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TRANSPORT PHYSIOLOGY

Adaptive response of equine intestinal Na^+/glucose co-transporter (SGLT1) to an increase in dietary soluble carbohydrate

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Abstract Experimental and epidemiological evidence suggests that consumption of hydrolyzable carbohydrate, hCHO (grain), by horses is an important risk factor for colic, a common cause of equine mortality. It is unknown whether the small intestinal capacity to digest hCHO and/or to absorb monosaccharides is limiting, or even if horses can adapt to increased carbohydrate load. We investigated changes in the brush-border membrane carbohydrate digestive enzymes and glucose absorptive capacity of horse small intestine in response to increased hCHO. Expression of the Na^+/glucose co-transporter, SGLT1, was assessed by Western blotting, immunohistochemistry, Northern blotting, QPCR, and Na^+-dependent D-glucose transport. Glucose transport rates, SGLT1 protein, and mRNA expression were all 2-fold higher in the jejunum and 3- to 5-fold higher in the ileum of horses maintained on a hCHO-enriched diet compared to pasture forage. Activity of the disaccharidases was unaltered by diet. In a well-controlled study, we determined SGLT1 expression in the duodenal and ileal biopsies of horses switched, gradually over a 2-month period, from low (<1.0 g/kg bwt/day) to high hCHO (6.0 g/kg bwt/day) diets of known composition. We show that SGLT1 expression is enhanced, with time, 2-fold in the duodenum and 3.3-fold in the ileum. The study has important implications for dietary management of the horse.

Keywords Glucose transport · Diet · Horse · Adaptation · Small intestine

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Introduction

The horse is a non-ruminant herbivore whose natural diet is pasture forage (grass). It possesses a voluminous and elaborate large intestine with a microbial population uniquely adapted [10] to ferment dietary plant fiber to monocarboxylates, commonly referred to as short chain fatty acids (SCFA), acetate, propionate, and butyrate. A significant proportion of the horse's energy is provided by SCFA absorbed from the cecum and the colon [3]. However, to provide enough energy for the demands of work and performance, domestic horses are fed diets supplemented with readily digestible hydrolyzable carbohydrates (hCHO)

generally in the form of grain; commonly referred to as concentrate diets. Diet, especially grain feeding and recent dietary change (within the previous 2-week period), have been identified by epidemiological and clinical studies as important risk factors for colic, the major cause of equine mortality [8, 17, 18, 20, 27, 42].

It is proposed that, when horses are introduced abruptly to diets containing high levels of hCHO (>0.4% of body weight), a substantial proportion of starch reaches the large bowel [25, 30]. It is then rapidly fermented to metabolites, such as lactic acid, that cause drastic alterations in the cecal/colonic pH, perturb the microbial populations, and dispose the horse to intestinal dysfunction such as colic [16]. The digestive physiology of the horse and its ability to adapt to carbohydrate load are therefore of vital consequence.

Dietary hCHO are broken down in the small intestine of non-ruminant species by pancreatic α-amylase and the brush-border membrane-associated disaccharidase, maltase, into D-glucose. The D-glucose is then transported across the brush-border membrane of the intestinal enterocytes by the Na^+/glucose co-transporter, SGLT1. Glucose then exits the cell across the basolateral membrane by the Na^+-independent monosaccharide transporter, GLUT2 [38]. In many herbivorous and omnivorous species (but not in carnivores), there is an enhancement in the expression of intestinal SGLT1 and the capacity to absorb monosaccharides in response to increased dietary carbohydrate levels [6, 15, 28, 39]. This increase is also observed in response to introduction of glucose and a range of monosaccharides into the small intestinal lumen [24, 40].

It has been shown that glucose is absorbed in the small intestine of the horse, and that this nutrient can fulfill some of the animal's basal energy requirements [2, 35]. However, until recently, there was very little information on the mechanism and intestinal site of monosaccharide absorption in the equine small intestine. We showed previously that, in horses maintained on pasture forage, the major site of monosaccharide absorption is in the proximal intestine (duodenum>jejunum), with lower expression of SGLT1 in the ileum [11]. Furthermore, we determined that the disaccharidases, sucrase, lactase, and maltase are expressed in the equine small intestine; maltase having a much higher activity compared to other species [11].

Here, we determined the expression profile of SGLT1 along the small intestine of horses maintained, long term (months), on diets supplemented with concentrate feeds rich in hCHO (e.g., oats, corn). We then assessed any changes that may have occurred in SGLT1 expression, compared to horses maintained on forage pasture [11], in response to increased dietary hCHO. Subsequently, we investigated the effect on SGLT1 expression of a switch from a hay-only diet to diets containing increasing levels of concentrate in horses under strictly controlled conditions.

Materials and methods

Animals and collection of tissue

Intestinal tissue from six adult (7- to 12-year-old) mares and geldings of various breeds, maintained routinely on diets supplemented with concentrate feeds rich in hCHO (e.g., oats, corn), were collected from the University of Liverpool Large Animal Hospital, immediately after death. The animals were euthanized for conditions other than gastro-intestinal-related problems. Sections of small intestine approximately 20 cm in length were removed from the proximal (30 cm distal to the pylorus), mid (half way along the small intestine), and distal (30 cm proximal to the ileo-cecal junction) intestine. Sections were opened longitudinally, rinsed in ice-cold 0.9% (*w*/*v*) NaCl pH 7.4, and blotted with paper towels to remove excess mucous. Samples of the tissue were fixed for histological examinations and immunohistochemical studies. The mucosa from the remainder of the tissue was then removed by scraping. Mucosal scrapings were wrapped in aluminum foil and frozen immediately in liquid nitrogen. Following transportation to the laboratory, frozen tissue samples were stored at −80°C until use. Histological examination of samples of fixed tissue confirmed that they were intact, with epithelial cells attached.

