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Regulation of D-Fructose Transporter GLUT5 in the Ileum of Spontaneously Hypertensive Rats

A. Mate[1], A. Barfull[2], Á.M. Hermosa[1], J.M. Planas[2], C.M. Vázquez[1]
[1]Department of Physiology and Zoology, Faculty of Pharmacy, University of Seville, C/Profesor García González 2, E-41012 Seville, Spain
[2]Department of Physiology-Division IV, Faculty of Pharmacy, University of Barcelona, Avda. Joan XXIII s/n, E-08028 Barcelona, Spain

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Abstract. Abnormalities in carbohydrate metabolism and the insulin resistance status have been associated with hypertension. We have previously described alterations in the sodium-coupled sugar absorption in an experimental model of hypertension; in the present work, we studied the regulation of the sodium-independent, GLUT5-facilitated D-fructose intestinal transport in this pathology. Spontaneously hypertensive rats (SHR) and their normotensive, genetic control Wistar-Kyoto (WKY) rats, were used. Kinetic studies, carried out in ileal brush-border membrane vesicles (BBMVs), revealed a significant reduction ($P < 0.05$) in the maximal rate of transport (V_{max}) for D-fructose in SHR, which, on the other hand, showed unaltered values for the Michaelis constant (K_m) and the diffusion constant (K_d). Immunoblotting analysis revealed the existence of lower ($P < 0.05$) levels of GLUT5 in apical membranes from SHR, this reduction being similar to that of V_{max}. Similarly, Northern blot studies on the abundance of GLUT5 mRNA from ileal enterocytes showed a decrease ($P < 0.05$) in hypertensive rats, following the same pattern mentioned above. Therefore, the impaired D-fructose intestinal absorption is another feature of SHR, and this decrease in D-fructose uptake correlates with a reduction in the abundance of the apical GLUT5 transporter, which is controlled at a transcriptional level.

Key words: Ileum — Kinetics — Northern blot — SHR — Sugar absorption — Western blot

Correspondence to: C.M. Vázquez; email: vazquez@us.es

Introduction

Arterial hypertension is one of the major diseases affecting individuals in the world, and its pathophysiology is associated with disturbances in the ion transport across cellular membranes (Orlov et al., 1999). In this sense, altered cellular sodium transport has been observed in patients suffering from essential hypertension (Rosskopf, Dusing & Siffert, 1993; Strazzullo, Galletti & Barba, 2003), as well as in different cell types of hypertensive rats. Specifically, it has been suggested that abnormalities in the sodium transport across intestinal cells may be present in spontaneously hypertensive rats (SHR) (Lubcke & Barbezat, 1988; Mu, Hansson & Lundgren, 1995).

Dietary carbohydrates are digested in the intestine and break down into monosaccharides, mainly D-glucose, D-galactose and D-fructose. D-Glucose and D-galactose are actively transported across the intestinal brush-border membrane by the Na^+-glucose cotransporter, SGLT1, whereas D-fructose is transported by the fructose facilitative transporter, GLUT5 (Wood & Trayhurn, 2003). Previous studies in our laboratory have shown that the capacity of the small intestine to transport D-glucose and D-galactose is decreased in hypertensive rats (Sanchez-Aguayo et al., 2001a, b), and although the physiological significance of these observations remain unknown, they point out that the intestine might play an important role in the pathophysiology of arterial hypertension.

Whether the observed changes in the intestinal transport of sugars in hypertensive rats are specific for Na^+-dependent monosaccharide cotransporters remained unanswered before this study. Therefore, the aim of the present work was to evaluate D-fructose transport in the ileum of hypertensive and

normotensive rats. In addition, Western and Northern blot assays have also been performed in order to study the regulation of GLUT5 transporter expression in the ileum of these rats.

Materials and Methods

Animals

Male SHR and Wistar-Kyoto (WKY) rats were obtained at the age of 7–8 weeks from Harlan IBERICA, S.A. (Barcelona, Spain) and kept on a 12-hour light/12-hour dark cycle with free access to water and a standard, fructose-free diet (Panlab, Barcelona, Spain). All experiments were carried out using 12- to 14-week-old rats, which were always killed in the early morning. All procedures followed are in accordance with the local ethics committee for animal experimentation.

