Cyclic AMP Stimulates Fructose Transport in Neonatal Rat Small Intestine$^{1,2}$

Xue-Lin Cui, Chris Ananian, Edwin Perez, Aidy Strenger, Annie V. Beuve, and Ronaldo P. Ferraris$^{3}$

Department of Pharmacology and Physiology, UMDNJ-New Jersey Medical School, Newark, NJ 07103-2714

ABSTRACT Intestinal fructose transporter (GLUT5)$^4$ located in the brush border membrane of epithelial cells, and then is released from the epithelial cells into the blood by another facilitative glucose transporter, GLUT2 located in the basolateral membrane (1). Under normal conditions, fructose absorption rates and GLUT5 mRNA abundance are very low during the suckling and early weaning stages of rat intestinal development (2–4). Intestinal fructose transport is significantly enhanced only after weaning is completed at ≥28 d of age (5). There is also evidence that in rabbits (6) and humans (7) intestinal fructose absorption becomes noteworthy only in late developmental stages. High breath hydrogen levels, which indicate intestinal carbohydrate malabsorption, are more than 2-fold greater in 1-y-old infants compared with 3- and 5-y-old children consuming fruit juice, and levels increase markedly in proportion to the amount of fructose consumed by all age groups (7).

Consumption of a high-fructose (HF) diet (8) or intestinal perfusion in vivo with a HF solution for 4–8 h (9) precociously and markedly increases GLUT5 activity and mRNA abundance in rats as young as 16–18 d of age. The activity and mRNA abundance of the brush border sodium-glucose cotransporter (SGLT1) are not affected. Actinomycin D, a transcription inhibitor, inhibits the dietary fructose-induced enhancement of fructose transport and GLUT5 mRNA expression, whereas cycloheximide, a protein synthesis inhibitor, inhibits only fructose transport (9). Hence, precocious induction by diet likely requires de novo mRNA and protein synthesis. However, the signal transduction mechanisms regulating GLUT5 expression during development are not known.

To determine the intracellular factors that can potentially modulate increases in GLUT5 expression and activity, we collected RNA from fructose- and glucose-perfused intestines and evaluated the differential expression of ~5000 genes using a rat microarray chip (10) (Cui and Ferraris, unpublished).
observations). Along with GLUT5, the mRNA expression of the key gluconeogenic enzymes, glucose-6-phosphatase and fructose-1,6 bisphosphatase, increased significantly in fructose-perfused intestines, suggesting a link between intracellular fructose and gluconeogenesis. Fructose-1,6 bisphosphatase activity is indirectly regulated by cAMP, which modulates levels of fructose-2,6 bisphosphate, an important metabolic intermediate determining the rates of gluconeogenesis and glycolysis. It is therefore possible that cAMP can affect fructose transport indirectly by its effect on gluconeogenesis rates and fructose metabolism. Moreover, there are 2 cAMP response elements in the human GLUT5 promoter (11), and cAMP may play a more direct role in GLUT5 synthesis or activity. We therefore tested the hypothesis that cAMP and protein kinase A (PKA) mediate the fructose-induced increase in fructose transport rate and GLUT5 mRNA expression.

**MATERIALS AND METHODS**

**Hypothesis and design.** To test our hypothesis, the following experiments were designed (Fig. 1). First, the effect of 8-bromo-cAMP, a cell permeable cAMP analog more resistant to phosphodiesterases than cAMP, was selected to prove the direct effect of cAMP on GLUT5 mRNA expression and/or activity. Second, the effect of endogenous changes in cAMP concentrations on GLUT5 was evaluated using dideoxyadenosine (DDA), a specific inhibitor of adenylyl cyclase, and 3-isobutyl-1-methylxanthine (IBMX), a "broad-spectrum" inhibitor of phosphodiesterase. Third, the role of PKA was assessed using PKA inhibitor (PKI) and N-[2-[(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide-2HCl (H89), 2 specific PKA inhibitors. Unless otherwise specified, all signaling mediators were purchased from BIOMOL Research Laboratories.