Furthermore, six mature (5- to 12-year-old) standardbred geldings with mean bodyweight (±SD) 453±28 kg were fed a controlled all-hay diet (2% of body weight (bwt) as timothy hay supplemented with vitamins and minerals) for 3 months (diet period 1) before harvest (biopsy 1 [B1]) of duodenal and ileal (at the junction of jejunum and ileum) mucosal biopsies via a laparoscopic technique [4] (Fig. 1 and Table 1). The biopsy samples were rinsed in saline and frozen immediately in liquid nitrogen. Horses were then introduced to a diet consisting of 60% hay, 40% grain (2/3 oats, 1/3 corn; 3.3 g starch/kg bwt/day; diet period 2). Biopsies (duodenal and ileal) were removed after 1 week (B2) and 1 month (B3) and frozen as above. At this time, horses were switched to 40% hay, 60% grain (6.0 g starch/kg bwt/day) for a further month (diet period 3) before duodenal

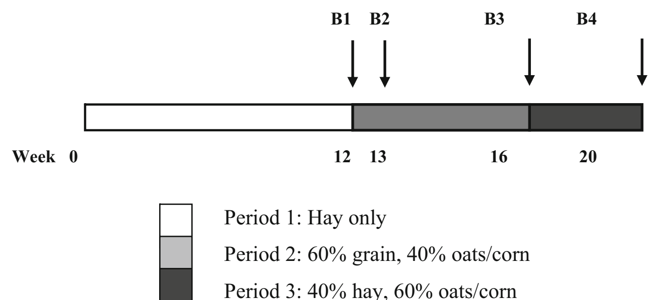


Fig. 1 Schematic of experimental design for the controlled feeding study. *B1*, *B2*, *B3*, and *B4* indicate timing of small intestinal biopsy collection

Table 1 Estimated dietary intake per horse on a daily basis and calculated dietary macronutrients during each phase of the study

	Dietary period	1	2	3
Feed intake, kg/day	Hay	10.5±0.9	6.2±0.6	4.2±0.3
	Oats	0	2.5±0.3	3.9±0.4
	Cracked corn	0	1.5±0.2	2.3±0.3
Nutrient intake, % of dry matter intake	Crude protein	11.0±0.9	11.4±0.8	11.3±1.0
	Acid detergent fiber	35.0±1.2	24.5±1.6	19.9±0.9
	Neutral detergent fiber	44.1±2.1	39.9±1.7	32.5±1.6
	WSC	4.9±0.9	2.3±0.9	1.5±0.9
	Starch	2.1±0.9	18.1±0.9	32.9±0.9
	Ether extract	2.7±0.9	3.3±0.9	3.6±0.9

Data (n=4 samples per dietary period) are expressed as mean±S.E.M.
WSC water-soluble carbohydrates

and ileal biopsies were removed (B4). The hay and grain ration was divided into three equal portions and fed at 07.00, 13.00 and 18.00 h. Horses were housed in box stalls with a minimum of 3 h/day turnout into a dry lot. Animals had free access to water and salt block at all times. All surgical procedures and animal treatments were handled according to the practices of humane animal care with the approval of the University of Guelph Animal Care Committee.

Isolation of brush-border membrane vesicles

Brush-border membrane vesicles (BBMV) were prepared from equine small intestinal mucosal scrapings using a method based on that of Shirazi-Beechey et al. [37] as described [21]. The final pellet, containing purified BBMV, was homogenized in 300 mM mannitol, 20 mM HEPES/Tris, pH 7.4, 0.1 mM $MgSO_4$, and 0.02% (*w*/*v*) NaN_3 buffer by passing through a 27-gauge needle several times. The BBMV were divided into aliquots and stored in liquid nitrogen until use. All steps in the procedure were carried out at 4°C. The protein concentration in the BBMVs was estimated by its ability to bind Coomassie blue according to the Bio-Rad assay technique. Bovine γ-globulin was used as the standard [11].

Enzyme assays

Maltase and sucrase activities were measured at 38°C in BBMV and cellular homogenates as described previously [11], using maltose or sucrose as substrates, respectively. D-Glucose released, as the result of substrate hydrolysis, was measured using a commercial kit (R-Biopharm RHÔNE Ltd., Glasgow, UK).

Measurement of monosaccharide transport

Na^+-dependent glucose uptake into BBMV was measured as described [11, 37]. The uptake of D-glucose was initiated by the addition of 100 µl of incubation medium containing 100 mM NaSCN (or KSCN), 100 mM mannitol, 20 mM HEPES/Tris, pH 7.4, 0.1 mM $MgSO_4$, 0.02% NaN_3, and 0.1 mM D-[$U^{14}C$]glucose (GE Healthcare, Little Chalfont, UK) to BBMV (100 µg protein). The reaction was stopped after 3 s by addition of 1 ml of ice-cold stop buffer, containing 150 mM KSCN, 20 mM HEPES/Tris, pH 7.4, 0.1 mM $MgSO_4$, 0.02% (*w*/*v*) NaN_3, and 0.1 mM phlorizin. A 0.9-ml portion of the reaction mixture was removed and filtered under vacuum through a 0.22-µm pore cellulose acetate/nitrate filter (GSTF02500, Millipore, Watford, UK). The filter was washed with 5×1 ml stop buffer, placed in a vial containing 4 ml of scintillation fluid (Scintisafe, Fisher Scientific, UK), and the radioactivity retained on the filter measured using a LS 6500 multi-purpose scintillation counter (Beckmann-Coulter, High Wycombe, UK). All uptakes were measured in triplicate. For kinetic studies, the initial rate, 3 s, of D-glucose uptake was measured at glucose concentrations of 0.01–5 mM D-glucose. To assess the activity of any potential facilitative glucose transporter, the initial rate of uptake of 1 mM 2-deoxy-D-glucopyranoside, a specific substrate of Na^+-independent D-glucose transport isoforms, into BBMV was determined at 38°C in incubation medium consisting of 300 mm mannitol, 20 mM Hepes/Tris, pH 7.4, 0.1 mM Mg SO_4, and 0.02% (*w*/*v*) NaN_3 in the presence and absence of 50 µM cytochlasin B as described [21].