Enterocyte Isolation

The ileum was quickly excised and washed in ice-cold saline buffer then divided into two pieces to isolate enterocytes and obtain brush-border membrane vesicles (BBMVs). Ileal enterocytes were isolated as described by Ferrer et al. (1994) with some modifications. Briefly, pieces of ileum were placed in a medium containing (in mmol/L) 80 NaCl, 3 K_2HPO_4, 20 Tris/HCl, 37 mannitol, 0.1 EGTA, 27 trisodium citrate and 1 mg/mL bovine serum albumin (BSA) at pH 7.4. To ensure that cells were obtained from the whole villus, including the crypts, the tissue pieces were shaken for 90 min and filtered through a nylon gauze (50 μm pore size). The filtered suspension was centrifuged three times at 800 × *g* at 4°C, and the enterocytes obtained were used for extraction of total RNA.

Preparation of Brush-Border Membrane Vesicles (BBMVs)

The mucosa was scraped from the underlying layer of the rest of the ileum with a glass slide and used for BBMV preparation by a $MgCl_2$ precipitation method, as previously described in detail (Sanchez-Aguayo et al., 2001a). The final pellets containing purified BBMVs were resuspended in a loading buffer (in mmol/L, 300 mannitol, 0.1 $MgSO_4$ and 20 HEPES/Tris, pH 7.4) and stored in liquid nitrogen until use (for a period no longer than 7 days). Preparation of BBMVs was always run in parallel on the same day from both animal strains.

Protein and Enzyme Activity Determinations

Protein determination was carried out by the method of Bradford (1976), using a bovine gamma-globulin as a standard. The membrane preparation was evaluated by measuring the specific activities of marker enzymes. Sucrase was used as the marker enzyme for BBMVs and estimated by the method of Dahlqvist (1964). The basolateral membrane marker enzyme, Na^+-K^+-ATPase, was measured according to Colas & Maroux (1980). Succinate dehydrogenase (Pennington, 1961) and acid phosphatase (Absolom, 1986), respective markers of mitochondria and lysosomes, were also determined as previously described. All enzyme activities were measured at 37°C.

D-Fructose Transport

Prior to D-fructose uptake, Na^+-dependent D-glucose transport was measured using a rapid filtration technique (Sanchez-Aguayo et al., 2001a), in order to control the functional integrity of the isolated BBMVs and to determine the intravesicular volume. Briefly, 5 to 10 μL of BBMVs were combined with 100 μL of an incubation medium comprised of (mmol/L) 100 NaSCN, 100 mannitol, 0.1 $MgSO_4$, 20 HEPES/Tris, pH 7.4, and 0.1 D-[^{14}C]glucose (specific activity = 306 mCi/mmol). For measurements of D-fructose uptake, the incubation medium was comprised of the same loading buffer inside the vesicles plus 1 mmol/L D-[^{14}C]fructose (316 mCi/mmol). Inhibition studies of D-fructose transport (1 mmol/L) were carried out in the presence of D-glucose (100 mmol/L), D-galactose (100 mmol/L), cytochalasin B (50 μmol/L), phlorizin (250 μmol/L) and D-fructose (100 and 300 mmol/L). For kinetic analysis, total fluxes were analyzed by non-linear regression using the Enzfitter program (Biosoft, Cambridge, UK), with D-fructose concentrations ranging 0.1–200 mmol/L.

SDS-PAGE and Western Blot Analysis

Similar amounts of protein (50 μg) of BBMVs from SHR and WKY rats were solubilized in Laemmli sample buffer and resolved by 8% SDS-PAGE (Laemmli, 1970). Proteins were then electrotransferred onto nitrocellulose membranes and immunoblotted as previously described (Mate et al., 2001b). Immunoblotting was performed using antisera kindly donated by Dr. T. Asano (University of Tokyo, Japan), which were raised in rabbits against the synthesized peptide corresponding to the COOH-terminal domain of rat GLUT5 (residues 490–502). The anti-GLUT5 antibody was detected by the enhanced chemiluminiscence (ECL) method according to the supplier's protocol and using a peroxidase-conjugated anti-rabbit IgG as a secondary antibody. In order to reject the possibility of having altered results due to manipulation, the membranes were incubated with a mouse anti-actin monoclonal antibody following the same protocol.

