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RESEARCH PAPER

Glucose absorption by isolated, vascularly perfused rat intestine: A significant paracellular contribution augmented by SGLT1 inhibition

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Abstract

Aim: Intestinal glucose transport involves SGLT1 in the apical membrane of enterocytes and GLUT2 in the basolateral membrane. In vivo studies have shown that absorption rates appear to exceed the theoretical capacity of these transporters, suggesting that glucose transport may occur via additional pathways, which could include passive mechanisms. The aim of the study was to investigate glucose absorption in an in vitro model, which has proven useful for endocrine studies.

Methods: We studied both transcellular and paracellular glucose absorption in the isolated vascularly perfused rat small intestine. Glucose absorbed from the lumen was traced with ^{14}C-D-glucose, allowing sensitive and accurate quantification. SGLT1 and GLUT2 activities were blocked with phlorizin and phloretin. ^{14}C-D-mannitol was used as an indicator of paracellular absorption.

Results: Our results indicate that glucose absorption in this model involves two transport mechanisms: transport mediated by SGLT1/GLUT2 and a paracellular transport mechanism. Glucose absorption was reduced by 60% when SGLT1 transport was blocked and by 80% when GLUT2 was blocked. After combined luminal SGLT1 and GLUT2 blockade, ~30% of glucose absorption remained. D-mannitol absorption was greater in the proximal small intestine compared to the distal small intestine. Unexpectedly, mannitol absorption increased markedly when SGLT1 transport was blocked.

Conclusion: In this model, glucose absorption occurs via both active transcellular and passive paracellular transport, particularly in the proximal intestine, which is important for the understanding of, for example, hormone secretion related to glucose absorption. Interference with SGLT1 activity may lead to enhanced paracellular transport, pointing to a role in the regulation of the latter.

KEYWORDS

glucose transporter 2, intestinal-glucose-absorption, intestine, paracellular transport, permeability, sodium glucose co-transporter 1, transcellular transport

1 | INTRODUCTION

The absorptive functions of the small intestine have been studied extensively over several decades. Glucose, derived from digested carbohydrates, is absorbed across the small intestinal mucosa, which is composed of the epithelial lining of the intestine, which rests on the lamina propria from where the glucose is cleared by the vascular system.[1,2] The general concept is that it is absorbed transcellularly by the enterocytes. Thus, in the 1960s, Crane[3,4] described a transepithelial glucose transporter, later named sodium–glucose transporter 1 (SGLT1). Subsequent research established the role of this transporter in glucose absorption.[5] Thus, the transfer of glucose across the gut epithelium involves secondary active transport via SGLT1 located in the apical membrane of the enterocytes, followed by facilitated transport across the basolateral membrane by glucose transporter 2 (GLUT2). However, some studies have shown that the rate of intestinal glucose absorption may exceed the transport capacity of SGLT1, which saturates when the luminal glucose concentration reaches ~30 mmol/L.[6–9] This observation has led to the development of different hypotheses regarding how glucose is absorbed. Pappenheimer et al. proposed that glucose is also transported paracellularly,[10,11] while Kellett et al. proposed that GLUT2 might be translocated from the basolateral to the apical membrane of enterocytes to assist SGLT1 with glucose transfer from the lumen to the cytosol.[12–16] Moreover, the specific methodology as well as glucose concentration employed may lead to variation in results.[17] Thus, it was important to establish how glucose is absorbed in our specific model for a better understanding of intestinal endocrine secretion. For the study of how nutrient absorption regulates the endocrine secretions of the gut, we have used isolated vascularly perfused preparations of rodent small intestines. This experimental model provides clear advantages compared to other in vitro models by preserving the polarity of the epithelial cells as well as the entire transport pathways and by ensuring adequate vascular perfusion of the mucosa.[18,19] We found a clear association between absorption rates of, for example, glucose and secretion of hormones like glucagon-like peptide-1.[20,21] However, to establish a causal relationship between the two, knowledge of the routes of transportation of glucose across the epithelial cells became essential. The overall aim of the study was to investigate transcellular and paracellular intestinal glucose absorption to understand how they operate in this perfusion model of entero-endocrine secretion.

2 | RESULTS

2.1 | Glucose absorption from the intestine is non-saturable

In the initial dose–response experiments, glucose concentration in the venous effluent was measured by a handheld glucometer. D-glucose was administered intra-luminally, and the concentration was increased every 15 min: 0% (baseline, isotonic saline), 1% (w/v) (55 mmol/L), 5% (278 mmol/L), 10% (550 mmol/L), and 20% (1100 mmol/L). Glucose absorption increased in response to rising luminal glucose concentrations (Figure 1A). Total glucose absorption during each 15-min luminal infusion (arteriovenous concentration difference × flow) for 1% (55 mmol/L) luminal glucose was 51.6 ± 4.2 μmol/15 min ($p = 0.02$); for 5% (275 mmol/L) glucose it increased to 88.31 ± 5.6 μmol/15 min ($p < 0.01$); for 10% (550 mmol/L) glucose it was 193.1 ± 14.7 μmol/15 min ($p < 0.01$), and for 20% (1100 mmol/L) 616.7 ± 78.2 μmol/15 min ($p < 0.05$) (Figure 1B).

Thus, the glucose absorption from the intestine did not saturate at the high glucose loads, suggesting that diffusive mechanisms are also involved. Glucose absorption reached a plateau at each luminal glucose load, indicating that a steady-state absorption rate was attained. However, greater luminal concentrations still resulted in increased absorption, suggesting that maximal absorption capacity depends on the luminal glucose concentration.

2.2 | Total glucose absorption was the same in response to repeated stimulation with the same luminal glucose concentration

We were interested in studying glucose absorption at lower luminal concentrations. Here, the lumen was stimulated with two consecutive luminal stimulations of 100 mmol/L D-glucose separated by a baseline period (luminal saline infusion) (Figure 1D) and the total glucose absorption was calculated for the two 15-min stimulation periods. Total glucose absorption was similar in the first and second stimulation period (149.7 and 141.8 μmol/15 min, $p = 0.72$) (Figure 1E). This experiment formed the basis for the following experiments since consecutive luminal glucose stimulations could be expected to induce comparable absorption responses. Significant differences in absorption rates would therefore be due to interventions during the second stimulation. At

