glucose much greater than the K_m of SGLT1. Additional work suggests the presence of immunoreactive GLUT2 in the brush border membrane of enterocytes (20). In our own work, we have observed variations in K_m along the intestinal tract; this observation suggests the presence of more than one transporter (21–23). Additional studies using phlorizin, a competitive inhibitor of SGLT1, exhibited a dramatic decrease in transporter-mediated glucose uptake but failed to achieve complete inhibition of transport, despite increasing doses of phlorizin (0.05–0.2 mM), which further suggests the presence of an additional glucose transporter in the brush border membrane (23). Additionally, in a cell culture model using several different intestinal epithelial cell lines, we have observed a rapid augmentation of glucose uptake, which is blunted by GLUT2 inhibition when the cells are exposed to high concentrations of glucose (24).

In this current study, we hypothesized that activation of SGLT1 leads to translocation of pre-formed GLUT2 to the apical membrane through a PKC-mediated signaling pathway via the cytoskeletal, microtubular architecture, where GLUT2 then functions to augment apical uptake of glucose.

METHODS

After approval by our Institutional Animal Care and Use Committee and in accordance with the guidelines of the National Institutes of Health for the humane use and care of laboratory animals, we studied transporter-mediated glucose uptake in jejunal segments of 250–300 g

Lewis rats (Harlan, Indianapolis, IN) between 8:00 am and 10:00 am. Adherence to this schedule was undertaken to avoid the known, diurnal patterns in hexose transporter expression and function which would confound our results (3,4,25,26). Transporter-mediated glucose uptake was measured using a modification of the everted sleeve technique as described previously (23,27).

In brief, normal, nondiabetic Lewis rats were anesthetized using 2% inhaled isoflurane for induction followed by intraperitoneal injection of 50 mg/kg sodium pentobarbital. Rats were then subjected to a mid-ventral celiotomy, and intestinal segments from the proximal jejunum (5 cm from the ligament of Treitz) were flushed, everted, mounted, and secured rapidly to metal rods with 5-0 silk ties and kept in chilled (4°C), oxygenated (95% O₂/5%CO₂) mammalian Ringer's 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). When ready for study, the sleeves were preincubated in oxygenated, glucose-free, mammalian Ringer's solution at 38°C for 5 min. Because our preliminary work in cell culture (24) suggested that transport of glucose was augmented by a GLUT2-dependent mechanism at glucose concentrations $\geq$ 20 mM and in vivo studies suggested that augmented jejunal absorption occurred only at greater concentrations of luminal glucose, transport studies were conducted by placing the sleeves in a flat-bottom test tube with 8 ml of mammalian Ringer's solution which had iso-osmotic replacement of NaCl with 1, 20 or 50 mM d-glucose. Because our cell culture work (24) showed that the augmentation of glucose absorption took $\geq$ 2 min, sleeves were incubated for either a short duration (1 min) or a greater duration (5 min) at 38°C with a stir-bar at the bottom of the test tube spun at 1,200 rpm to promote mixing of the test solution and to ameliorate any effects of the "unstirred layer". After incubation, sleeves were rinsed immediately with chilled (4°C), glucose-free Ringer's solution for 20 s to stop further transporter-mediated uptake and placed in a glass, liquid scintillation, counting vials with 0.5 ml of tissue solubilizer (Solvable; Perkin-Elmer, Boston, MA) and incubated at 50°C overnight.

Radiolabeled stereospecific glucose in the incubation solution was used to measure transporter-mediated and non-transporter-mediated glucose uptake. ¹⁴C-d-glucose (1.0 uCi/8ml) was used as a marker for transporter-mediated glucose uptake; ³H-l-glucose (a glucose enantiomer that is not transported by the stereospecific glucose transporters; 1.8 uCi/8ml) was used to account and correct for both passive glucose diffusion and glucose adherent to the mucosal surface. Once the tissues were solubilized, 15 ml of counting cocktail (Opti-Flour, Perkin-Elmer, Shelton, CT) was added to the glass vial, and counts were obtained using dual isotope, liquid scintillation counting on a Beckman LS6000SC counter (Fullerton, CA). Transporter mediated glucose uptake was calculated as total glucose uptake (total uptake [¹⁴C-glucose] minus adherence and passive uptake [³H-glucose]) for each sample and expressed in nmol·cm⁻¹ per duration of incubation.