RNA Extraction and Northern Blot Assays

Whole RNA was extracted from isolated enterocytes as described by Chomczynski & Sacchi (1987). RNA was obtained after phenol extraction and alcoholic precipitation and measured by spectrophotometric analysis at 260 and 280 nm to evaluate the purity and concentration of RNA. Samples were electrophoresed in a formaldehyde-agarose gel (15 μg total RNA/lane), stained with ethidium bromide to verify RNA integrity and equivalence of loading and transferred to a nylon membrane (NytranN, 0.45 μm, Schleicher & Schuell, Dassel, Germany). Membranes were prehybridized (1 h, 68°C) and hybridized (1 h, 68°C) using a commercial solution (ExpressHyb Hybridization Solution, Clontech, Palo Alto, CA). Blots were washed with 2 × sodium chloride/sodium citrate (SSC)/0.05% sodium dodecylsulfate (SDS) at room temperature (5 × 10 min) and 0.1 × SSC/0.1% SDS at 50°C (3 × 20 min). Specific RNA was detected using a 2.2 kb EcoRI fragment encoding a rat jejunal GLUT5 transporter (provided by Dr. G. I. Bell, University of Chicago, Illinois, USA). Probes were labeled with α-^{32}P-dCTP by random priming (Random Primer DNA Labeling Mix, Biological Industries Co., Kibbutz, Israel). Membranes were stripped off and rehybridized with a) a plasmid encoding for the 18S ribosomic protein and b) a 1.1 kb PstI fragment encoding a chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Autoradiography was carried out at −80°C and autoradiograms were quantified by scanning densitometry.

Chemicals

All unlabelled reagents and anti-actin monoclonal antibody were obtained from Sigma Chemical (Madrid, Spain). D-[U-^{14}C]fruc-

tose, D-[U-^{14}C]glucose, [α-^{32}P]dCTP and the ECL reagent were obtained from Amersham International (Madrid, Spain). The membrane filters were obtained from Millipore (Barcelona, Spain).

Statistical Analysis

Results are expressed as means ± SE. Time- and strain-dependent differences were subjected to two-way analysis of variance (ANOVA). When significance was found, the unpaired, two-tailed Student's *t*-test was used, and differences were considered significant at $P < 0.05$. All other comparisons were performed by the unpaired *t*-test.

Results

Characterization of BBMVs

In the final BBMV preparation, sucrase activity was highly enriched in both SHR and WKY rats (15 ± 2- and 16 ± 2-fold over the original homogenate, respectively; $n = 6$). The enrichment of Na^+-K^+-ATPase (2.3 ± 0.6- and 2.7 ± 0.1-fold, respectively; $n = 6$), acid phosphatase (1.1 ± 0.2- and 1.7 ± 0.3-fold, respectively; $n = 6$) and succinate dehydrogenase (1.4 ± 0.2- and 1.6 ± 0.3-fold, respectively; $n = 6$) were low, indicating very little basolateral, lysosomal and mitochondrial contamination, respectively, in both experimental groups. No significant differences were observed in the specific activities of Na^+-K^+-ATPase, acid phosphatase and succinate dehydrogenase. However, the specific activity of the brush-border marker enzyme, sucrase, was significantly lower in ileal BBMVs from SHR than those from WKY rats (192 ± 26 vs 301 ± 23 nmol glucose/min per mg protein, respectively; $n = 6$, $P < 0.05$).

The transport capability of the BBMVs was assessed by determining D-glucose uptake at 5 s incubation time. The presence of a Na^+ gradient ($Na_o^+ > Na_i^+$) induced a transient accumulation of D-glucose in both groups of animals, which confirmed the functionality of BBMVs (*data not shown*). In addition, the intravesicular volume, estimated from D-glucose distribution at the equilibrium (incubation time = 30 min), was similar in normotensive and hypertensive rats (0.36 ± 0.06 vs 0.36 ± 0.03 μL/mg protein, respectively).

