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Research Article

INFLUENCE OF HEXOSES ON INTESTINAL PHOSPHATE TRANSPORT USING EVERTED GUT SACS OF MICE

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ABSTRACT

Background & objectives: Active transports of intestinal glucose and phosphate (Pi) is shown to be sodium dependent. Earlier reports show an inhibitory role for glucose on intestinal Pi transport . Information on other hexoses is scanty. So this study is conducted to see the effects of hexoses like galactose , 3-O-methyl glucose and fructose on phosphate transport.

Materials and methods: Everted gut sacs from proximal intestine were prepared from Swiss male albino mice. The sacs were filled with 0. 5ml of serosal fluid and placed in mucosal medium of same composition. After incubation of an hour, the amount of Pi lost from mucosal medium and serosal gain of phosphate are estimated. To test the effect of hexoses on Pi transport sugars were added (5.5mM) to the incubation medium (fructose 5.5 and 10mM).To evaluate the inhibitory role of fructose on Pi transport , Succinate and fumerate were added to the serosal compartment. Moreover intraperitoneal injection of mannoheptulose (MH) was given to mice 30 minutes prior to experimentation to load the tissue with MH.

Results: Pi uptake from brush border is reduced significantly by glucose, galactose and 3-0-methyl glucose but not by fructose. Fructose significantly attenuated Pi release process. Fumarate, succinate and preloading with MH lifted this inhibitory effect of fructose partly but significantly (significance $p<0.001$).

Conclusion: Sodium dependent transports of glucose, galactose, 3-O-methyl glucose that are mediated through a common carrier inhibits brush border Pi uptake. Fructose may be transported differently and its phosphorylation in the intestinal wall may be causing inhibition on Pi release. Succinate and fumarate breaks down phosphorylated fructose and action of MH is inexplicable.

INTRODUCTION

Phosphate homeostasis is maintained by cross talk between intestinal phosphate (Pi) absorption and renal Pi excretion. Until recently, the accepted view has been that Pi balance is achieved predominantly through the control of phosphate reabsorption in proximal tubule. However, evidence now suggests that intestinal Pi absorption plays a considerably larger role in Pi homeostasis than previously recognized.

Pathways responsible for intestinal phosphate transport

Despite our detailed knowledge of the renal mechanisms of Pi transport, we know far less about the pathways responsible for Pi absorption across the intestinal tract (Grace & Joanne, 2015).

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What we know about the intestinal Pi transport using brush border membrane vesicles is that there exists a sodium-phosphate co-transport occurring at the intestinal brush border (Berner et al, 1976). The phosphate uptake across the brush border membrane (BBM) is considered to be secondary active as the transport of phosphate into the enterocyte is coupled to the flux of sodium ions and is driven by the Na+ gradient (Baumann et al, 1975, Harrisson & Harrison,1963) . The Na+ dependent phosphate transporters present at BBM utilise the Na+ gradient established by the Na+/K+ ATP ase at the baso-lateral membrane (Lee et al,1986, Loghman-Adham et al,1993). NaPi-IIb is the key player in the intestinal transport of phosphate (Sabagh et al, 2009) and 1,25 dihydroxy-cholecalciferol enhances intestinal Pi absorption via increased NaPi-11b expression (Ganong, 2010). Recent studies using NaPi-11b knock out mice have confirmed the role of this transporter in intestinal phosphate absorption and also revealed a potential role for its involvement in regulating phosphate homeostasis.

The ubiquitously expressed type 111 transporters, Pit1, Pit 2 have also been detected in the intestine although they have not yet been shown to functionally contribute to intestinal Pi absorption (Marks et al, 2010). In humans it is likely that during fasting and with low dietary Pi concentration Pi absorption is mediated by Na Pi -11b but when phosphate levels are elevated postprandially, transport could occur via sodium independent, transcellular or paracellular pathway (Borowitz & Ghishan, 1989).Interestingly this pathway is not regulated by classical regulators of Pi transport like 1, 25 $(OH)_2$ D_3 or dietary phosphate load (Lee et al,1986).

Effect of hexoses on intestinal phosphate transport

Hexoses like glucose, 3-O-methyl glucose and galactose are also transported across the intestine by the similar sodium dependent mechanisms mediated possibly through a common carrier (Crane.1960, Jorgenson et al,1961).It was Robert K. Crane (1960) who presented for the first time his discovery of Na-glucose transport as the mechanism for intestinal glucose absorption. As the absorptive mechanism of phosphate and hexoses (sodium dependent secondary active transports) are similar an inhibitory action of these hexoses is expected on phosphate transport at the intestinal brush border level.With the brush border vesicles prepared from chick intestine , an inhibition on phosphate uptake by glucose was demonstrated by Peterlik et al (1981a).