Western blot analysis

The abundance of SGLT1 protein in the BBMV was determined by Western blotting as described previously [11]. Protein components of BBMV were separated by SDS-polyacrylamide gel electrophoresis on 8% (*w*/*v*) polyacrylamide mini-gels, containing 0.1% (*w*/*v*) SDS, and electrotransferred to PVDF membrane (Immun-Blot, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). The PVDF membranes were blocked by incubating for 1 h in TTBS (140 mM NaCl, 40 mM Tris/HCl, pH 7.4, 0.05% (*v*/*v*) Tween 20) containing 5% (*w*/*v*) non-fat dried milk. The membranes were then incubated for 1 h at room temperature with SGLT1 antibody diluted 1:2,000 in TTBS containing 1% (*w*/*v*) non-fat dried milk. The antibody to SGLT1 was raised in rabbits (custom synthesis) to a

synthetic peptide corresponding to amino acids 402–420 (STLFTMDIYTKIRKKASEK), a conserved intracellular loop region of SGLT1. Equine SGLT1 shares a high degree of homology with other species over this region [11]. This antipeptide antibody which identifies the same specific immunoreactive band as the antibody raised to the recombinant SGLT1 protein [45] has been successfully used in a number of our studies during the past 14 years [24, 28]. Immunoreactive bands were detected by incubation for 1 h with affinity purified horseradish peroxidase-linked swine anti-rabbit secondary antibody (DAKO Ltd., Cambridge, UK) diluted 1:2,000 in TTBS solution, and visualized using an enhanced chemiluminescence system (GE Healthcare), according to the manufacturer's instructions. The intensity of the immunoreactive bands detected in the BBMV was quantified using scanning densitometry (Phoretix 1D Quantifier).

The PVDF membranes were stripped by three 10-min washes in 137 mM NaCl, 20 mM glycine/HCl, pH 2.5 and then probed with monoclonal antibodies raised against villin (Abcam, Cambridge, UK) and β-actin (Sigma-Aldrich) as controls.

Immunohistochemical localization of equine intestinal glucose transporters

Immunohistochemistry was carried out based on previous procedures [14, 28] with some modifications. Equine small intestinal tissue sections fixed for 4 h in 4% (*w*/*v*) paraformaldehyde/phosphate buffered saline (PBS) were paraffin wax embedded and sectioned at a thickness of 5–7 μm onto poly-L-lysine-coated slides. The slides were dewaxed in 100% xylene three times for 10 min each, dehydrated twice in 100% ethanol for 10 min, and twice in 70% ethanol for 5 min. Slides were then rehydrated twice in doubled distilled H_2O for 5 min each. Slides were immersed in antigen retrieval buffer (1 M Tris/HCl pH 10) and autoclaved twice at 121°C and 15 psi for 15 min each. Subsequently, the slides were washed three times for 5 min in PBS followed by 1-h incubation in blocking solution (3% BSA, 0.1% NaN_3, and 2% donkey serum/PBS) at 25°C in a humidified chamber. Sections were incubated overnight at 4°C with primary antibody either against SGLT1, GLUT2 (C-terminus), or GLUT2 (residues 40–55), all diluted 1:200. We have cloned and sequenced cDNA encoding for equine GLUT2 (accession number AJ715983) and have deduced the amino acid sequence (CAG29734). The antibody to equine GLUT2 was raised in rabbits (custom synthesis) to a synthetic peptide (AAVEMEFLGATETA) corresponding to amino acids 523–536 of equine GLUT2 C-terminus region. There is a high degree of homology in this region between rat, human, and horse GLUT2. A commercial antibody (Chemicon [now part of Millipore], UK) raised against a peptide consisting of 16 amino acids (PQEVIISHYRHVLGVP) and residues 40–55 of rat GLUT2 (which is identical to the equine sequence) was also used. The antibody to SGLT1 was the same used in Western blot analysis (see above). The slides were then washed in PBS five times for 5 min and incubated with FITC-conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:500. Finally, the slides were washed with PBS five times for 5 min and mounted in Vectashield hard set mounting media with DAPI (Vector Laboratories, Burlington, CA, USA). The immunostaining was visualized using an epifluorescence microscope (Nikon, UK) and images were captured with Hamamatsu digital camera (C4742-95). Control sections were subjected to the same protocol except omitting the primary antibodies for SGLT1 and GLUT2.

RNA isolation, qPCR, and Northern blot analysis

Total RNA isolated from intestinal tissue using the RNeasy Mini Kit with on-column DNase 1 digestion (Qiagen, Crawley, UK) was used for cDNA synthesis using Supercript III reverse transcriptase (Invitrogen) and oligo$(dT)_{12–18}$ primers. cDNA was purified using QiaQuick PCR purification kit (Qiagen) and qPCR assays were performed using 25 ng of cDNA per reaction. PCR primers for the amplification of the Na^+/glucose co-transporter (SGLT1, *SLC5A1*), along with β-actin (ACTB), were designed using Primer Express (Applied Biosystems, Warrington, UK) and purchased from Eurogentec (Belgium) (Table 2). Each qPCR reaction contained 5 μl of cDNA template (25 ng), 1× JumpStart Taq ReadyMix with SYBR green (Sigma-Aldrich, Poole, UK), and 300 nM of each primer in a total volume of 25 μl. QPCR cycling was performed as follows: initial denaturation at 95°C for 2 min followed by 30–40 cycles of 95°C for 15 s, 60°C for 60 s.

Table 2 Equine real-time PCR primers

Gene	Accession number	Primer	Sequence
SGLT1	NM_001081872	Sense	TGTCGGGCTGTGGGCTAT
		Anti-sense	TTCGTCCTGCGAGGAAGAAG
ACTB	NM_001081838	Sense	TCACGGAGCGTGGCTACAG
		Anti-sense	CCTTGATGTCACGCACGATT

All reactions were performed in triplicate using a Rotor-Gene 3000 (Corbett Research). Relative amounts of mRNA were normalized to β-actin mRNA within each sample.

For Northern blot analysis, total RNA samples, 3 µg per lane, were separated on 1% agarose gels containing 0.66 M formaldehyde and transferred to nylon membrane (Hybond N+, GE Healthcare). RNA integrity and equality of loading were determined by methylene blue staining. Northern blotting was performed as described, using ^{32}P-labelled equine SGLT1 cDNA (nucleotides 1406–2025) as the probe [11]. The intensity of the total area of all transcripts was quantified using scanning densitometry (Phoretix 1D Quantifier, Non-linear Dynamics Ltd., Newcastle upon Tyne, UK).