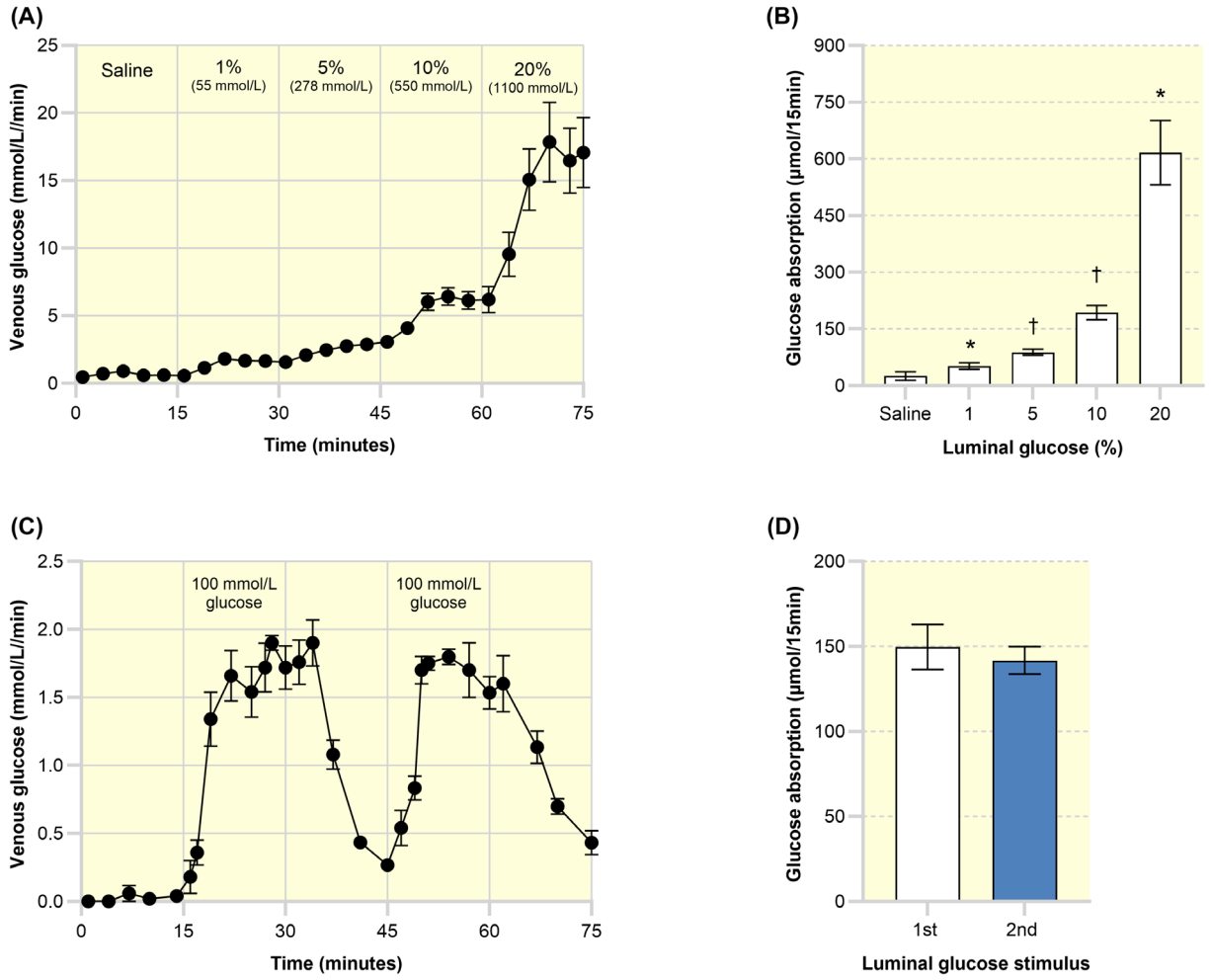

FIGURE 1 Glucose absorption by the small intestine. Venous (mmol/L/min) in the venous effluent following luminal infusion of increasing concentrations of glucose (1%–20%) (A). Total glucose absorption is compared to the baseline (luminal saline) by one-way ANOVA for repeated measurements followed by Bonferroni's multiple comparison test (B). Venous glucose (mmol/L/min) during two luminal glucose (100 mmol/L) stimulation periods separated by a baseline (luminal saline infusion) (C). Total glucose absorption (15-min stimulation period) is compared by paired *t*-test analysis (D) $n = 4$. Data are shown as mean ± SEM. $p < 0.5$ is considered significant. *$p < 0.05$, †$p < 0.01$.

a concentration of <100 mmol/L glucose in the lumen, the sensitivity of the glucometer for measurements of glucose increases in the venous effluent was inadequate, and for further experiments, we traced glucose absorption by adding radioactively labeled glucose (^{14}C-D-glucose).

2.3 | Blocking SGLT1 at the apical membrane impairs glucose absorption

To assess the role of apical SGLT1 activity for glucose absorption, phlorizin was administered to the lumen before repeating the luminal glucose stimulation (Figure 2A). During the first luminal glucose (10 mmol/L) stimulation, total absorption was 60.7 µmol/15 min. Luminal administration of phlorizin at 1 µmol/L (around 250-fold higher than the IC50 of phlorizin on SGLT1) suppressed total glucose absorption by ~70% (to 19.5 ± 1.0 µmol/15 min, $p = 0.03$, $n = 4$) (Figure 2B). At 100 mmol/L luminal glucose, total glucose absorption decreased by ~60%, from 429.8 to 184.1 µmol/15 min ($p < 0.001$, $n = 8$) during luminal phlorizin administration (Figure 2C,D). Thus, at these concentrations of glucose, blocking SGLT1 and thereby secondary active transport of glucose, 30%–40% of total glucose absorption was not mediated by SGLT1, justifying the search for additional routes of absorption.

SGLT1 is not expressed at the basolateral membrane,[22] we did not expect to see changes in glucose absorption during intra-arterial administration of phlorizin (Figure 2E). Total glucose absorption in the first stimulation period was 488.5 and 453.8 µmol/15 min in the second (Figure 2F, $p = 0.09$). Thus, the effects of phlorizin were clearly associated with luminal administration, as expected. This experiment also confirmed that stimulation