Transporter-mediated glucose uptake in normal rat jejunum was studied under six conditions: control, SGLT1 inhibition, GLUT2 inhibition, combined SGLT1 and GLUT2 inhibition, PKC inhibition, and disruption of intracellular microtubular structure (n=6 rats each); specific antagonists were added directly to the preincubation and incubation solutions. Using doses proven effective previously (28–34), phlorizin (0.2 mM) was used to inhibit SGLT1, phloretin (1 mM) was used to inhibit GLUT2, calphostin C (50 nM) was used to inhibit PKC activity, and nocodazole (10 μ M) was used to inhibit intracellular translocation. Interim review of our results after jejunal sleeves had been tested in 1 and 20 mM glucose for 1 min incubation lead to the following changes in our experimental protocol: the concentration of calphostin C was increased to 500 nM and an additional PKC inhibitor (chelerythrine 50 μ M) was tested separately (35,36). These changes applied to all

sleeves tested in 50 mM glucose for 1 min and for all glucose concentrations (1, 20, & 50 mM) in which everted sleeves were incubated for 5 min.

Phlorizin, phloretin, calphostin C, and chelerythrine were solubilized in ethanol with 100- μ l aliquots used to obtain the above concentrations. Vehicle control studies with 100 μ l of ethanol alone were conducted and did not show any difference in transporter-mediated glucose uptake (data not shown). Calphostin C was protected from light until it was added to the preincubation and incubation solutions. Nocodazole required solubilization in dimethyl sulfoxide (DMSO). Vehicle studies with 100 μ l of DMSO in the incubation solution showed a significant decrease in transporter-mediated glucose uptake; however, 1 μ l of DMSO had no effect on transporter-mediated glucose uptake (data not shown). Therefore, nocodazole was solubilized in DMSO and 1- μ l aliquots were added to the preincubation and incubation solutions to obtain the above concentration. All agents were added to preincubation and incubation solutions at least 5 min before the sleeves were exposed to the solutions.

Nonlinear regression of transporter-mediated uptake at 1, 20, and 50 mM glucose was used to calculate K_m values of the Michaelis-Menton kinetic equation (GraphPad Prism version 4.03).

Statistical Analysis

Statistical analysis was performed using the Kruskal-Wallis test to compare nonparametric data between groups, while the Wilcoxon rank sums test was used to compare directly the non-parametric datasets. P values were corrected according to the Bonferroni method, and a corrected p value of ≤ 0.05 was considered significant. All data are reported as the mean values $\pm$ standard error of the mean unless specified otherwise; n values are number of rats.

RESULTS

When the everted jejunal sleeves were exposed to increasing concentrations of luminal glucose for 1 minute, transporter-mediated uptake displayed a characteristic, saturable, single transporter pattern (figure 1A-control). Transporter-mediated uptake of glucose was increased from the uptake at 1 mM glucose in the 20 mM experiments, but there was no further increase at 50 mM glucose. The calculated K_m was 4.0 ± 2.4 which correlates with the K_m reported previously for SGLT1.

We then measured transporter-mediated uptake of glucose at these same concentrations of glucose (1, 20, and 50 mM) but for 5 minutes exposure. We were expecting to see an augmentation of transporter-mediated glucose uptake at the 50 mM glucose exposure that would be much greater than the uptake at 20 mM glucose suggesting recruitment of another transporter different from SGLT1. We found that the pattern of transporter-mediated uptake, however, again resembled that of a saturable, single transporter system without a marked increase in transporter-mediated uptake at 50 mM glucose as we had expected, and the K_m was 8.9 ± 7.6 , again consistent with SGLT1 as the transporter and not different statistically to the K_m for transporter-mediated transport at the 1 minute duration of exposure.

Inhibition of SGLT1 and/or GLUT2

When the jejunal everted sleeves were exposed to glucose concentrations of 1, 20, and 50 mM for the short, 1 min duration, addition of the SGLT1 inhibitor, phlorizin resulted in marked inhibition of transporter-mediated glucose uptake at all glucose concentrations (figure 1A). Treatment with phloretin, the GLUT2 inhibitor, also decreased transporter-mediated glucose uptake for the 1 minute duration by approximately 50%. The combination of both phlorizin and phloretin further decreased glucose uptake and inhibited transporter-mediated uptake almost completely.

When the duration of incubation was increased to 5 minutes (figure 1B), very similar effects were seen. Phlorizin and phloretin alone each inhibited glucose uptake at all concentrations of glucose, and the combination of both inhibitors lead to a further decrease in glucose uptake.