Statistics

Data are expressed as mean±SEM and statistical comparisons are made, where appropriate, using either two-way ANOVA, one-way repeated measure ANOVA (with Bonferroni post test) or with unpaired two-tailed Student's *t* test (GraphPad Prism 5). Results were considered significant if $P<0.05$.

Results

Disaccharidase activity in the small intestine of horses maintained on a concentrate diet

We have previously measured the specific activities of sucrase and maltase in duodenum, jejunum, and ileum of horses maintained on pasture forage [11]. This study indicates that the specific activities of these disaccharidases in duodenum, jejunum, and ileum of horses on concentrate diets (Table 3) are similar to those maintained on grass [11].

Glucose transport in the small intestine of horses maintained on a concentrate diet

We demonstrated previously that in equine small intestine D-glucose is transported across the brush-border membrane into enterocytes by a Na^+-dependent process with characteristics of that of SGLT1 [11, 37]. Furthermore, in horses maintained on a forage pasture diet, the highest rates of transport were in the duodenum>jejunum and very little in the ileum [11]. The initial rates of Na^+-dependent D-glucose transport in BBMVs isolated from the intestines of horses maintained, for months, on concentrate feeds are shown in Fig. 2a. Rates of D-glucose uptake in the duodenum (180.0±28.5 pmol/s/mg protein) were identical to those reported previously in horses fed pasture forage [11]. However, horses on a concentrate diet had 1.9-fold higher (215.5±17.4 vs. 115.9±33.0; $P=0.04$) rates of uptake in the jejunum and 3.7-fold higher rates in the ileum (139.5±7.6 vs. 38.4±16.4; $P=0.005$) than the forage pasture-fed animals. Kinetic analysis indicated that the increase in transport rates observed in the intestine of animals maintained on a concentrate diet compared to those on pasture forage was due to an enhancement in V_{max}, and not the K_m. The K_m values were 0.49±0.06 mmol/l [21] vs. 0.47±0.14 mmol/l in grass- and concentrate-fed animals, respectively. The V_{max} values in animals maintained on concentrate diets were 2,413±147, 2,883±400, and 1,707±61 pmol/s/mg protein in duodenum, jejunum, and ileum, respectively. Compared to V_{max} values in animals on pasture forage [11], there was 3.5-fold increase in the ileum and 2-fold increase in the jejunum with no change in the V_{max} value in the duodenum. This suggests that the increase in transport rates may be due to an enhancement in the number of SGLT1 molecules (see below) and not the affinity of the transporter for its substrate.

There was no cytochalasin B-sensitive 2-deoxy-D-glucose transport detected in BBMV samples, indicating the absence of GLUT2 from the BBMVs.

SGLT1 protein and mRNA abundance in the intestine of horses fed a concentrate diet

To determine if the increase in D-glucose transport seen in the jejunum and ileum of concentrate-fed horses is due to an increase in SGLT1 protein abundance, as suggested by the kinetic analysis, Western blot analysis was carried out (Fig. 2b). SGLT1 protein abundance in the duodenum of concentrate-fed horses were identical (750.0±250 vs. 714±91.0; $P=0.867$) to those measured in the duodenum of grass-fed horses as reported previously [11]. However, it was 2-fold higher (876±199 vs. 429±78; $P=0.028$) in the jejunum and 5.1-fold higher (551±124 vs. 107±70; $P=0.011$) in the ileum of concentrate-fed animals (Fig. 2b) vs. grass-fed animals. The data confirm that the increase in glucose transport in the intestine of the concentrate-fed animals is due to an increase in the number of SGLT1 protein molecules. The levels of the structural proteins villin and β-actin were identical in animals fed on either

Table 3 Disaccharidase activity in BBMV isolated from the intestine of concentrate-fed horses (mean±S.E.M.)

Enzyme	Region	Specific activity (µmol/min/mg protein)
Sucrase	Duodenum	0.187±0.023
	Jejunum	0.470±0.116
	Ileum	0.273±0.061
Maltase	Duodenum	0.317±0.089
	Jejunum	0.929±0.187
	Ileum	0.697±0.151

grass or concentrates indicating that the increase in SGLT1 is not due to a general enhancement in intestinal structure. This was further confirmed by morphometric analysis which showed that villus heights and widths were similar in each region of the intestine, independent of diet (data not shown). It is noteworthy that the activities of sucrase and maltase (Table 3) were similar in the intestine of concentrate-fed horses and those on forage pasture measured previously [11].

The expression of SGLT1 mRNA in the intestine of horses fed concentrate diets was investigated by Northern blot analysis and quantitative PCR (Fig. 2c,d). We used Northern blot analysis to be able to compare our present data with our previous work in which this technique was used to measure SGLT1 mRNA expression in the intestinal tissues of horses maintained on pasture forage [11]. Densitometric analyses of Northern blot autoradiograms indicated that levels of SGLT1 mRNA in the duodenum of horses maintained on concentrate diets were similar to those on grass diets (1467±271 vs. 1301±145; P=0.6) [11]. However, mean SGLT1 mRNA levels were increased 1.7-fold in the jejunum (1739±163 vs. 1025±130; P=0.006) and 3.1-fold in the ileum (1155±168 vs. 374±81; P=0.0004) of concentrate-fed horses compared to grass-fed animals. Northern blot data (Fig. 2c) was confirmed by QPCR analyses (Fig. 2d). SGLT1 mRNA levels, normalized to β-actin (ACTB), were similar in the duodenum of concentrate- and grass-fed animals (1.27±0.22 vs. 1.10±0.14; P=0.517), but were increased by 1.9-fold in the jejunum (1.52±0.38 vs. 0.81±0.16; P=0.116) and 3.2-fold in the ileum (1.12±0.25 vs. 0.35±0.06; P=0.015) of horses maintained long term on diets containing concentrates. There was a good quantitative correlation between rates of glucose transport, SGLT1 protein, and mRNA abundance in individual animals, implying that dietary carbohydrate regulation of equine intestinal SGLT1 expression is predominantly controlled at the level of mRNA abundance.