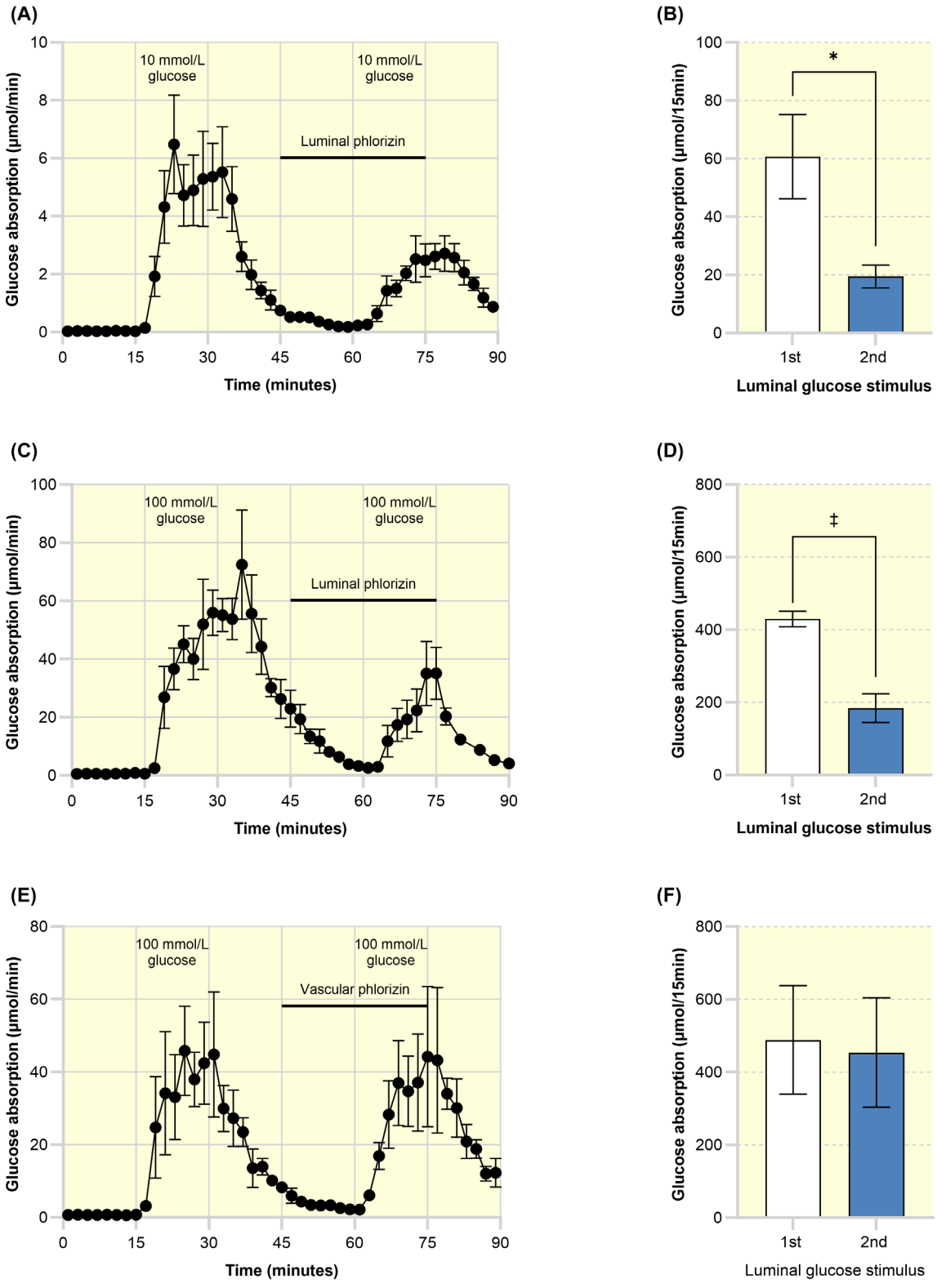

FIGURE 2 Luminal phlorizin inhibits intestinal glucose absorption. (A) Glucose absorption traced with ^{14}C-D-glucose (µmol/min) during luminal infusion of 10 mmol/L glucose with 1 mmol/L luminal phlorizin, (B) total glucose absorption before and after luminal phlorizin infusion. (C) 100 mmol/L luminal glucose with luminal phlorizin, (D) total glucose absorption before and after luminal phlorizin infusion. (E) 100 mmol/L luminal glucose with 1 mmol/L intra-arterial phlorizin. (F) Total glucose absorption during 15-min periods with or without phlorizin. Total glucose absorption with or without phlorizin infusion was compared by paired *t*-test analysis. Data are shown as mean ± SEM. *p*-values <0.05 was considered significant. *$p < 0.05$, $^{‡}p < 0.001$.

at the luminal and basolateral sites of the intestine could be differentiated.

2.4 | Intestinal glucose absorption continues despite GLUT2 blockage

GLUT2 mediates the facilitated transport of glucose at the basolateral membrane; phloretin is a known blocker of GLUT2.[18,23] Blocking GLUT2 at the basolateral membrane by intra-arterial phloretin infusion (1 mmol/L) (Figure 3A,C) tended to reduce (non-significantly) glucose absorption by ~80% (from 70.1 to 13.2 μmol/15 min, $p=0.15$) at 10 mmol/L luminal glucose (Figure 3B). At 100 mmol/L luminal glucose, blocking GLUT2 resulted in a 70% decrease (from 470.2 to 150.1 μmol/15 min, $p=0.03$) in total glucose absorption (Figure 3D). Thus, GLUT2 blockade impairs glucose absorption, presumably by preventing the exit of intracellularly accumulating glucose across the basolateral membranes; however, some glucose absorption remains.

It has been suggested that GLUT2 may be inserted into the apical membrane to facilitate glucose transfer over the apical.[15,16] This putative mechanism was tested by investigating total glucose absorption during intra-luminal infusion of phloretin (Figure 3E). We also tested intraluminal infusion of phlorizin and phloretin in combination (Figure 3F). Luminal administration of the GLUT2 inhibitor reduced total glucose absorption by ~55% (from 526.8 to 239.9 μmol/15 min, $p=0.047$) (Figure 3F), which might support a contribution by apically inserted GLUT2—or alternatively that phloretin is itself transported across the epithelium allowing it to block GLUT2 expressed at the basolateral membranes of the cell.[24] Phloretin has been shown to be absorbed by the intestinal wall,[25–27] but it is not known whether absorbed intracellular phloretin can block basolateral GLUT2. Importantly, simultaneous luminal administration of phlorizin and phloretin only reduced total glucose absorption by ~60% (from 398.6 to 152.7 μmol/15 min, $p=0.005$) (Figure 3G,H) similar to the inhibition by phlorizin alone, excluding GLUT2 as a pathway for additional glucose absorption during phlorizin blockade. The simplest interpretation of the inhibitory effect of phloretin alone under these conditions is that both compounds about equally block SGLT1, as suggested previously.[28]

2.5 | Mannitol is absorbed by the small intestine independently of luminal glucose concentration

Mannitol is the same size as glucose, but mannitol is not a substrate for the glucose transporters; thus, it has been used in several studies to study the leakiness of the intestine. We used ^{14}C-D-mannitol to study paracellular glucose absorption (Figure 4A). Total absorption of mannitol following luminal infusion of 100 mmol/L D-mannitol traced with ^{14}C-D-mannitol was 129.6 μmol/15 min, which tended to increase, although not significantly, to 403.1 μmol/15 min when the lumen was stimulated with 100 mmol/L mannitol and 100 mmol/L glucose traced with ^{14}C-D-mannitol simultaneously (Figure 4B). The marked tendency to increased mannitol absorption after the addition of glucose excludes competitive interaction between glucose and mannitol (which would be expected to decrease mannitol absorption) but would be compatible with increased diffusive permeability.

2.6 | Mannitol absorption is most prominent in the proximal small intestine

It has been described that the proximal small intestine is "leakier" than the distal part.[21] We tested differences in mannitol absorption in two segments of the small intestine. Mannitol absorption was greater in the proximal (upper 30–45 cm) compared to the distal (lower 30–45 cm) small intestine in response to luminal stimulation with 10 mmol/L D-mannitol (Figure 4C,D). Total mannitol absorption was 74.0 μmol/15 min in the proximal and 22.4 μmol/15 min in the distal small intestine. These results confirm that intestinal leakiness is greater in the proximal intestine, possibly due to greater expression of pore-forming tight junctions[29,30] and/or SGLT1-induced solvent drag. In the second luminal stimulation period, 10 mmol/L D-glucose was traced with ^{14}C-D-mannitol. There was no difference in recorded radioactivity (^{14}C-D-mannitol) between the two stimulations, neither in the proximal (95.3 μmol/15 min, $p=0.45$, $n=5$) nor the distal (26.5 μmol/15 min, $p=0.48$, $n=6$) small intestine (Figure 4D). The similar amounts of absorbed tracer, whether the luminal solute was glucose or mannitol, suggest that the transport was independent of mediated transport, tracing paracellular transport of both mannitol and glucose.

2.7 | SGLT1 blocking induced paracellular mannitol absorption

To investigate any dependency of mannitol absorption on SGLT1 activity, we performed perfusions stimulating the lumen with mannitol while blocking SGLT1 with luminally infused phlorizin (Figure 5A) and canagliflozin (Figure 5C). Surprisingly, blocking SGLT1 markedly increased mannitol absorption. Luminal phlorizin increased mannitol absorption sevenfold, from 30