D-Fructose Transport into Ileal BBMVs

Figure 1 shows the time course of D-fructose uptake into ileal BBMVs prepared from normotensive and hypertensive rats. Uptake values were linear up to 15 s of incubation in both groups. On the other hand, D-fructose uptake was significantly lower in SHR than in WKY rats up to 2 min of incubation. From this time, no differences were observed between the two rat groups, and the equilibrium was reached at 30 min.

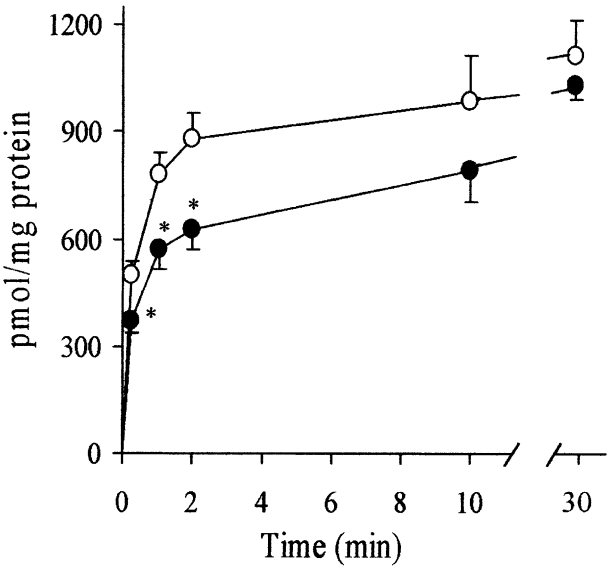

Fig. 1. Time course of D-fructose uptake in BBMVs prepared from ileal WKY (*empty symbols*) and SHR (*filled symbols*). Values represent means ± SE of at least ten different preparations. *$P < 0.05$.

Kinetic analysis in the range 0.1–200 mmol/L D-fructose is shown in Fig. 2. In both groups of animals, total fluxes were best fitted to a kinetic model comprised of one saturable component plus a non-saturable one. The calculated kinetic parameters (Table 1) revealed a 53% decrease in the V_{max} in the ileum from SHR compared with WKY rats. However, no differences were noted in the K_m and K_d values between both rat strains.

Neither D-glucose, D-galactose, cytochalasin B nor phlorizin inhibit D-fructose uptake (Fig. 3). The transport was only inhibited by the presence of high concentration of D-fructose (50–55% at 100 mmol/L and 80–85% at 300 mmol/L in both groups of animals).

Immunoblotting

The anti-GLUT5 antibody recognized both in SHR and WKY rats an immunoreactive protein of about 58 kDa that was blocked by preabsorption with the antigenic peptide (Fig. 4*A*). To assure equivalence of loading and absence of artifacts due to manipulation, blots were normalized with an anti-actin antibody, which recognized a single band of 45 kDa (Fig. 4*B*). The relative abundance of GLUT5 declined by 51% in BBMVs from SHR when compared to those from WKY rats (Fig. 4*C*).

Northern Blotting

Purity of the total RNA solution was assessed by measuring absorbance at 260 and 280 nm. In all cases, the ratio 260:280 was higher than 1.8 (*data not*