**Animals.** Adult male and female Sprague-Dawley rats weighing ~200 g were purchased from Taconic and bred. Rats were housed in a temperature-controlled room (22–24°C) with a 12-h light:dark cycle in the research animal facility, and allowed to consume water and nonpurified diet ad libitum (Purina Mills). After the female rats became pregnant, they were separated from the male rats and carefully monitored until the pups were born. The time and date of birth were recorded; age at birth was considered 0. Mid-weaning rat pups were used in this experiment. The pups were kept with their dams until they were 20–22 d old. They were then randomly separated into 2 or 4 groups according to experimental needs. All of the procedures conducted in this study were approved by the Institutional Animal Care and Use Committee, UMDNJ-New Jersey Medical School.

**Perfusion model.** The rat intestinal perfusion procedure was conducted following the method of Jiang and Ferraris (9). The composition of the perfusion solution was as follows (in mmol/L): 78 NaCl; 4.7 KCl; 2.5 CaCl2; 5 H2O; 1.2 MgSO4; 19 NaHCO3; 2.2 KH2PO4; and 100 glucose or fructose. The concentration of sugar was based on previous findings that the magnitude and time course of enhancement of GLUT5 mRNA abundance and fructose transport rates with 100 mmol/L fructose perfusion were the same as those observed when pups were fed 65% fructose pellets (9). Intestinal luminal sugar concentration in pups fed pellets containing 65% sugars can exceed 100 mmol/L (12). All perfusions, unless otherwise indicated, lasted for 4 h, after which sugar uptakes and transporter mRNA abundance were measured.

**Fructose and glucose uptake measurements.** Measurements of glucose and fructose uptake rates were performed according to the technique of Karasov and Diamond (13) as modified by Jiang and Ferraris (9). All radiolabeled sugars were purchased from NEN. For each rat, 2 sleeves were used to measure uptake rates of each test solute. The mean was calculated to represent the glucose or fructose absorption in that rat. Fructose and glucose uptake rates were determined at 50 mmol/L, which yields near maximum velocity (Vmax) rates, so that any change in uptake rate will be due mainly to a change in Vmax (13).

**Effect of 8-bromo-cAMP on sugar absorption.** Fourteen pups were randomly divided into 2 groups (n = 7/group). Each group was perfused with regular Ringer solution with or without 8-bromo-cAMP (10 μmol/L). This concentration of 8-bromo-cAMP was consistently demonstrated to alter intestinal sugar absorption in rats and mice (14–16).

**Effect of DDA on sugar absorption.** In this experiment, rat pups (n = 16) were randomly divided into the following 4 groups (n = 4 pups/group): 1) 100 mmol/L fructose; 2) 100 mmol/L fructose + DDA (125 μmol/L); 3) 100 mmol/L glucose; and 4) 100 mmol/L glucose + DDA. This or a lower concentration of DDA was shown to inhibit adenylyl cyclase in the guinea pig intestine (17,18) and human platelets (19).

**Effect of IBMX on sugar absorption.** In preliminary experiments, 50–100 μmol/L IBMX (20,21) was used to inhibit intestinal sugar absorption in rats and mice (17,18) in vivo. Rats were therefore divided into 2 groups (n = 7/group) and perfused with or without IBMX (50 μmol/L) for 5 min. This brief duration ensured almost 100% survival and is sufficient because brief applications of 1–100 μmol/L IBMX can increase intracellular concentrations of cAMP for several hours after the application (23), ensuring that cAMP will build up in the intracellular tissue. Therefore, all pups were perfused initially with IBMX + Krebs-Ringer bicarbonate (KRB) (control: KRB alone) for 5 min, followed by KRB for 4 h.