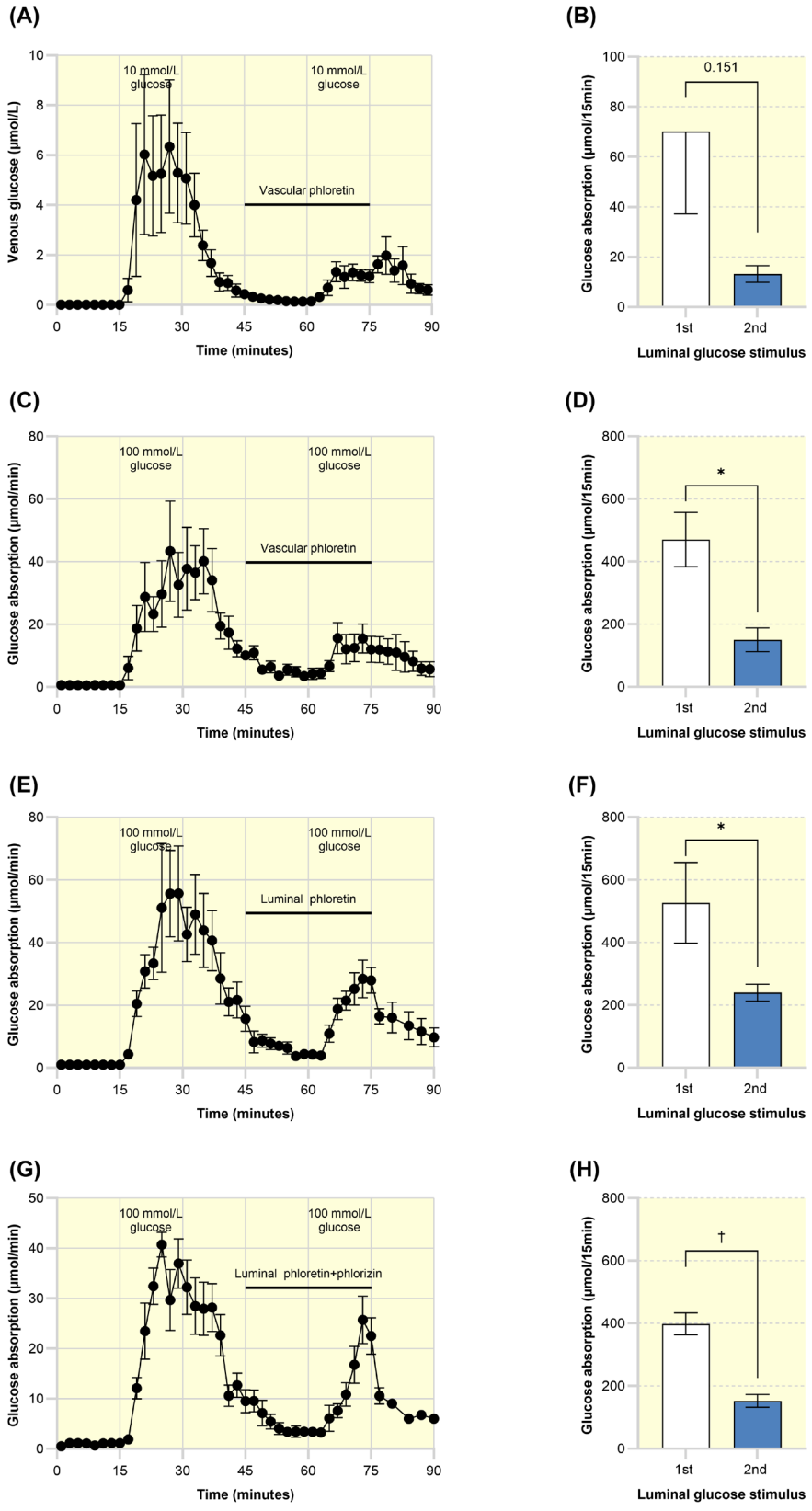

FIGURE 3 Vascular infusion of phloretin inhibits glucose absorption. Glucose absorption (µmol/min) traced with ^{14}C-D-glucose during (A) 10 mmol/L and intra-arterial infused phloretin, (B) total glucose absorption in 15-min period. (C) 100 mmol/L luminal glucose with intra-arterial phloretin, (D) total glucose absorption in 15-min periods before and after intra-arterial phloretin. (E) 100 mmol/L luminal glucose with intra-luminal phloretin, (F) total glucose absorption before and after luminal phloretin infusion. (G) 100 mmol/L luminal glucose with combined intra-luminal phlorizin and phloretin infusion. (H) Total 15-min glucose absorption at baseline and during glucose-transporter inhibitor infusion. Total glucose absoprtion was compared by paired *t*-test. Data are shown as mean ± SEM; $n = 4–7$. $p < 0.05$ was considered significant. $^{*}p < 0.05$, $^{\dagger}p < 0.01$.

to 206.4 µmol/15 min ($p = 0.009$) (Figure 5B). Similarly, blocking SGLT1 with luminally infused canagliflozin (60 µmol/L, 100-fold higher than the IC50 of canagliflozin on SGLT1 in the intestine) induced a 1.9-fold increase in total mannitol absorption from 65.4 to 126 µmol/15 min ($p = 0.02$) (Figure 5D). These results suggest that blocking transcellular glucose transport may increase paracellular transport.