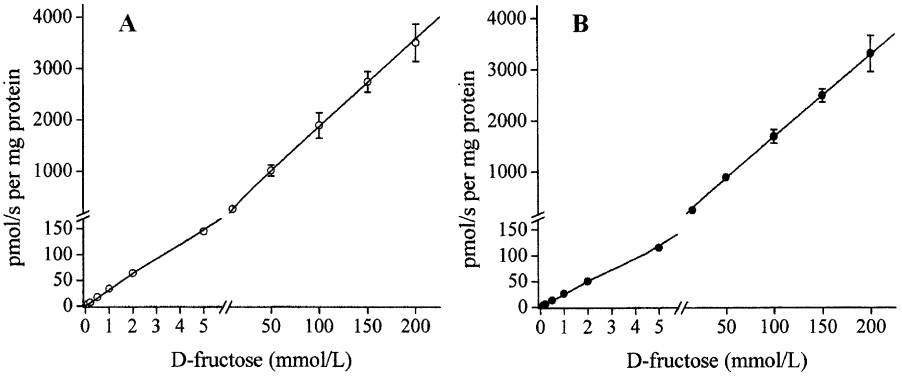

Fig. 2. Kinetics of D-fructose uptake in ileal BBMVs prepared from WKY (*A*) and SHR (*B*). Total fluxes were measured at 15 s incubation at the indicated D-fructose concentrations. Kinetic analysis was performed as described in Materials and Methods. Values represent means ± SE of four independent experiments. When not given, SE bars are smaller than the symbol used.

Table 1. Kinetic parameters of D-fructose uptake in ileal BBMVs prepared from WKY and SHR

Parameter	WKY	SHR
V_{max} (pmol/s per mg protein)	196 ± 12	92 ± 10*
K_m (mM)	11 ± 3	8 ± 3
K_d (nL/s per mg protein)	17 ± 2	16 ± 1

Vesicles were incubated for 15 s with concentrations ranging from 0.1 to 200 mM D-fructose. Values (V_{max}, maximal rate of transport; K_m, Michaelis constant; K_d, diffusion constant) represent means ± SE of four different preparations. *$P < 0.05$.

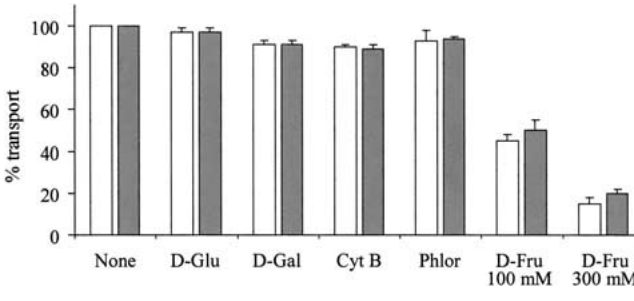

Fig. 3. Effect of different substrates on the uptake of 1 mmol/L D-fructose in ileal BBMVs prepared from WKY (*white bars*) and SHR (*gray bars*). Uptake was measured at 15 s incubation, in the presence of D-glucose (100 mmol/L), D-galactose (100 mmol/L), cytochalasin B (50 μmol/L), phlorizin (250 μmol/L) and D-fructose (100 or 300 mmol/L). Values represent means ± SE of four different preparations, and are expressed as a percentage of the values obtained in the absence of inhibitors.

shown), indicating a high purity and low contamination by protein fractions. Hybridization with a specific probe for GLUT5 showed a distinct band of 2.7 kb in both WKY and SHR (Fig. 5*A*). There was a decrease of 52% in the abundance of the GLUT5 mRNA levels in the SHR group when compared to the WKY (Fig. 5*D*). Each well contained equivalent amounts of total RNA, as assessed by rehybridizing the same blot with ribosomal 18S RNA (Fig. 5*B*), and with a GAPDH probe (Fig. 5*C*).

Discussion

We have previously reported that the capacity of the small intestine to absorb D-glucose and D-galactose was impaired in hypertensive rats (Sanchez-Aguayo et al., 2001a, b), which might be due in part to a lower expression of Na^+-glucose cotransporter (SGLT1) protein at the level of intestinal apical membranes (Mate et al., 2001a). These results are extensible to the kidney, where a decrease in the proximal tubular reabsorption of monosaccharides was found in SHR compared with WKY rats (Mate et al., 2000; Mate et al., 2001b).