**Effect of PKA inhibitors on sugar absorption.** In the PKA inhibition experiment, mid-weaning pups were randomly divided into 4 groups (n = 5–6 pups/group): 1) 100 mmol/L fructose; 2) 100 mmol/L fructose + PKI (360 nmol/L); 3) 100 mmol/L glucose; and 4) 100 mmol/L glucose + PKI. The reported Kᵢ, of PKI for PKA was 36 nmol/L (24) and 100 mmol/L is sufficient for PKI to inhibit the activity of PKA in intestinal cell culture (25). In the present study, a concentration of PKI that was 10 times that of the Kᵢ, was used because preliminary work showed that pups survived 360 nmol/L PKI concentrations for over 4 h. The experiment was then repeated, using a different type of PKA inhibitor, H89, at a concentration of 500 mmol/L. The Kᵢ, for H89 is 48 nmol/L (26,27). The effective concentration of H89 in intestinal tissues ranges from 200 (28) to 1000 mmol/L (29). A concentration ~10 times the Kᵢ, was used, for consistency with the PKI experiment and because preliminary work showed pups to survive 4 h of perfusion of 500 mmol/L H89. The following design (n = 6–8 pups/group) was used: 1) 100 mmol/L fructose + dimethyl sulfoxide (DMSO; the vehicle used to dissolve H89); 2) 100 mmol/L fructose + H89 (500 mmol/L); 3) 100 mmol/L glucose + DMSO; 4) 100 mmol/L glucose + H89. In other perfusion studies in which no vehicle was specified, the inhibitor was sufficiently soluble in KRB.
Determination of cAMP content in intestinal tissues. After the intestine was perfused for 4 h, an ~10 cm segment of jejunum 25 cm distal to the ligament of Treitz was immediately dissected, snap-frozen, and stored at −80°C. ~400 mg of the frozen intestinal tissue was homogenized (Polytron) in ice-cold PBS (pH 7.4) containing 0.5 mmol/L IBMX. The homogenate was centrifuged at 2000 × g for 10 min at 4°C. The amount of cAMP in the supernatant was measured by RIA, as previously described and adapted for cAMP (30,31).

Northern blot analysis. At the end of each perfusion, ~10 cm of jejunum 15 cm from the ligament of Treitz was quickly frozen in liquid nitrogen and then stored at −80°C for later GLUT5, GLUT2, and SGLT1 mRNA and 18s rRNA (loading and transfer control) abundance measurement by Northern blotting (32). Total RNA in the small intestines was isolated using a RNeasy Midi Kit (QIAGEN).

Statistical analysis. Data are presented as means ± SEM. A one-way ANOVA was first used to determine the significance of the difference in relative mRNA abundance among groups with different treatments. If there was a significant difference, Fisher’s Protected Least Significant Difference test was used to determine the particular effect that caused that difference. An unpaired Student’s t test was used to determine the difference between fructose-perfused and glucose-perfused groups, or drug treated and untreated groups. P < 0.05 was considered significant. Statistical analysis used the STATVIEW program (Abacus Concepts).

RESULTS

8-Bromo-cAMP perfusion. Fructose uptake was significantly higher in the small intestine perfused with 8-bromo-cAMP (Fig. 2). In contrast, the glucose uptake rate was independent of 8-bromo-cAMP perfusion. However, 8-bromo-cAMP did not affect the expression of GLUT5, GLUT2, and SGLT1 mRNA in the small intestinal tissues (not shown).

DDA perfusion. After 4 h, fructose uptake rate was markedly greater in the small intestine perfused with fructose compared with that perfused with glucose (Fig. 3). Simultaneous perfusion of DDA and fructose significantly inhibited the increase in fructose uptake rate induced by fructose perfusion. There was no significant difference in fructose uptake between the intestines perfused with glucose and glucose + DDA, indicating that DDA had no effect on transport properties of the intestine. There were also no differences in glucose uptake rate among the 4 groups (Fig. 3).

The expression of GLUT5 mRNA in the small intestines perfused with fructose and in those perfused with fructose + DDA was significantly higher than in those perfused with glucose and glucose + DDA (P < 0.01) (Fig. 4). The fructose-induced increase in GLUT5 mRNA expression was not inhibited by DDA. These results suggest that the increase in GLUT5 mRNA is independent of cAMP levels. The expression of GLUT2 and SGLT1 mRNA in the small intestine was independent of sugar solution and unaffected by DDA. GLUT5 and GLUT2 mRNA were typically found as single bands, but SGLT1 appeared often as 3 bands (4.5, 3.5, and 1.0 kb) as was found by other workers (33). We selected for evaluation only the strongest and clearest band (4.5 kb; integrating or averaging all 3 bands produce the same result).