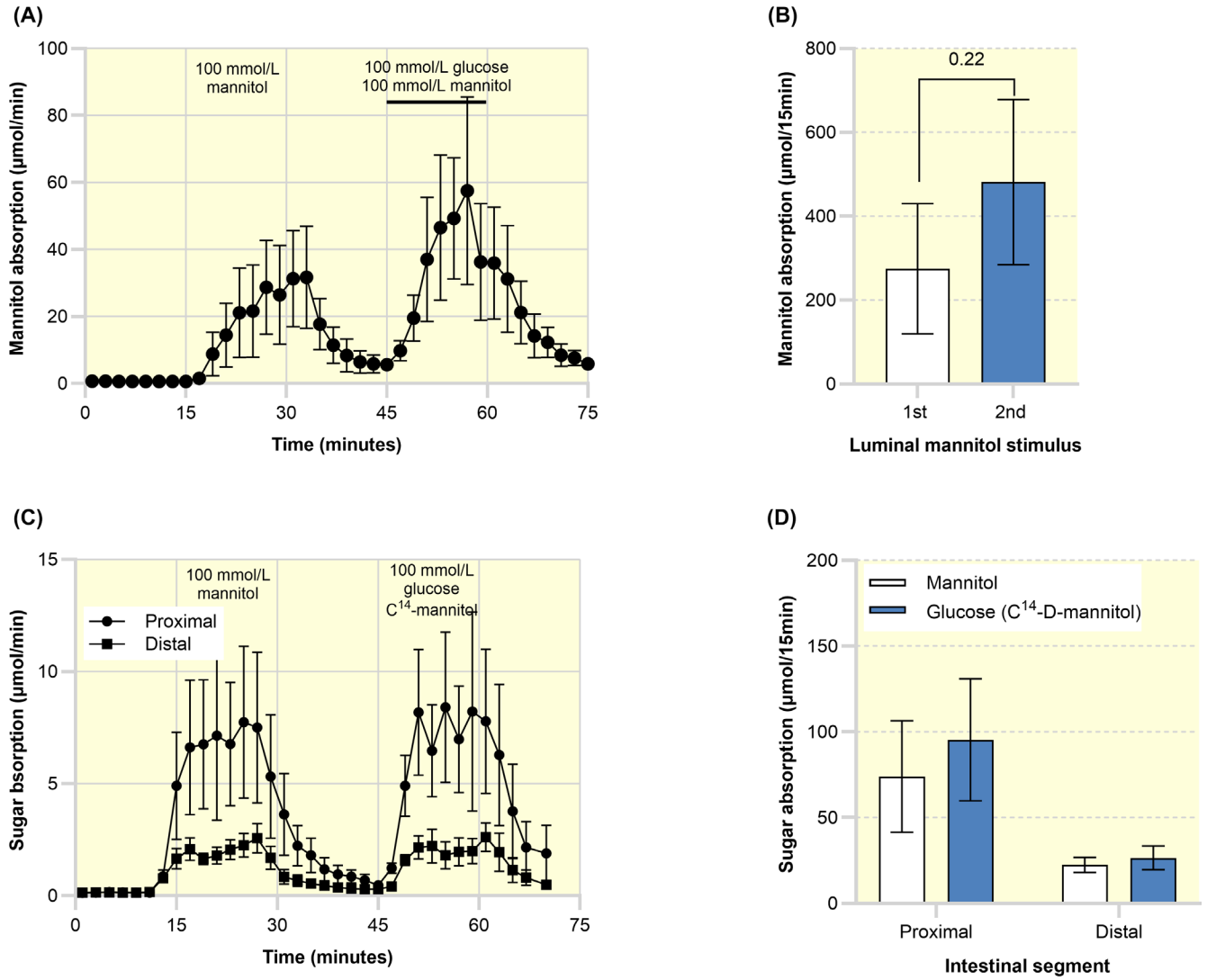

FIGURE 4 Small intestinal mannitol absorption. Mannitol absorption (µmol/min) traced with ^{14}C-D-mannitol during luminal infusions of 100 mmol/L mannitol followed by combined infusion of 100 mmol/L mannitol and 100 mmol/L glucose traced with ^{14}C-D-mannitol (A). Total mannitol absorption was compared between the two 15-min luminal infusions by paired *t*-test analysis (B), $n = 6$. Mannitol absorption (µmol/min) was measured in the proximal (round; bold) and distal (square; bold) small intestine after luminal stimulation of 10 mmol/L mannitol followed by 10 mmol/L glucose, both traced with ^{14}C-D-mannitol (C). Total mannitol absorption in each segment was compared between the two 15-min luminal infusions by paired *t*-test analysis (D), $n = 5$. Data are shown as mean ± SEM. $p < 0.05$ was considered significant.

3 | DISCUSSION

During our studies of nutrient-induced stimulation of the secretion of gut hormones from the small intestine, we realized that it is unclear to what extent glucose absorption relies on diffusive glucose uptake, which would not be expected to stimulate enteroendocrine hormone secretion.[20] Further, the amount of paracellular glucose absorption may be very specific to the methodology and model employed. Therefore, like our studies of hormone secretion, the current study is based on isolated vascularly perfused rat small intestine preparations, which appeared capable of absorbing glucose at physiological rates. We initially investigated the absorption capacity of the rat small intestine in response to luminal administration of large amounts of glucose (corresponding to those introduced intra-intestinally in vivo or during oral glucose tolerance tests with rapid gastric emptying—for glucose tolerance testing, gavage with 2 g/kg of glucose is frequently used in rodents). Studies in humans have shown that luminal glucose concentrations can reach 300 mmol/L postprandially.[11] In our perfusion experiments, we observed that the absorption of glucose was not saturated even at very high luminal glucose concentrations. Thus, during stimulation with increasing glucose concentrations from 0 to 1100 mmol/L, dose-dependent increases in the

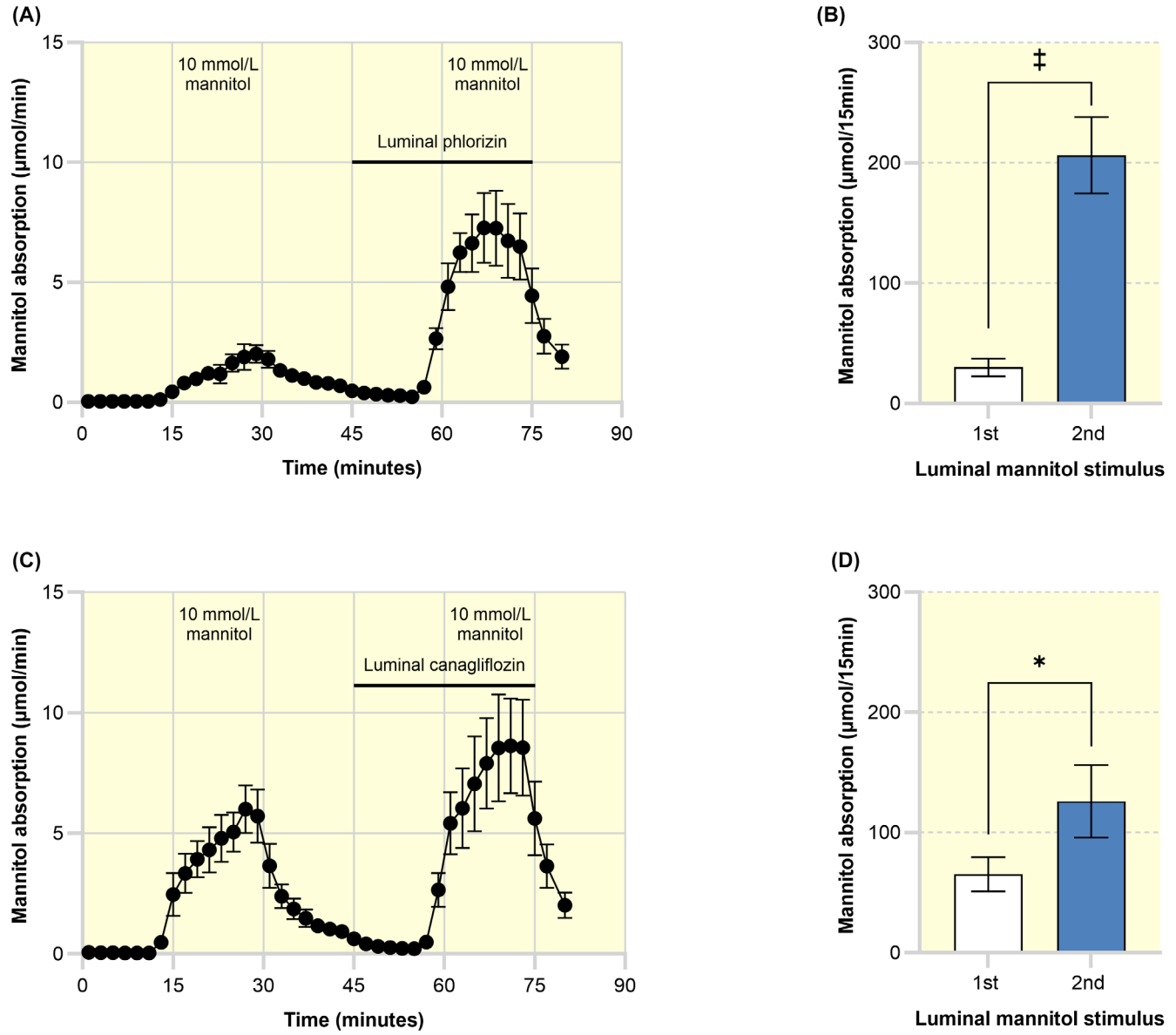

FIGURE 5 Mannitol absorption during SGLT1 blocking. Mannitol absorption (µmol/min) traced with ^{14}C-D-mannitol after luminal infusion of 10 mmol/L mannitol with intra-luminally infused phlorizin (A–B) or intra-luminal canagliflozin (60 µmol/L) (C–D). Total mannitol absorption during baseline infusion and during glucose-transporter inhibitor infusion was compared by paired *t* test. $n = 5$. Data are presented as mean ± SEM. $p < 0.05$ was considered significant. *$p < 0.05$, ‡$p < 0.001$.

concentration of glucose were noted in the venous effluent (Figure 1A–C), consistent with the existence of glucose transport mechanisms other than the well-established SGLT1/GLUT2 pathway.