These studies question not only the previous idea of glucose being an energy substrate but also raises the possibility of a deleterious effect of glucose on phosphate transport. Similar interference of Pi transport by glucose was demonstrated in renal tubules also (Corman et al, 1978).Information on the role of other hexoses on Pi transport is scanty. So experiments are carried out in an attempt to clarify the interaction of sugars with phosphate transport .The role of glucose and other sugars like , 3-O-methyl glucose and galactose is explored here. The role of fructose on Pi transport is also investigated in this study as there are reports to show deleterious effect of dietary fructose on intestinal calcium absorption associated with marked vitamin D insufficiency (Veronique et al, 2010, Armbrecht et al, 2003).

Objectives of the study

- To investigate the inhibitory effect of fructose on intestinal Pi transport of everted gut sacs of mice.
- To evaluate the inhibitory action of fructose on Pi transport
- To study if other hexoses could interact with Pi transport like that of fructose.

MATERIALS AND METHODS

This study is carried out using everted gut sacs of mice and fructose is added at two different concentrations in the incubation medium to check its interference with phosphate transport. To investigate the effects of other hexoses like glucose, galactose and 3-O-methyl glucose on Pi transport, these sugars are added to incubation medium at 5.5 mM. Male albino mice of 3 months old (weighing 24 to 30 gm.) were fed on a standard laboratory diet obtained from Gold Mohur laboratory feeds, Bangalore (calcium 1%, phosphate 0.6%) for a week prior to experimentation.

Everted gut sacs of 6cm length were prepared from the proximal part of the intestine after over night fasting and killing the mice under ether anesthesia. Method of Wilson and Wiseman (1954) was used for preparing the everted gut sacs and the procedure is given below.

Procedure for preparing everted gut sacs

Intestine extending from pyloric end to the ileo-caecal junction was removed carefully from the mice killed under ether anesthesia after the overnight fasting. Fat and mesenteric attachments were removed .This separated segment was immediately chilled and flushed extensively and slowly with ice-cold 0.9% saline using a syringe equipped with a blunt needle**.** To evert the gut , a stainless steel rod (300 mm x 1.5mm) was used with a deep groove on the distal end. This rod was pushed into the lumen of the gut gently and after tying the ileal end of the gut to the deepened groove of this smooth rod , pyloric end was pushed towards this ; until it appeared at the distal opening of the intestine and the eversion was completed by rolling the proximal half of the intestine on the rod. The everted intestine was then slipped off the steel rod and was placed in ice cold saline in a petri dish. For the proximal segment, the first 6 cm segment from the pyloric end was used. The distal end was tied with a ligature and a ligature was placed loosely around the pyloric end ready for tying. This 6 cm segment was weighed quickly.

The sacs were filled with 0.5 ml of the incubation medium (serosal fluid) using a micro-syringe (Gastight syringe 1750, Hamilton Co: USA) and the ligature was tightened. Everted gut sac was placed in 25 ml Erlenmayer flasks with 5 ml of mucosal fluid. After oxygenation of the flasks with 100% O_2 for 1 minute they were tightly closed and incubated in a metabolic shaker bath (Techno India Ltd, Pune, India) with constant shaking at a frequency of 90-100 oscillations/min. for 1 hr at 37^0 C. The incubation medium was of sodium phosphate buffer that contained (mM) : NaCl, 135; KCl, 11 and $CaCl_2$, 0.04 dissolved in 2 mM phosphate buffer at 7.4 pH.

After 1hr of incubation, these sacs were removed from the flasks, blotted and weighed again. The serosal fluid was emptied from the sacs and this serosal fluid and the mucosal fluid from the flasks were used for the estimation of phosphate. Volume changes were taken into consideration in the chemical estimation. The initial serosal fluid content was determined as the difference between the weight of the empty sac and filled everted sac before incubation. The final serosal fluid content was calculated by subtracting the weight of the empty sac from that of the filled sac after incubation. The initial and final weights of empty sacs did not differ significantly. The fall in phosphate content of the mucosal medium is characterized as phosphate uptake by the sac while rise of Pi content of the serosal fluid is considered as phosphate release. Statistical comparisons were carried out using Student's t-test. All the values are expressed as mean ± S.E.M of six observations in each group. Uptake and release of Pi are expressed as µmol/gm tissue wet weight/hr.