In this work, we have studied the transport of D-fructose in the ileum of hypertensive and normotensive rats, analyzing the kinetic properties and the regulation of the fructose transporter, GLUT5. A 53% reduction in the maximal transport capacity (V_{max}) was obtained in ileal BBMVs prepared from SHR when compared to WKY rats. The V_{max} value for D-fructose transport determined in the ileum from normotensive rats, 196 pmol/s per mg protein, is lower than that previously reported (Sigrist-Nelson & Hopfer, 1974; Crouzoulon & Korieh, 1991), which may be explained by the rat strain or the small intestinal region used and/or by the different degree of purity of BBMVs. On the other hand, the affinity of the D-fructose transporter (i.e., the K_m values), was similar in both groups and within the range of previous reports (Burant et al., 1992; Miyamoto et al., 1994; Sugawara-Yokoo et al., 1999; Corpe et al., 2002). These results are not due to variations in the purity or size of vesicle preparations, since the enrichment of sucrase activity and the uptake of Na^+-dependent D-glucose at equilibrium (30 min), were similar for normotensive and hypertensive rats.

The effect of the presence of different sugars on D-fructose transport confirmed, in both animal groups, previous results, which demonstrated that the transport of D-fructose across ileal BBMVs is highly specific (Sigrist-Nelson & Hopfer, 1974; Burant et al.,

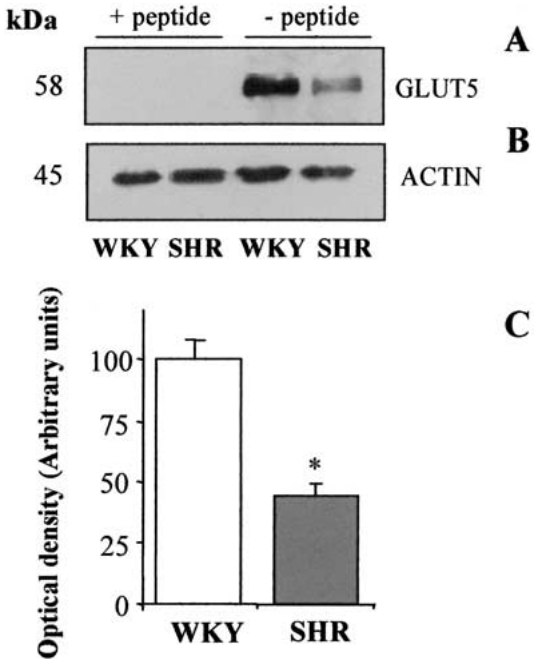

Fig. 4. (*A*) Western blot analysis of GLUT5 in BBMVs obtained from ileum of WKY and SHR. The antibody recognized an immunoreactive protein of about 58 kDa. When the antibody was previously adsorbed with its antigenic peptide (1 mg/mL), no signal was detected. (*B*) Immunoreactive signal obtained when the membrane used in (*A*) was washed and incubated with control, anti-actin antibody. The antibody recognized a single band of 45 kDa without significant abundance differences between BBMVs from WKY and SHR. (*C*) Relative abundance of GLUT5 measured by optical densitometry. Values represent means ± SE of three separate experiments. $*P < 0.05$.

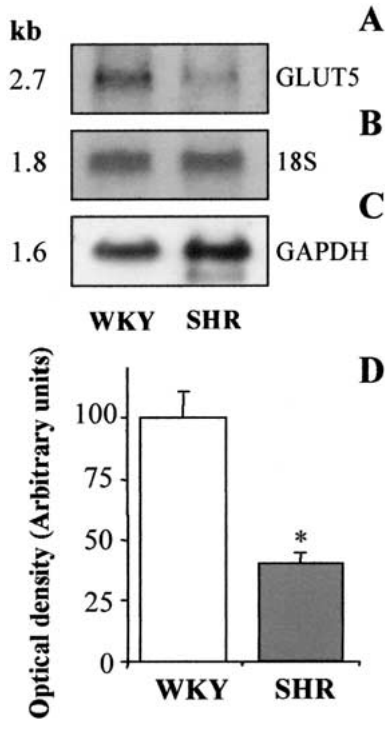

Fig. 5. (*A*) Northern blot analysis of GLUT5 from isolated ileal enterocytes of WKY and SHR. A single transcript of about 2.7 kb was detected in every sample. Hybridization of the same blot with a probe for 18S ribosomal protein (*B*) and for GAPDH (*C*) showed no significant differences in the amount of total RNA loaded. (*D*) Relative abundance of the specific mRNA for GLUT5 normalized by 18S rRNA and measured by optical densitometry. Values represent means ± SE of three separate experiments. $*P < 0.05$.