Fructose perfusion and intestinal cAMP concentrations. The relative concentration of cAMP in the fructose-perfused intestine was greater than that in the glucose-perfused intestine [HF: 127 ± 7%; high-glucose (HG): 100 ± 9%, P < 0.05]. Moreover, cAMP concentration decreased significantly in the intestine perfused with fructose plus DDA (HF + DDA: 90 ± 17%) compared with that in the intestine perfused with fructose alone. There was no difference in cAMP concentrations between the intestines perfused with glucose plus vehicle and glucose plus DDA (HG + DDA: 85 ± 9%).

IBMX perfusion. There was a significant difference (P < 0.05) in relative fructose uptake rate between the intestines perfused with (128 ± 8%) and without IBMX (100 ± 10%). For relative glucose uptake rate, there was no difference between the 2 groups (without IBMX perfusion: 100 ± 28%; with IBMX: 107 ± 29%).

The mRNA abundance of GLUT5 (without IBMX perfusion: 100 ± 34%; with IBMX: 93 ± 37%), GLUT2 (100 ± 29; 116 ± 40%), and SGLT1 (100 ± 24%; 105 ± 34%) were not altered (P > 0.50) by IBMX perfusion.

PKI and H89 perfusion. The fructose-induced increase in fructose uptake rate was not blocked by either PKI or H89.
Moreover, neither PKI nor H89 altered the glucose uptake rate among the groups. GLUT5 mRNA abundance increased markedly in the fructose and fructose/H89 PKI intestines compared with those in the glucose and glucose/H11001 PKI intestines. Hence, PKI did not inhibit the fructose-induced increase in GLUT5 mRNA expression in the small intestine. However, SGLT1 and GLUT2 mRNA abundance did not differ among the 4 groups (Fig. 7).

(Figs. 5 and 6). Moreover, neither PKI nor H89 altered the glucose uptake rate among the groups.

GLUT5 mRNA abundance increased markedly in the fructose and fructose + PKI intestines compared with those in the glucose and glucose + PKI intestines. Hence, PKI did not inhibit the fructose-induced increase in GLUT5 mRNA expression in the small intestine. However, SGLT1 and GLUT2 mRNA abundance did not differ among the 4 groups (Fig. 7).
Cyclic AMP stimulates intestinal fructose uptake in neonatal rats. Our main finding is that cyclic AMP mediates in part the fructose-induced increase in fructose transport in the neonatal rat small intestine during early development. First, we demonstrated that 8-bromo-cAMP significantly increases fructose uptake rate in vitro in the small intestine. Second, inhibition of adenyl cyclase and therefore of endogenous cAMP synthesis blocks the increase in fructose uptake induced by luminal fructose. Third, we observed that the concentration of cAMP increases when the small intestine is perfused with fructose. Finally, the fructose-related increase in cAMP concentration is prevented by inhibiting adenyl cyclase. These effects on fructose transport are specific, because glucose uptake is not affected.

There was a previous report of cAMP increasing intestinal fructose transport. Dibutyryl cAMP and forskolin, which stimulate adenyl cyclase, markedly increased fructose uptake and GLUT5 protein in Caco-2 cells, a human colon cancer cell line that differentiates spontaneously in culture into cells with the properties of small intestine enterocytes (11,34). The mechanisms underlying this increase in fructose uptake include increases in GLUT5 mRNA transcription rates and in GLUT5 mRNA stability; the effects of cAMP are limited only to differentiated Caco2 cells and do not affect the undifferentiated cells.