Approval of the Ethical Committee of the institution was taken for carrying out this study.

Plasma phosphate in mice was found to be 2.14+0.02 mMol/L. For this purpose blood was collected from the retro-orbital

sinus of the eyes after giving light ether anesthesia. The method of Chen et al (1956) was employed to measure phosphate concentration in mucosal and serosal compartments and the method of Strom (1949) was used in the estimation of lactic acid. In experiments for the evaluation of the inhibitory effect of fructose on intestinal Pi transport using succinate and fumarate, lactate was analyzed and is expressed as µmol/gm. tissue wet weight/hr. The total quantity of lactic acid in the mucosal and serosal fluid was taken as the amount of lactic acid released.

Additives to the incubation medium:

To test the effect of fructose and other hexoses on phosphate transport, all sugars except fructose were added at a concentration of 5.5mM and fructose of 5.5 mM &10 mM was added to both mucosal and serosal compartment prior to incubation. To evaluate the effect of fructose on Pi transport metabolic intermediates succinate (20 mM) and fumarate (20mM) were added to the serosal compartment. For the same purpose, preloading of the intestinal tissue was done in mice with mannoheptulose, a known inhibitor of enzyme hexokinase (Coore & Randle, 1964). For preloading the control group of mice received only saline while the test group was administered intra- peritonealy 1 ml of 20mM of mannoheptulose in saline 30 minutes prior to experimentation**.** Enterocytes were isolated mechanically by vibrating the drained gut sacs for performing the trypan blue test for viability (Karsenty et al, 1985). These cells were then incubated with 0.2% trypan blue solution at 37^{o}C to check the viability.80 to 90% of the mucosal cells showed the exclusion of the stain showing their viability.

Chemicals: All chemicals were of analar grade. Sugars of galactose, 3-O-methylglucose and succinate , fumarate were purchased from Sigma Chemical Company, St. Louis, MO,USA.

RESULTS

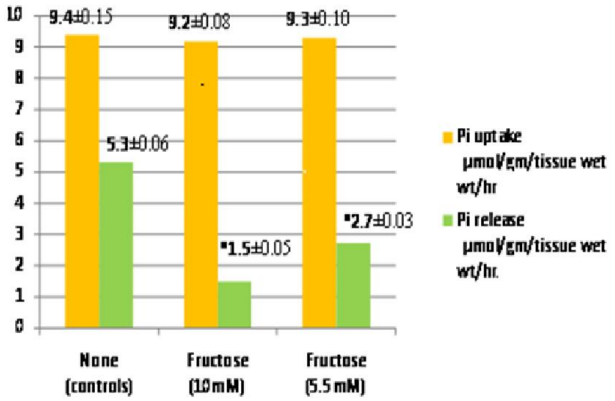

Fig 1. Effect of fructose on Pi transport of everted gut sacs of mice (Mean and S.E.M.)

Figure:1: Fructose was added to the incubation medium at 5.5 mM and 10 mM as indicated below the bars .Controls do not have fructose in the incubation medium(none/controls). Presence of fructose affected Pi release only and this inhibition is significant (P< 0.001, t-test) at a dose dependent manner while leaving Pi uptake uninterrupted. Each value is the mean of six observations. Mean value and its S.E.M. are given respectively on the top of the corresponding bars. Pi uptake and release are represented as µmoles /gm tissue wet weight /hr. *Marked values are significantly different from that of the control value (P< 0.001, t-test).

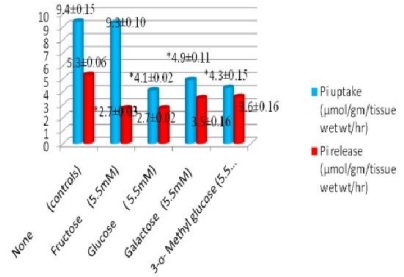

Fig. 2. Effect of hexoses on Pi transport of everted gut sacs of mice (Mean and S.E.M)

Figure 2: All sugars are added to the incubation medium at 5.5 mM. Each value is the mean of six observations. * Marked values are significantly different from that of control value (P< 0.001, t-test). With fructose Pi release only is inhibited significantly and Pi uptake is uninterrupted. While with glucose, galactose and 3-o-methyl glucose Pi uptake is significantly (P< 0.001, t-test) inhibited at the brush border membrane side. Pi release is proportionately reduced.