At each of the chosen luminal glucose loads (instilled at a flow rate of 2.5 mL/min while the luminal volume of the perfused intestinal segment approximated 11 cm^3), the absorption rate seemed to plateau after 5 min of infusion, indicating that glucose absorption was taking place at a steady state. However, challenging the intestine further with increasing concentrations of glucose gave rise to new plateaus of absorption. We expected SGLT1-mediated transport to be saturated already at ~30 mmol/L,[31] but this was not the case. We therefore conclude that luminal glucose exceeding these concentrations might utilize different routes of absorption other than secondary active transport by SGLT1. Continuously increasing exposure of distal segments of the perfused intestine during instillation of the higher doses (via the proximal opening) might create a similar result, but the instillation flow was sufficient to fill the intestinal lumen in <4 min, so we assume that most of the epithelial surface was exposed to equally high concentrations during the 15 min stimulation periods in spite of concurrent absorption. In addition, as

clearly demonstrated, the absorption capacity decreased markedly in the distal half of the small intestine, reducing the absorption-induced fall in luminal concentration.

We expected that the effective absorption of glucose at lower luminal concentrations would be due mainly to the SGLT1/GLUT2 system. In subsequent experiments, we therefore analyzed the transporter-mediated intestinal glucose absorption by blocking the two known transporters, SGLT1 and GLUT2, while maintaining luminal concentrations at 100 mmol/L. For these studies, we were able to use a paired experimental design since glucose absorption rates, measured directly in the venous effluent from the preparation, were comparable between two consecutive luminal glucose stimulation periods. To block glucose transport, phlorizin was used as a SGLT1 inhibitor (IC50 = 290 nM) and phloretin was used as a GLUT2 inhibitor (IC50 = 1 μM[24,32] and 50 μM on SGLT1[26]). As suspected, inhibiting SGLT1 with 1 mmol/L phlorizin in the lumen resulted in decreased glucose absorption. Absorption was, however, not eliminated, suggesting the existence of glucose transport mechanisms working in parallel with SGLT1. Attesting to the specificity of the setup, intra-vascular phlorizin infusion did not change glucose absorption, illustrating that the perfusion model successfully discriminates between luminal and vascular stimulations. Next, we investigated the effect of blocking GLUT2 at the basolateral membrane by intra-vascular phloretin infusion, which resulted in decreased glucose absorption. Again, however, some absorption remained. Thus, we were unable to eliminate intestinal glucose absorption by blocking the transporters SGLT1 and GLUT2, whether at the apical or the basolateral membrane. The results support the notion that at luminal concentrations up to 100 mmol/L,[15] glucose absorption is mediated by two components, where at least 30% of glucose absorption occurs via other routes than SGLT1/GLUT2. These effects of the inhibitors are in agreement with those reported by Skopec et al.[33] using an in vivo model in rats relying on measurements of disappearance rates of labeled 3-O-methyl glucose.

In the early 2000s, it was reported that GLUT2 might be translocated to the apical membrane when the luminal glucose concentration is high, thereby contributing to facilitated glucose absorption from the lumen to the enterocytes, and it was shown that GLUT2 translocation is somehow dependent on SGLT1 activity.[15,34] We tested this putative mechanism using the isolated vascularly perfused small intestine by stimulating the lumen with glucose and phloretin. Luminally infused phloretin did indeed immediately decrease glucose absorption (by ~55%), supporting the existence of a phloretin-sensitive luminal absorption of glucose, potentially via apically inserted GLUT2. Apically inserted GLUT2 has been suggested to account for all absorption that is otherwise considered "passive" or "diffusive"—some investigators have suggested that such passive transport is responsible for up to 75% of total glucose absorption.[15] If GLUT2 is inserted into the apical membrane and this is, in fact, the mechanism contributing to the diffusive mode of glucose absorption that we and others[35] have observed, intestinal glucose absorption should be eliminated by simultaneously blocking these two transporters at the apical membrane. However, though luminal double blockade markedly lowered glucose absorption, the reduction did not exceed that resulting from SGLT1 blockade alone, which is unsupportive of a role of apically located GLUT2 for glucose absorption. Notably, phloretin is possibly not an ideal GLUT2 blocker—it has been reported to inhibit also SGLT1 activity.[36] GLUT2 has been identified at the apical membrane by western blot of membrane vesicles and by confocal microscopy; however, it was only present in enterocytes from people living with obesity, and it was absent in lean individuals.[34,37] Importantly, an increase in the number of apical GLUT2 was observed at luminal glucose concentrations >30 mmol/L, and this number was doubled by 100 mmol/L luminal glucose.[12] That same study showed complete inhibition of glucose absorption by co-administration of phlorizin and cytochalasin B, which block the passive intracellular transport, leaving no room for a paracellular component of absorption.[34] However, a more recent study[22] found GLUT2 to be located only at the basolateral membrane, confirmed by intestinal immuno-staining. Röder et al.[22] detected apical GLUT2 by Western blot but also detected considerable amounts of other basolateral markers in the apical membrane preparations and thus assumed that detectable GLUT2 was due to basolateral contamination. In addition to this, there was no increase in GLUT2 protein density after a glucose gavage, which would be expected with proposed models of GLUT2 trafficking.[16] Further, it was found that *glut2* KO mice accumulate glucose in intestinal tissue after an intragastric glucose bolus, indicating that GLUT2 is important for substrate efflux from intestinal cells into the circulation, not essential for influx from the lumen. Our finding that a major diffusive component exists is in line with this study and others showing similar oral glucose tolerance in *glut2* KO mice and wildtypes.[38] Naftalin in 2014,[39] based on theoretical considerations, deduced that GLUT2 translocation would result in decreased rather than increased paracellular transport. Moreover, in humans with Fanconi-Bickel syndrome, who have inactivating *glut2* mutations, there is no intestinal glucose malabsorption but severe glucosuria resulting from impaired renal glucose re-absorption.[40] Taken together, it seems unlikely

that GLUT2 translocation would be able to explain the non-SGLT1-mediated glucose transport observed in this study.

Passive glucose absorption has been observed to be accompanied by high rates of water absorption, suggesting that glucose in the intercellular spaces might provide a solvent drag that could be responsible for the additional absorption of nutrients.[10,41] In agreement, electron microscopy of glucose-perfused segments of hamster small intestine revealed large dilatations within tight junctions; thus, Na^+-coupled transcellular transport from the lumen to the cytosol of enterocytes not only provides a driving force for the absorption of fluids and nutrients, but also triggers the widening of intercellular junctions.[10,31] Solvent drag was discussed critically as a mechanism for paracellular transport by Karasov in 2017.[17] We understand solvent drag as the acceleration of flux of a permeable solute by moving the solvent. It is not immediately apparent how increased solvent flow, which should be driven by the *transcellular* fluxes of glucose and sodium, could explain the greatly increased absorption we observe during high luminal glucose (up to 617 μmol/15 min; Figure 1B) compared to the SGLT1-mediated transport (phlorizin-dependent) which amounted to 246 μmol/15 min (430–184 μmol/15 cm; Figure 2B) at 100 mmol/L glucose. Our experiments with the unspecific blocker phlorizin, but also with the much more specific SGLT1/2 blocker, canagliflozin, in which the non-transporter absorption of mannitol increased acutely, suggest that changes may occur in the permeability of the tight junctions for molecules like glucose and mannitol, and that such changes explain the increased non-transporter-mediated absorption during activation of SGLT1. Whether the SGLT1 activation and the increased permeability are causally related remains to be studied.