Inhibition of mechanisms of intracellular GLUT2 translocation

Two inhibitors of PKC activity, calphostin C and chelerythrine, were used to inhibit PKC activity in attempt to disrupt potential intracellular translocation of GLUT2 to the apical membrane of the enterocytes. When evaluated at either 1 min (figure 1A) or 5 min (figure 1B) of exposure to glucose at the lesser concentrations of glucose (1 and 20 mM), as well as at the greatest concentration (50 mM), no consistent effect on suppressing glucose uptake was seen with either concentration of calphostin C or with chelerythrine. Similarly, when nocodazole was used to disrupt the cellular cytostructure within the enterocyte by depolymerizing the microtubules, no consistent effect was noted on glucose uptake at either the lesser or greater durations of exposure to either the lesser or greater concentrations of glucose.

DISCUSSION

Formerly, the presence of GLUT2 in the apical membrane was associated with diabetes and was presumed to be a pathologic event in response to increasing blood concentrations of glucose (37). Currently, some groups still dismiss the theory of a rapid, reversible translocation of GLUT2 to the apical membrane, citing previous work that demonstrated GLUT2 only in the basolateral membrane using immunohistochemical techniques targeting the C-terminal of GLUT2 (38–40). More recent data, however, using immunohistochemical assays targeting the extracellular loop of GLUT2, have demonstrated its presence at both the basolateral and brush border apical membranes of the enterocyte (16,20). Additional studies have shown that activities of the membrane-bound transporter SGLT1 and intracellular enzyme PKC appear to be integral, inter-related initiators of an intracellular signaling pathway that appears to regulate the translocation of GLUT2 into the apical membrane; this pathway establishes the rapid increase in transporter-mediated uptake of glucose when the intraluminal concentration of glucose is high, such as early after a meal. Indeed, in the presence of high concentrations of intraluminal glucose (≥ 50 mM) that far exceed the K_m of SGLT1 (~ 3 –5 mM) and that saturate all SGLT1-mediated uptake of glucose, the combined activities of SGLT1 and PKC appear to be necessary to initiate translocation of pre-formed, cytoplasmic stores of GLUT2 rapidly to the apical membrane. This translocation of GLUT2, which has a lesser affinity but a much greater V_{max} and thus a greater capacity for glucose absorption, allows for the marked increase in absorption of luminal glucose at concentrations that are far greater than the K_m of SGLT1. Once the intraluminal concentration of glucose decreases, GLUT2 appears to be either degraded rapidly or internalized rapidly such that it does not maintain a constant presence in large amounts in the apical membrane (17,18,20).

Our initial experiments were designed to reproduce with an *in vitro*, full-thickness, tissue-based model the augmented, transporter-mediated uptake of glucose at concentrations > 20 mM in the lumen when the everted sleeves were exposed for short (1 min) and greater (5 min) durations. Our ultimate goal is to have an *in vivo*, *in vitro*, and cell culture-based methodology to study the phenomenon of GLUT2 translocation. To our surprise however, we were unable to show augmentation in transporter-mediated uptake, and the K_m of the transport measured at 1 minute and 5 minutes of exposure were similar to one another and similar to that reported by others for SGLT1. Therefore, under the constraints of our experimental preparation and conditions, we were unable to provide evidence for recruitment of another glucose transporter with a greater K_m (as expected for GLUT2)

within the 5 minutes of exposure, an effect that differs from our preliminary observations in cell culture in several intestinal epithelial cell lines (24, preliminary data).

Our subsequent experiments were designed to examine this model of intestinal uptake of glucose for evidence of GLUT2-mediated uptake of glucose under basal conditions and the role of SGLT1, PKC, and the intracellular, cytoskeletal microtubular structure on GLUT2-mediated uptake of glucose. Prior work *in vivo* has suggested that SGLT1 activity is necessary for GLUT2 activation and translocation (41,42); inhibition of SGLT1 by phlorizin should, therefore, disrupt uptake of glucose by SGLT1 and GLUT2 and prevent the translocation of GLUT2 to the apical membrane. Our studies with phlorizin in this everted sleeve model inhibited markedly the transporter-mediated uptake of glucose in the jejunum. The small amount of persistent glucose uptake in the jejunum could be due to either uninhibited SGLT1 or to GLUT2 (or another transporter) expressed constitutively in the apical membrane. While most groups comment on the rapid translocation of GLUT2 to and from the apical membrane *in vivo* in response to glucose, few studies have commented on the presence of GLUT2 in the apical membrane in “unstimulated,” nondiabetic enterocytes; the presence of GLUT2 in the apical membrane of the enterocyte in the absence of a high concentration of luminal glucose has been demonstrated in several studies using western blots and could be the explanation for the phlorizin-insensitive absorption in our experiment. (16,18–20,28,37,42). Another potential explanation for the phlorizin-insensitive glucose transport observed in our experiment is that GLUT2 was present in the apical membrane as a result of taste receptor-induced GLUT2 translocation, another recently described pathway for GLUT2 translocation (43,44). Treatment of everted sleeves with phloretin, which inhibits GLUT2 selectively, should not affect SGLT1-mediated glucose uptake but should inhibit glucose uptake by GLUT2, if GLUT2 was present in the apical membrane. Our findings of partial inhibition of transporter-mediated glucose uptake appear to confirm this concept in these experiments with the short duration of exposure to glucose (1 min). Although we would expect a greater K_m (possibly > 30 mM based on others work (7)) and therefore an increase in transporter-mediated uptake at concentrations of glucose greater than 20 mM, we could not demonstrate greater transporter-mediated uptake at 50 mM glucose solutions. We can only attempt to explain these findings by suggesting that constitutive GLUT2 in the apical membrane may have a lesser K_m similar to that of SGLT1; if true, this hypothesis would explain our findings.