Adaptation in intestinal SGLT1 expression

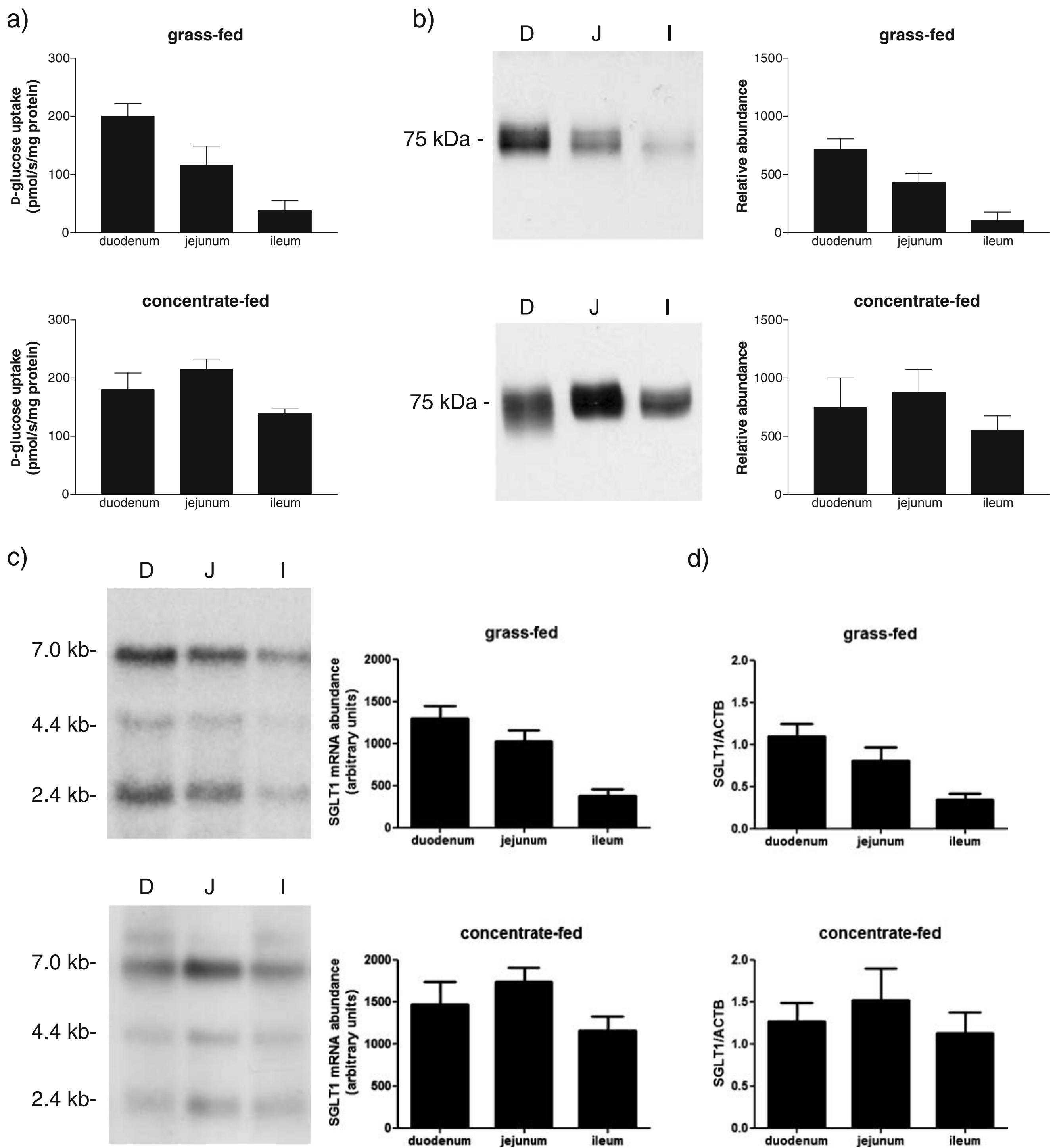
To determine the effects of the level of concentrate feeding on the magnitude and time course of SGLT1 induction, horses maintained on a hay-only diet were placed on diets containing increasing levels of hCHO in the form of oats and corn. Mucosal biopsies were removed from the duodenum and ileum for analysis of SGLT1 expression by QPCR (Fig. 3).

In dietary period 1 of the experiment when horses were on a forage only diet, SGLT1 mRNA abundance was higher in the duodenum (1.28±0.16 U; Fig. 3a) than in the ileum (0.49±0.14 U; Fig. 3b) in agreement with our previous results [11] and results from this study (Fig. 2d). After 1 week of concentrate feeding (dietary period 2) there was no change in SGLT1 expression in the duodenum (1.28±0.16 vs. 1.71±0.17; P=0.102; Fig. 3a) but a 2-fold increase (0.57±0.14 vs. 1.15±0.11; P=0.0072) in SGLT1 expression in the ileum (B1 vs. B2; Fig. 3b). After a further 1 month on the same level of concentrate (B3), SGLT1 expression in the duodenum was significantly increased to nearly twice the original level (1.28±0.16 vs. 2.31±0.39, P=0.048; Fig. 3a), but ileal SGLT1 expression remained at B2 levels (0.57±0.14 vs. 1.23±0.17, P=0.0125) (B1 vs. B3; Fig. 3b). When the hCHO content of the diet was increased to 6.0 g/kg bwt/day for a further month (B4), duodenal SGLT1 expression (2.39±0.44) was unchanged relative to that observed at the end of dietary period 3 (B3 vs. B4; Fig. 3a). There was, however, a further increase in ileal SGLT1 to 3.3-fold higher than the level in B1 (0.57±0.14 vs. 1.91±0.38; P=0.0067; Fig. 3b). Therefore, it appears that the equine intestinal glucose transporter expression is enhanced, with time (see "Discussion" section below), in response to increased hCHO. This is accomplished through enhancement in SGLT1 expression not only in the proximal but predominantly in the distal small intestine.