We used the transfer of mannitol, a non-metabolizable carbohydrate of approximately the same size as glucose, which is not transported by SGLT1 or GLUT2, as a surrogate measure of paracellular glucose absorption.[42] Mannitol was absorbed from the small intestine in our model, even at low luminal concentrations. This absorption occurred independently of luminal glucose infusion, indicating that mannitol and glucose do not compete for transport, since mannitol absorption did not decrease when glucose was infused luminally and vice versa. These findings support the notion of a passive route of absorption. The "leakiness" of the small intestine is determined by the structure and expression pattern of the tight junctions and has also previously been suspected to depend on SGLT1 expression.[43] Both tight junction protein and SGLT1 expression are higher in the proximal compared to the distal small intestine.[21,29,43,44] Therefore, we investigated mannitol absorption in both the proximal and distal small intestine. As expected, mannitol absorption from the proximal segment by far exceeded that of the distal segment, consistent with a more leaky epithelium in the proximal small intestine. Importantly, there was no difference in reported radioactivity (^{14}C) when stimulating the lumen with mannitol traced with ^{14}C-D-mannitol compared to glucose infusion traced with ^{14}C-D-mannitol, indicating a similar leak for these molecules at these concentrations. To further investigate any influence of SGLT1 activity on mannitol absorption, we applied luminal phlorizin during luminal mannitol administration, and this surprisingly led to a marked increase in mannitol absorption. To test whether this represented an unknown unspecific effect of phlorizin or a more general effect of SGLT1 blockade, we repeated the experiment with canagliflozin, a different but highly specific dual SGLT1/SGLT2 inhibitor. Indeed, canagliflozin also induced a major increase in mannitol absorption. Given that mannitol absorption is paracellular, these results suggest that paracellular uptake is promoted by SGLT1 activation or blocking.

The contribution of transcellular and paracellular transport of glucose is debated. Based on a series of human studies, Schwartz 1995[45] strongly opposed the concept of paracellular transport but had uninterpretable data, with L-glucose apparently showing "paracellular" behavior. Karasov[33] critically reviewed the field and concluded that paracellular transport is likely to be found in most vertebrates and to be essential in several species (e.g., birds) that depend on the rapid uptake of large amounts of ingested glucose and concluded that the important issue was the quantitative contribution in vivo in the species in question. With our data strongly supporting an important contribution of phlorizin-independent transport at high luminal concentrations, the question arises of how translatable our model is. The vascularly perfused intestinal preparation is fully viable, respires, exhibits appropriate motility, and has perfect morphology post-perfusion.[18] As already mentioned, the endocrine secretion also seems to reliably reflect the in vivo responses. These include aspects related to the polarization of the mucosa (the ability to study luminal and basolateral stimulation separately), paracrine interactions, and in particular, a single-pass circulation, which preserves venous drainage of substrates, products, and metabolites that might otherwise have obstructed/inhibited dynamic processes (as in Ussing chamber preparations). It has been mentioned that anesthesia may seriously affect intestinal glucose transport.[46] Our donor animals were, of course, anesthetized, but the donor animal is killed immediately after isolation of the organ and establishment of artificial circulation, and 40–60 min of perfusion precedes the

first transport studies, which furthermore did not change during the course of the up to 200 min perfusion experiments. As mentioned, although not reported here, our preparations show apparently normal motor activity and react to motor stimuli, such as acetylcholine, substance P, and serotonin.[47] Again, although not addressed here, it is assumed that the enteric nervous system, which would be responsible for generating the basic motility of the gut wall, is fully maintained in the preparation, judging from its motor activity.

Regarding the evaluation of transporter-mediated glucose transport, the best evidence would seem to derive from a comparison with other models of absorption. In the present experiments with 100 mmol/L glucose luminally, which must be considered close to physiological, we had a phlorizin-inhibitable absorption of 246 μmol/15 min. Karasov[17] and Pappenheimer[11] present data corresponding to 0.25 to 1 μmol/cm/min. According to our steady-state data, derived from a gut segment with a length of ~90 cm, we have mediated absorption rates corresponding to 0.5 μmol/cm/min. In other words, quite comparable values. A very important argument for the translatability of our results is the finding of very large regional differences in mediated as well as non-mediated transport. Returning to the original purposes of our studies, the endocrine secretion, it was of interest that the entire secretory GLP-1 response to luminal glucose disappeared after phlorizin,[20] as well as the omission of luminal sodium, indicating that secretion was entirely dependent on the absorption process; indeed, it could also be elicited by non-metabolized analogs like 3-O-methyl glucose,[20] showing similar sensitivity to phlorizin.

4 | MATERIALS AND METHODS

4.1 | Model considerations

For the isolated perfused rat small intestine preparation, the intestine is surgically isolated and then vascularly perfused in situ, while the lumen is perfused with glucose or other agents. This experimental model maintains the polarization of the epithelium intact, preserves the vascular bed and enteric neural plexus, and allows for repeated stimulation with luminal and vascular stimuli in a single-pass system. Importantly, the intestine remains viable with adequate respiration and metabolism and stays responsive for hours.[18]

For our experiments, absorption rates of glucose/mannitol were determined based on perfusion flow and substrate concentrations in the venous effluent. To enhance sensitivity, glucose and mannitol were traced using ^{14}C-D-glucose and ^{14}C-D-mannitol. This allowed measurements of total glucose absorption before and after the introduction of the different blockers of glucose transporters at physiologically relevant glucose concentrations.

4.2 | Animals and ethical considerations

Animal studies were conducted with permission from the Danish National Committee for Animal Research (2018-15-0201-01397), from the local animal use committee (SUND, EMED, P19-435) and in accordance with the guidelines of Danish legislation governing animal experiments. Male Wistar rats (~250 g) were purchased from Janvier (Saint Berthevin Cedex, France), housed in pairs in an air-conditioned (21°C) and humidity-controlled (55%) room, and kept in a 12:12 h light: dark cycle with ad libitum access to standard chow and drinking water. Rats were acclimatized for at least 1 week. Adult male rats were used to avoid the influence of female reproductive hormones on gut function.[48]

4.3 | Rat small intestine perfusions

4.3.1 | Surgical procedure

On the day of the experiments, non-fasted rats (~300 g) were anesthetized with a subcutaneous injection of Hypnorm/Midazolam (0.3 mL/100 g BW). When proper anesthesia was established, which was confirmed by the absence of reflexes, the rat was placed on a 37°C heat plate. The abdomen was opened, and after ligation of the relevant vasculature, the colon was excised, leaving the distal ileum open. A plastic tube was inserted into the lumen of the proximal intestine (~2 cm distal to the stomach), and the intestinal contents were carefully flushed out using pre-warmed (37°C) isotonic saline.

When perfusing only the proximal half of the small intestine (upper 30–45 cm), the vasculature supplying the distal small intestine was ligated, and the lower part was excised. For perfusion of the distal half of the small intestine (lower 30–45 cm), the vasculature supplying the upper small intestine was ligated, and a luminal tube was placed ~45 cm from the stomach.

Throughout the experimental procedure, a constant luminal flow of heated saline (37°C) was maintained at a low rate (0.5 mL/min) using a syringe pump (AL-1000 programmable syringe pump, WPI, UK).

For total small intestinal as well as upper and lower small intestine, a metal catheter was inserted into the upper superior mesenteric artery for arterial perfusion

with heated (37°C) perfusion buffer at a constant flow rate of 7.5 mL/min. Another catheter was inserted into the portal vein for the collection of the venous effluent. Once perfusion flow was established, indicated by a pale appearance of the intestine and a clear venous effluent flow of ≥4 mL/min, the rat was euthanized by perforation of the diaphragm. The preparation was then allowed to equilibrate for 25 min before the experimental protocol was initiated.