We were also interested in studying the intracellular pathways regulating GLUT2 activity, as well as GLUT2 translocation in response to high luminal concentrations of glucose. When we targeted the role of PKC with the PKC inhibitors calphostin C and chelerythrine, we anticipated that uptake of glucose would be similar to that of the phloretin-treated sleeves if SGLT1 signaling through PKC was, in fact, necessary for GLUT2 activation and translocation to the apical membrane. We found no inhibition of transporter-mediated uptake during the short duration of exposure (1 min) to glucose concentrations greater than 1 mM and were somewhat surprised that inhibition of PKC activity did not decrease glucose absorption at the greater duration of incubation (5 min).

We further complemented these experiments by exploring the role of the integrity of the cytoskeleton in the apical translocation of GLUT2 by using nocodazole which depolymerizes intracellular microtubules. We hypothesized that if GLUT2 is translocated to the apical membrane, then disruption of the microtubule structure should prevent this translocation and blunt transporter-mediated glucose uptake similar to that expected with inhibition of PKC by calphostin C, chelerythrine, and by inhibition of GLUT2 by phloretin. Our results with nocodazole were similar to those seen with PKC inhibition, and no effect was noted in transporter-mediated uptake.

One other goal of our study was to use this *in vitro* technique of full thickness everted sleeves to study this presumed dynamic process of rapid (within minutes) apical translocation of glucose transporters. Unfortunately, our study shows that this model is not a useful preparation to study rapid translocation of glucose transport for several reasons. One reason is that the time necessary to harvest and prepare the everted sleeves and to equilibrate the tissue in the solution may be associated with translocation (or degradation) of GLUT2 out of the apical membrane. Indeed, this phenomenon has been blamed as the cause by which the role of GLUT2 in the apical membrane has been overlooked (7). Second, a 5 minute exposure to high concentrations of glucose, although satisfactory to see translocation in cell culture, may be too short a duration in everted sleeves; indeed, *in vivo*, this phenomenon appears to take 10 to 15 minutes. Longer exposures in the everted sleeve technique have not proved reliable in our extensive preliminary studies, because glucose uptake decreases markedly over time.

CONCLUSION

While these data, under the experimental conditions used, do not support conclusively the presence of an intracellular signaling pathway mediated by PKC that promotes translocation of additional glucose transporters (GLUT2) to the apical membrane, it is clear that inhibition of SGLT1 activity with phlorizin and/or GLUT2 with phloretin has an inhibitory effect on transporter-mediated glucose uptake. The partial inhibition of transporter-mediated glucose uptake in the presence of GLUT2 inhibition implicates GLUT2 as being involved in apical glucose transport at the “luminal” concentrations tested, while the inhibition of GLUT2 activity by SGLT1 inhibitor phlorizin suggests that SGLT1 activity is required for GLUT2 uptake of glucose. These data show that GLUT2 be constitutively active at the apical membrane of nondiabetic rats under basal conditions with a K_m not significantly different from that of SGLT1.

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Figure 1.

A) Effects on transporter mediated glucose uptake of inhibition of SGLT1 (phlorizin 0.2 mM), GLUT2 (phloretin 1 mM), both SGLT1 and GLUT2 (phlorizin 0.2 mM and phloretin 1 mM), PKC activity (calphostin C 50 or 500 nM or chelerythrine 50 μ M), and disruption of the microtubular system (nocodazole 10 μ M) in jejunum using everted sleeves incubated for 1 min. B) same as (A) except everted sleeves incubated for 5 minutes and calphostin C 500 nM.