Expression of intestinal glucose transporters in equine small intestine

We carried out immunohistochemistry to determine the expression of intestinal glucose transporters, SGLT1 and GLUT2, in the small intestinal tissues of horses maintained on concentrate diets. Figure 4a shows specific reaction of the GLUT2 antibody (targeted to the C-terminus region of equine GLUT2) with the basolateral domain of equine enterocytes. Figure 4b demonstrates that the antibody targeted to the extracellular loop between transmembrane

Fig. 2 Effect of diet on SGLT1 activity and expression. **a** The initial (3 s) rate of 0.1 mmol/l D-glucose transport into BBMV, measured at 38°C in the presence of 100 mmol/l NaSCN, as described in "Materials and methods" section. All uptakes were measured in triplicate and values are presented as mean±SEM (n=6–10). **b** Protein components of BBMV (20 μg of protein/lane) isolated from duodenum (D), jejunum (J), and ileum (I) were separated on 8% polyacrylamide gels, electrotransferred to PVDF membranes, and Western blotted using a SGLT1 polyclonal antibody, as described in "Materials and methods" section. Results of a typical Western blot showing the presence of a 75-kDa SGLT1 protein, along with densitometric analysis of Western blots carried out using Phoretix 1D. Data are expressed as mean±SEM (n=6–10) in arbitrary units. **c** Total RNA (3 μg per lane) was separated on 1% agarose denaturing gels, transferred to nylon membrane, and probed with a ^{32}P-labelled SGLT1 cDNA probe, as described in "Materials and methods" section. Results of a typical northern blot showing SGLT1 transcripts with combined densitometric analyses of Northern blots carried out using Phoretix 1D. Data expressed as mean±SEM (n=6–10) in arbitrary units. **d** Steady-state levels of SGLT1 mRNA were determined by QPCR normalized to β-actin (ACTB) expression. All values are expressed relative to SGLT1 in the duodenum of horses on grass diets as means±SEM. Data were generated in triplicate (n=6–10)



regions 1 and 2 of equine GLUT2 also locates this glucose transporter entirely to the basolateral membrane. The signal is specific since there is no labelling when primary antibodies are omitted (see Supplementary Figure 1, c and d). Figure 4c and d (100× magnification) demonstrates that there is no GLUT2 on the brush-border membrane of enterocytes using either of the GLUT2 antibodies. In contrast, using an antibody to SGLT1, a continuous labelling of the brush-border membrane is observed (Fig. 4e).

Supplementary Fig. 1a and b (10× magnification) shows GLUT 2 expression on the basolateral membrane of a number of villi detected by both GLUT2 antibodies; in addition, the figure indicates that villi were intact with the cells attached (Supplementary Fig. 1, a and b). This pattern

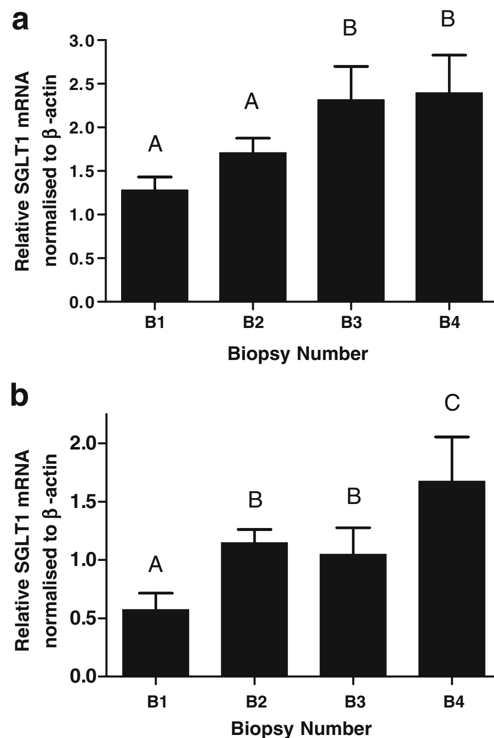


Fig. 3 Effect of dietary hCHO on SGLT1 expression. Horses on hay-only diets (*B1*) were switched to 60% hay, 40% grain (3.3 g starch/kg bwt/day). Biopsies (duodenal and ileal) were removed after 1 week (*B2*), and 1 month (*B3*). At this time, horses were switched to 40% hay, 60% grain (6.0 g starch/kg bwt/day) for a further month before duodenal and ileal biopsies were removed (*B4*). Steady-state levels of SGLT1 mRNA were determined by QPCR in RNA isolated from duodenal (**a**) and ileal (**b**) biopsies; data were normalized to ACTB expression. All values are expressed relative to SGLT1 in the duodenum of horses on hay-only diets (1) as means±SEM. Data were generated in triplicate, with $n=6$ animals in each group. Statistically significant results ($P<0.05$) are indicated by different letters above *error bars* (*ABC*) based on one-way ANOVA with the Bonferroni post test

of expression for SGLT1 and GLUT2 was similar in all regions of the small intestine of horses maintained on the concentrate diet.

Discussion

Nutrient transporters, expressed on the apical membrane of intestinal absorptive cells, are directly exposed to an environment which changes significantly with diet, and consequently the expression of many is adaptively regulated by dietary substrates. The horses' natural diet, grass, undergoes seasonal variation in its soluble (hydrolyzable) carbohydrate content [19]. However, this variation is much less than in the natural diet of most omnivores, and is certainly less than the difference between grass and the high grain (starch) diets fed to many horses in managed environments. It has been proposed that the equine intestine may have a slower or blunted adaptive response to dietary change [7], which may be an important consideration for the development of dietary-induced intestinal dysfunction in horses.

It is not known however if the slower adaptive response is in the digestion or the absorption of dietary carbohydrates. Starch is mainly hydrolyzed in the small intestine by pancreatic α-amylase and the intestinal brush-border membrane disaccharidase, maltase, to glucose [38, 39]. We have shown that the activity of maltase is high in equine small intestine compared with that in other species [11]. It has been proposed that this ability has evolved to digest large quantities of disaccharides that are present in the horse's natural grass diet [22], and it is unlikely, therefore, that there is a deficiency in maltase activity limiting starch digestion in the horse [34]. However, it has been shown that the activity and the concentration of α-amylase in the equine intestine are low, compared to other species [23, 33]. It has been suggested that it is likely that the breakdown of starch into maltose, maltotriose, and α-dextrin may limit starch digestion in horses given diets containing large amounts of grain [32].

In this study, we compared the activity of disaccharidases and the activity/expression of the glucose transporter, SGLT1, along the length of the small intestine of horses that had been maintained on feeds rich in hCHO, with those of horses maintained on grass.