4.3.2 | Perfusion system

We used a *single-pass* perfusion system (Uniper UP-100, Hugo Electronics-Harvard apparatus, March-Hugstetten, Germany) which heats the perfusion buffer to 37°C and allows continuous recording of perfusion pressure (Hugo Sachs Electronics), processed by associated software (BDAS). The perfusion buffer was a modified Krebs-Ringer bicarbonate buffer containing 0.1% (w/v) bovine serum albumin (BSA) (Merck KGaA, Darmstadt, Germany), 5% (w/v) dextran T-70 to balance osmolarity (Pharmacosmos, Denmark), 3.5 mmol/L glucose, and 5 mmol/L of fumarate, glutamate, and pyruvate. The buffer was constantly gassed with 95% O_2/5% CO_2 to maximally increase pO_2 and maintain pH around 7.4. Arterial as well as venous perfusate samples were analyzed regularly for pH and partial pressures of oxygen and carbon dioxide (Radiometer Acid–Base laboratory).

4.3.3 | Perfusion protocol

Throughout the experiments, venous effluent was collected at 1-min intervals. Samples were immediately placed on ice and then stored at −20°C until analysis. Each protocol began with a 10–15-min basal period of luminal saline infusion (0.5 mL/min), followed by the administration of test substances either through the vascular circulation (intra-arterially) or into the intestinal lumen (intraluminally). Vascular infusions were delivered via a three-way valve for 15 min at a flow rate of 0.375 mL/min. Luminal stimuli were administered for 15 min, beginning with an initial infusion rate of 2.5 mL/min for the first 5 min (to replace previous solutions in the lumen, luminal volume of ~11 cm^3), followed by a 10-min infusion at a flow rate of 0.5 mL/min. All stimulation periods were separated by a 15–20-min basal saline infusion period to allow for washout of the test substances. After luminal administration of test substances, the lumen was flushed with saline using the bolus and maintenance rate specified above. Compounds administered into the intestinal lumen were diluted in isotonic saline, while those administered intra-arterially were diluted in perfusion buffer. Compounds with poor water solubility (such as phloretin and canagliflozin) were first dissolved in dimethyl sulfoxide (DMSO) and then further diluted in either saline or perfusion buffer. The final concentration of DMSO did not exceed 1%.

4.4 | Compounds

Phlorizin, an SGLT1 (and SGLT2) inhibitor, was administered to the intestinal lumen to block glucose transport through SGLT1 at the apical membrane. Phloretin, a GLUT2 inhibitor, was administered to the intestinal lumen to block glucose absorption through any apical GLUT2 and to the vasculature to block GLUT2 at the basolateral membrane. Canagliflozin, a dual SGLT1/2 inhibitor used in the treatment of type 2 diabetes, was administered to the lumen to block glucose transport through SGLT1 at the apical membrane. All test substances were purchased from the following sources: Sigma Aldrich, Brøndby Denmark (IBMX, Cat. No I5879; phloretin, Cat. No. P7912; phlorizin, Cat. No. P3449; D-glucose, Cat. No. G8270; D-mannitol, Cat. No. M4125), Perkin Elmer, USA (Glucose, D-[1-^{14}C], Cat. No. NEC043X; mannitol, D-[2-^{14}C], Cat. No. NEC852; Ultima Gold, Cat. No. 6013321). Canagliflozin (Invokana; Janssen-Cilag International NV, Beerse, Belgium).

4.5 | Glucose and mannitol measurements

Glucose concentration in the venous effluent was measured by a handheld glucometer (Accu-Chek Mobile, Hvidovre Denmark) in the first experiments. Because of the limited sensitivity of the glucose analysis by the glucometer, we also used tracers for absorption studies. Thus, ^{14}C-D-glucose and ^{14}C-D-mannitol were used as tracers of glucose and mannitol absorption in the perfusion effluent; 1 μL/mL glucose corresponding to 0.1 μCi/mL. The specific activity of ^{14}C-D-glucose or ^{14}C-D-mannitol (counts per minute (CPM)) was determined, by liquid scintillation counting, in 1 mL of the glucose solutions (10 and 100 mM) used for luminal administration (CPM/mL). Samples of the venous effluent were collected and mixed with scintillation fluid (Ultima Gold, Perkin Elmer, USA) and measured for radioactivity using a Quantulus GCT 6220 liquid scintillation counter from Perkin Elmer (USA) (combining 3 1-min samples) for 10 min. Radioactivity (counts) in each sample was then translated into glucose absorbed (mmol) on the basis of the specific activity of the glucose solutions employed (10 and 100 mmol/L) for luminal administration and the volume of the effluent fraction, assuming that the specific activity would remain constant. D-mannitol absorption was calculated in the same manner.

4.6 | Data presentation and statistical analysis

Data are presented as means ± SEM. Averaged basal and response outputs were calculated by averaging output values over the entire stimulation period (15 min luminal D-glucose administration). The absorption rate of unlabeled glucose (mmol/min) is the product of sugar concentration in the effluent (mmol/L) (after subtraction of the arterial glucose concentration of 3.5 mmol/L) and the flow rate (7.5 mL/min) in the perfusion experiment. Total glucose absorption of each stimulation period was calculated by summing the individual absorption (mmol/min) within a 15-min stimulation period. Statistical significance was assessed by paired *t*-test or, alternatively, by ANOVA analysis (as indicated in figure legends). Statistical tests were performed using GraphPad Prism 8 software (La Jolla, CA, USA). Graphs were constructed in GraphPad Prism. $p < 0.05$ was considered significant. Manuscript and materials submitted are in conformity with the Acta Physiologica guidelines.[49]

5 | CONCLUSION

In conclusion, we have analyzed and classified the major pathways of glucose absorption in the rat small intestine. The transport mechanisms depend on the luminal concentrations of glucose. At concentrations below 100 mmol/L, the major pathway is the classical saturable pathway, mediated by the secondary active transporter SGLT1 in the apical membrane and the facilitated transporter GLUT2 in the basolateral membrane. The second component is a paracellular route of absorption, which acts in a diffusive manner and therefore is unsaturable. At 100 mmol/L, which is physiologically relevant, the paracellular route contributes around 30% of absorption. However, the paracellular route appears to be active even at low luminal concentrations since we can detect trace amounts of mannitol in the venous effluent. Our results therefore suggest that absorption generally occurs as a combination of trans- and para-cellular transport, but with increased paracellular contribution at higher luminal concentrations.

Importantly, our findings suggest that SGLT1 activation or blockade enhances paracellular absorption. We speculate that SGLT-1-dependent signaling might modulate intestinal leakiness by regulating the tight junctions between enterocytes. We cannot currently provide a molecular mechanism for this effect, but the molecular nature of the tight junctions is under intense investigation,[50,51] not the least after the demonstration of paracellular transport of small peptides, and the results indicate that the passage is plastic and dynamic and may be regulated by, for instance, claudin phosphorylation. SGLT1 activation or perhaps sodium influx may acutely affect the channel properties.

AUTHOR CONTRIBUTIONS

Cecilie Bæch-Laursen: Conceptualization; writing – original draft; writing – review and editing; visualization; formal analysis; data curation; methodology; investigation. **Rune Kuhre Ehrenreich:** Conceptualization; methodology; writing – review and editing. **Ida Marie Modvig:** Methodology; investigation; supervision; writing – review and editing. **Simon Veedfald:** Investigation; methodology; writing – review and editing. **Jens Juul Holst:** Conceptualization; funding acquisition; writing – original draft; writing – review and editing; supervision.

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CONFLICT OF INTEREST STATEMENT

Authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Experimental protocols, materials, and raw data are available upon request.

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