The effects of cAMP on human colon cancer-derived Caco2 GLUT5 mRNA abundance contrast with our findings that cAMP has no effect on GLUT5 mRNA abundance in rat small intestine. This may be due to tissue differences because Caco2 cells typically retain both enterocyte and colocyte characteristics even after confluence (35). This may also be due to species differences, because a kinase belonging to a different signaling pathway, PKC, decreases glucose transport in oocytes expressing rabbit and rat, but increases glucose transport in oocytes expressing human SGLT1 (36). There are also striking differences in the regulation of sugar transporters and hydrolases between the Caco2 cell line and rodent small intestine, because Caco2 GLUT5 responds to both glucose and fructose (11), whereas neonatal rat (4) and adult mouse (37) intestinal GLUT5 responds only to fructose. Moreover, glucose markedly inhibits the transcription, mRNA stability, biosynthesis, and intracellular trafficking of sucrase isomaltase in Caco2 cells (38,39), whereas high-sugar diets stimulate activity and transcription of sucrase-isomaltase in rat and mouse small intestine (40,41). In STC-1, another intestinal cell line, glucose in the medium increases SGLT1 abundance, SGLT1 promoter activity, and intracellular cAMP concentrations. This glucose-induced activation of the SGLT1 promoter was mimicked by 8-bromo-cAMP and inhibited by H89 (42).

Our findings that cAMP has no effect on GLUT5 mRNA abundance indicate that cAMP does not regulate the fructose-induced fructose uptake at the transcriptional level, but at the post-transcriptional level, and that cAMP affects fructose transport via other pathways. The absence of an effect of cAMP on GLUT5 mRNA abundance is consistent with the absence of an effect of adenyl cyclase and PKA inhibitors on GLUT5 mRNA abundance.

There have been other reports of cAMP affecting only the activity but not mRNA abundance of intestinal nutrient transporters, paralleling its effect on GLUT5. Forskolin increases Na⁺-dependent glucose transport in mouse small intestine (43) and decreases H⁺-dependent dipeptide transport in Caco2 cells (44). The increase in glucose transport may be due to an increase in electrochemical gradient for Na⁺ because in lizard duodenum and rat hepatocytes, Na⁺-dependent L-alanine transport is stimulated by forskolin or dibutyryl cAMP, which hyperpolarize the plasma membrane (45,46). Dibutyryl cAMP increases glucose transport in mouse small intestine by PKA-mediated phosphorylation of SGLT1, leading to increases in the binding ability of the transporter to its substrate, glucose (14). Treatment of frog oocytes with 8-bromo-cAMP increases Na⁺-dependent glucose transport and the number of SGLT1 transporters in the plasma membrane (47). The mechanism underlying this increase in transport across the plasma membrane is a cAMP-dependent increase in intracellular transport of vesicles containing SGLT1 proteins and subsequent insertion of SGLT1 into the plasma membrane.

Cyclic AMP may specifically stimulate fructose transport directly without enhancing GLUT5 mRNA expression by reducing intracellular fructose concentrations in the enterocyte and increasing the chemical gradient for fructose transport. Increased intracellular concentrations of cAMP activate a protein kinase, which phosphorylates several enzymes common in fructose and glucose metabolism such as phosphofructokinase (48). Under such conditions, gluconeogenesis is favored, converting intracellular fructose to phosphorylated metabolites and eventually to glucose. Indeed, fructose perfusion triggers marked increases in mRNA abundance of genes widely known to regulate gluconeogenesis (10); Cui and Ferraris, unpublished observations]. Microarray and Northern blot analyses revealed that mRNA expression of some of these genes (glucose-6-phosphatase and fructose-1,6 bisphosphatase) increased mainly with fructose perfusion and did not change with glucose perfusion. More importantly, mRNA expression of a cAMP-regulated bifunctional enzyme, fructose-2,6 bisphosphatase, also increased. cAMP inhibits the synthesis and stimulates the breakdown of fructose-2,6 bisphosphate. Because fructose-2,6 bisphosphate is an inhibitor of fructose-1,6 bisphosphatase, increases in cAMP should increase fructose-6 phosphate concentrations and lead to eventual conversion to glucose via glucose-6-phosphate isomerase and glucose-6-phosphatase, another enzyme upregulated with fructose perfusion. Future studies will determine the activity and expression of these enzymes during fructose or cAMP perfusion. Dietary or luminal fructose itself can stimulate fructose transport specifically by increasing the activity of metabolic enzymes specific for fructose, such as fructokinase and triokinase (49), thereby reducing intracellular fructose concentrations. Although fructose feeding does increase the activity of these enzymes, it is not known whether the second messenger is cAMP.