Our data indicate that horses routinely fed diets containing grain (oats, corn, barley) have an increased capacity for D-glucose absorption compared to horses maintained on grass. This adaptation is through an increase in the expression of SGLT1 in the mid and distal intestine. SGLT1 protein levels are increased 2-fold in the jejunum and 4- to 5-fold in the ileum and this is reflected in a concomitant increase in the rates of glucose transport and SGLT1 mRNA abundance. This increase is not due to a general trophic response, as levels of the structural proteins β-actin and villin remained constant. A similar pattern of increase in mRNA, protein abundance, and glucose transport function occurs for the basolateral membrane glucose transporter, GLUT2, in the intestine of horses maintained on concentrate diets [36]. Collectively, the data indicate that, in response to increased dietary carbohydrate, there is a coordinated enhancement in the rate of glucose transport across the luminal and basolateral membrane of equine enterocytes resulting in enhanced transcellular transport of glucose from the lumen of the intestine into the blood.

To determine the effects of the level of concentrate feeding on the magnitude and time course of SGLT1 induction, in a controlled manner, horses maintained on a hay-only diet were placed on diets containing increasing

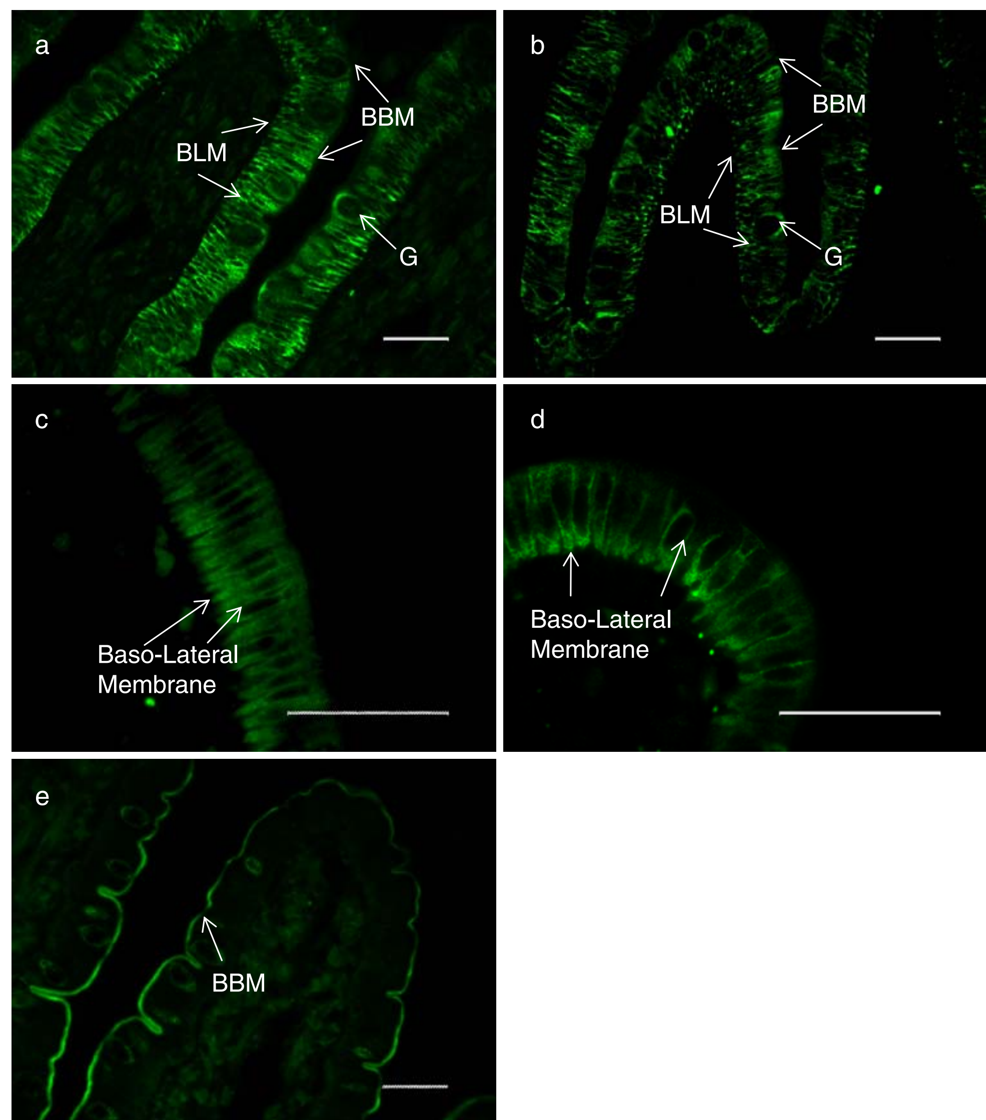


Fig. 4 Detection and localization of GLUT2 and SGLT1 in small intestine of horses maintained on concentrate diets. Immunofluorescent detection of GLUT2 in sections of horse jejunum, using either the antibody targeted to the C-terminus region of GLUT2 (**a**, ×40 magnification; **c**, ×100 magnification) or to the extracellular loop between transmembrane regions 1 and 2 of GLUT2 (**b**, ×40 magnification; **d**, ×100 magnification). **e** Immunofluorescent detection of SGLT1 (×40 magnification) in a section of equine jejunum. *Scale bar*=20 μm. *BLM* basolateral membrane, *BBM* brush-border membrane, *G* goblet cell

levels of hCHO. The results not only confirmed our previous findings [11] but also extended them to show that with time (1 month on 40% grain followed by a further month on 60% grain) there is a significant 2-fold and 3-fold increase in SGLT1 expression in the proximal and distal small intestine, respectively. During this period (and afterwards), the animals remained healthy and had no signs of any digestive diseases or abdominal pain associated with colic. It has been shown that a rapid dietary change disposes the horse to severe abdominal pain and digestive complications associated with colic [20, 25]. Our findings that the gradual increase in starch content of the diet to 6.0 g/kg bwt/day can be tolerated with no ill effects have practical applications in feeding management and recommendations. It indicates that dietary change should be carefully managed to minimize the risk of colic.