1992; Miyamoto et al., 1994; Inukai et al., 1995). Neither cytochalasin B (50 μM) nor phlorizin (250 μM) affected D-fructose transport. Among the sugars examined, only D-fructose inhibited its own transport, with a similar inhibition pattern in WKY and SHR. This result contradicts Rand et al. (1993), who reported that rat GLUT5 is capable to transport D-glucose in oocytes, although to a lower extent than D-fructose.

Western blot analysis was performed in order to know whether the observed changes in D-fructose transport were related to the amount of GLUT5 protein in ileal brush-border membranes. A single band of 58 kDa was detected in both groups of animals, which is within the range of Mr found in human (Burant et al., 1992), rabbit (Miyamoto et al., 1994), and rat (Sugawara-Yokoo et al., 1999) intestine, as well as in mouse (Corpe et al., 2002) and rat (Sugawara-Yokoo et al., 1999) kidney. Results of blot densitometry demonstrated that the abundance of D-fructose transporter is approximately 51% lower in ileal BBMVs isolated from SHR, a similar reduction to that of the V_{max}.

Since GLUT5 protein levels were diminished in ileal BBMVs from SHR, we then focused on the GLUT5 mRNA abundance. Northern blot analysis revealed the expression of a single, 2.7 kb transcript in the ileum of both WKY and SHR, consistent with previous work in rabbit ileum (Miyamoto et al., 1994), rat small intestine (Rand et al., 1993; Shu, David & Ferraris, 1998), and mouse intestine (Corpe et al., 2002). The levels of GLUT5 mRNA were decreased by 52% in hypertensive rats when normalized against 18S rRNA signals.

Our results therefore demonstrate that the capacity to absorb D-fructose is impaired in hypertensive rats. The results reported here for the ileal transport are extensible to the jejunum, where we found similar patterns for GLUT5 activity and expression (Barfull et al., 2001). The similarity in the magnitude of the decreases in GLUT5 mRNA, protein and activity suggests that there is a transcriptional downregulation of D-fructose transporter, GLUT5, in the intestine of these rats, which in turn induces a corresponding decrease in the levels of GLUT5 protein and in the transport of D-fructose. It has yet to be determined whether these abnormalities constitute a consequence or a genetically determined

feature of hypertension. Also, it has to be considered that, in addition to these biochemical and molecular changes, hypertensive rats also show a patchy loss of microvilli in some sections of the ileum (Sanchez-Aguayo et al., 2001a), which could be the origin of an important reduction of apical surface area with functional consequences. Moreover, the activity of sucrase in the ileal brush-border membrane was reduced by 36% in SHR, although the molecular regulation of this protein was not investigated.

The renin-angiotensin-aldosterone system (RAAS) is considered crucial in the pathogenesis of hypertension (Bertram et al., 2002; Cediel et al., 2003). Studying a possible link between alterations in GLUT5-mediated D-fructose transport and an activation of RAAS might be of particular importance in order to clarify whether alterations in fructose absorption are somehow related to the pathophysiology of hypertension. In this regard, Shiuchi et al. (2002) have recently related abnormalities in sugar transport to alterations in angiotensin II type 1 (AT1) receptor. Also, aldosterone is known to control the changes induced by low sodium intake in the activity of apical (SGLT1) and basolateral (GLUT2) hexose transporters in the chicken intestine, acting through cytosolic mineralocorticoid receptors (Garriga, Planas & Moreto, 2001). Although there is no clear evidence of altered aldosterone levels in hypertensive rats, they do show alterations in angiotensin II receptor levels (Johren et al., 2003). Therefore, the study of the effect of AT1 receptor blockade and/or angiotensin-converting enzyme (ACE) inhibitors on D-fructose uptake in SHR could be particularly interesting for this purpose.

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