It is interesting to note that glucagon and fasting both activate adenyl cyclase, which activates fructose 2,6 bisphosphatase, thus stimulating gluconeogenesis (48). Hence, fructose perfusion does stimulate the same metabolic pathways known to be stimulated by cAMP-mediated actions of glucagon.

Because there was insufficient amounts of neonatal intestinal tissue for simultaneous measurements of function, transporter protein levels, and transporter mRNA abundance, we could not determine whether the effect of cAMP was due to increased rate of fructose absorbed per GLUT5 transporter or to increased amounts of GLUT5 in the brush border membrane. However, a metabolic effect would much more likely be demonstrated in preparations measuring transepithelial fructose transport.

Increased activity per GLUT5 transporter might arise due to accelerated fructose metabolism. Another possibility is that GLUT5 itself may become phosphorylated, also leading to increased activity per transporter. Activity of GLUT4 in skel-
etial muscle is modulated by phosphorylation (50) but it is not known whether activity of other GLUTs can also be modulated in the same manner.

Our failure to observe effects from PKA inhibitors is surprising, and may be due to insufficient inhibitor concentrations or to other experimental conditions that did not inhibit PKA. These are unlikely scenarios, however, because 2 inhibitors with different modes of action each independently failed to prevent luminal fructose from stimulating fructose uptake. Both inhibitor concentrations were 10 times the K_i and exceeded or equaled concentrations already known to inhibit PKA in intestinal tissues (25,28,29). Our failure to observe cAMP effects on GLUT5 mRNA abundance may also be due to higher cAMP concentrations being required to alter GLUT5 mRNA abundance than those required to alter GLUT5 activity. For example, it is possible that the cAMP concentration used in our experiment was merely sufficient to trigger de novo transcription. The effect of cAMP on fructose uptake may also reflect only part of the effect of luminal fructose on fructose uptake because luminal fructose also affects GLUT5 mRNA abundance. These complex interactions between intracellular and luminal signals can be elucidated only in future work.

**Developmental aspects underlying regulation of glucose transport.** There has been no study on dietary regulation of glucose transport in neonatal rats except those from our laboratory, and we have consistently shown that intestinal glucose transport in developing pups, unlike that in adult rats, cannot be modulated by diet (4,8). Glucose transport in the neonate is higher than that in adults of many mammalian species (5,6,51), and this is likely due to the need to maintain high rates of glucose and galactose uptake from milk digestion. Dietary regulation of glucose absorption at the early developmental stage may not be beneficial because the neonatal diet does not vary markedly in sugar content. In contrast, glucose absorption is regulated by diet in adults consuming diets with varying carbohydrate content, particularly in omnivorous species. This may explain in part our previous findings of neonatal rat glucose transport independent of dietary sugar content (4,8), and our present findings of glucose transport independent of cAMP analogs and cAMP or PKA inhibitors. Similarly, glucose permeability has no effect on cAMP concentrations in the neonatal rat intestine. There have been reports of cAMP modulating intestinal glucose transport in adult rodents (43,52,53).

In conclusion, cyclic AMP modulates fructose transport in neonatal rats by mechanisms that do not involve alterations in mRNA abundance. This finding differs from previous work in which changes in fructose uptake were always linked to changes in GLUT5 mRNA abundance (54). However, the effects of cAMP and the mechanisms underlying those effects may vary with ontogenic development, along the crypt-villus axis, or among different stages of confluency or differentiation of epithelial cell lines (55), leading to conflicting results. In fact, the effect of dopamine and vasoactive intestinal peptide on cAMP synthesis differs between villous and crypt enterocytes (56). Thus, it will be difficult to demonstrate marked increases in cAMP such as those stimulated by luminal fructose, if those increases occurred only in villous cells.

**ACKNOWLEDGMENTS**

We thank C. Burant and M. Lee for the cDNA probes.

**LITERATURE CITED**