It has been proposed that, in response to high concentrations of lumenal glucose (or fructose), GLUT2 (typically a basolateral membrane glucose transporter) is translocated to the brush-border membrane of enterocytes where it can transport both glucose and fructose. The proposed mechanisms for GLUT2 translocation have been revised a number of times [21, 26]. However, as yet, no experimental evidence has been provided to confirm such a translocation. With the availability of modern imaging techniques and protein tagging procedures, it should be possible to determine the translocation of a protein, in response to a stimulus such as high extracellular glucose concentration, from an intracellular location to the apical plasma membrane. Such established techniques have been successfully used, for example, to determine the translocation of the glucose transporter isoform 4, GLUT4, from intracellular stores to the plasma membrane in response to insulin stimulation [29, 41].

In this study, we determined the expression of the GLUT2 protein, using immunohistochemistry, with intesti-

nal tissues obtained from horses on concentrate diets. Our data using two different antibodies targeted to either the C-terminus region or to the residues on the extracellular loop between transmembrane regions 1 and 2 of equine GLUT2 both show that GLUT2 is expressed only on the basolateral membrane of equine enterocytes and not on the brush-border membrane. This observation is supported by our functional studies in which there was no uptake of 2-deoxy-D-glucopyranoside, a specific substrate of Na^+-independent glucose transporters, into the same population of BBMV that could transport glucose in a Na^+-dependent manner. In addition, glucose uptake into BBMV was not inhibited by cytochlasin B, the specific inhibitor of GLUT transporters. The two GLUT2 antibodies have also been used for the immunohistochemical localization of GLUT2 in rat small intestine [1], and it has been concluded that the antibody targeted to the residues on the extracellular loop detects GLUT2 on the brush-border membrane of rat enterocytes [1]. The data presented, however, is open to interpretations that differ to the conclusions made [1]. In support of our findings, Cui et al. [9] have shown, using Western blotting, that GLUT 2 is not expressed on the brush-border membrane of rat enterocytes luminally perfused in vivo with either 100 mM fructose or 100 mM glucose for 4 h [9]. In our previous studies on intestinal expression of glucose transporters in a number of species, including humans, we also have shown that GLUT2 is only expressed on the basolateral membrane and not on the brush-border membrane [12]. As indicated (Wright et al. [46]) the presence of GLUT2 in the brush-border membrane would short circuit glucose transport via SGLT1. That is, that the accumulation of glucose in the enterocytes would be reduced or eliminated by the efflux of sugar out across the brush-border membrane by GLUT2, a bi-directional facilitative glucose transporter that transports glucose down its concentration gradient, either in or out of the cell [46].

It has been shown in a wide range of species (excluding carnivores) that SGLT1 expression is upregulated by monosaccharides, but not by starch [15, 24, 40, 44]. Our recent work has identified the underlying mechanism involved in enhancement of SGLT1 expression by dietary monosaccharides [28]. A heterodimeric guanine nucleotide binding protein (G-protein)-coupled receptor, consisting of sweet taste receptor 1 family subunits (T1R2+T1R3) and the α-subunit of the associated G-protein, gustducin, are required for upregulation of SGLT1 by lumenal monosaccharides [13, 28]. The receptor is expressed and associated with the luminal membrane of the enteroendocrine cells of the small intestine in mice and human and changes in the concentration of lumenal sugar are detected by this receptor [28]. This stimulates a signaling pathway resulting in secretion of gut hormones from the enteroendocrine cells, which, in a paracrine manner, enhance the expression of SGLT1 in the absorptive enterocytes [28]. Further work in our laboratory has demonstrated that the sweet taste receptor subunits and gustducin are also expressed in the equine small intestine (Dyer, Al-Rammahi, and Shirazi-Beechey, unpublished data). G-protein-coupled signaling mechanisms are known to be rapid processes, and we have recently shown that SGLT1 upregulation in response to increased dietary sugars occurs rapidly in mouse intestine. This leads to enhanced SGLT1 expression in the brush-border membrane of all villus enterocytes (Moran, Arora, and Shirazi-Beechey, unpublished data). Our observation that, in the horse small intestine, SGLT1 is slowly upregulated, with time, infers that this lag period may be due to some other factors such as the time required for α-amylase upregulation. Studies carried out in other species have indicated that, with time, there is an adaptive response in amylase concentration and synthesis to increases in hydrolyzable dietary carbohydrates [5]. Although the precise underlying mechanisms are not known, insulin and oral/blood glucose have been proposed to be mediators of pancreatic adaptation to dietary carbohydrates [5]. It is known that, in horse, amylase activity is upregulated in response to increased dietary hydrolysable carbohydrate intake. Kienzle et al. [23] have shown that the mean activity of amylase in jejunal chyme amounted to 22.3 U/g wet weight. This was considerably higher when horses were fed grain diets (30.8±15.4 U/g chyme) for 24 h rather than maintained on an all-hay diet (15±3.4 U/g). However, the increase was much smaller than in omnivorous species such as pigs or even in the carno-omnivorous dog.

Nothing is known about the underlying mechanism controlling equine pancreatic amylase upregulation to increased dietary carbohydrate. Since horses are considered to be more insulin resistant compared to omnivores [31], one may postulate that this factor may play a role for the delay in amylase upregulation in the horse. In support of this proposition, it has been shown that, in hyperinsulinemic diabetes (obese Zucker rat model), due to peripheral insulin resistance, there is a decrease in amylase content and synthesis (5- to 6-fold). Ciglitazone treatment, which reverses insulin resistance and normalizes glucose metabolism, restores amylase content and synthesis [43].

It is not known if, in horse, a longer term adaptation period is needed for enhancement in α-amylase activity in response to increased dietary hCHO [32]. Since SGLT1 expression is only upregulated by lumenal monosaccharides and not by starch, we propose that starch hydrolysis increases over time, to release glucose into the lumen of the intestine during the dietary change period (1 week to 1 month). The released glucose then enhances the expression of SGLT1 in first the proximal and then the distal small intestine. This

suggests that α-amylase activity in horse can be enhanced but requires a longer time for adaptation.

A better knowledge of the equine digestive system, at the molecular and cellular levels, and identification of the mechanisms underlying the intestinal adaptive processes in response to a dietary change will assist the development of rational strategies to modify the capacity of the intestine to digest and absorb dietary carbohydrates. This will facilitate a more scientifically based approach to designing feed formulation and management with the ultimate aim of enhancing the health of the horse and reducing the incidence of intestinal disorders such as colic.

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