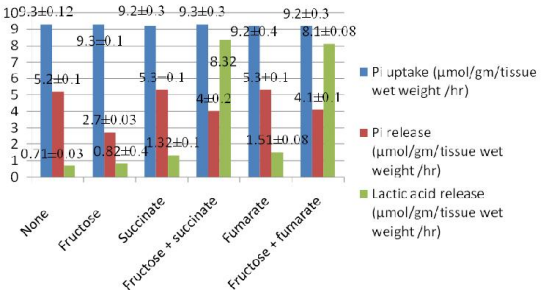

Fig. 3. Effect of succinate, fumarate on fructose inhibition of Pi release and on lactic acid release by everted gut sacs of mice (Mean and S.E.M)

Figure-3: Phosphate release with fructose (5.5mM) and succinate or fumarate (20 mM on serosal side) is significantly higher (P<0.001) than with fructose alone. The metabolite intermediates, Succinate and fumerate significantly elevated lactic acid output in presence of fructose (P<.001). Succinate (20mM), fumerate (20mM) were added to the serosal compartment. Each value is the mean of six observations.

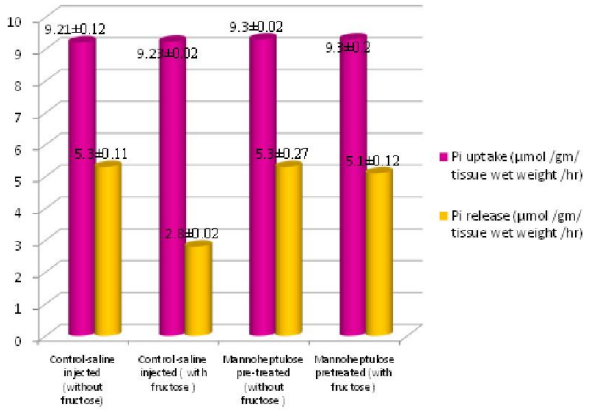

Fig 4. Effect of pre-treatment with mannoheptulose on fructose inhibition of phosphate transport of everted gut sacs of mice (Mean and SEM)

Fig 4 : For preloading with mannoheptulose the the test group was administered intra- peritoneally 1 ml of 20mM of mannoheptulose in saline 30 minutes prior to experimentation and the control group of mice received only saline. No significant difference was found between test and control group in phosphate uptake . But Phosphate release in the presence of fructose was significantly elevated ($P<0.001$) by pre-treatment with mannoheptulose. Control values obtained with saline pretreated ones did not differ from the relevant values obtained in other experiments.

Result contd

Effect of fructose on Pi transport is shown in Figure 1. Addition of fructose to the incubation medium did not affect the uptake of phosphate by the everted sacs but significantly decreased the Pi release by the sacs in a dose dependent manner. While the addition of other hexoses ie glucose, 3-O-methyl glucose and galactose exerted the inhibitory effect on the brush border process of Pi uptake only. As the Pi uptake process was affected, the release process (refer to Figure: 2) was reduced proportionately from the baso-lateral side in these experiments with hexoses in the incubation medium. Addition of succinate and fumerate did not affect phosphate transport by themselves, but they significantly lessened the inhibition of phosphate release caused by fructose (refer toFigure:3). Lactic acid release from the sacs with fructose and succinate /fumerate showed significant elevation ($P<.001$). Loading the tissue with mannoheptulose prior to experimentation did not affect the Pi uptake in the presence or absence of fructose. But mannoheptulose treated sacs showed significant elevation in phosphate release process in the presence of fructose (Figure: 4).

DISCUSSION

It is clearly shown from these experiments that fructose behaved differently from that of other hexoses glucose, galactose and 3-O-methyl glucose in influence on phosphate transport (refer to Figure :2). Presence of fructose inhibited only Pi release process from the enterocytes leaving the brush border process of Pi uptake uninterrupted. The two different concentrations of fructose at 5.5 and 10 mM in the incubation medium showed the similar pattern of inhibition on Pi release process in a dose dependent manner (Figure:1). All the other hexoses inhibited the Pi uptake process at the brush border side of the enterocyte only. What could be the factor contributing towards this difference in behavior of fructose?

Inhibitory mechanism of various hexoses on phosphate transport

Glucose inhibits phophate uptake lending support to the observations of Peterlik et al (1981a). Similar behavior was exhibited by galactose and 3-O-methyl glucose. All these hexoses are transported by sodium dependent mechanisms mediated possibly through a common carrier (Crane, 1960; Jorgenson et al., 1961). Crane's discovery of co-transport was the first ever proposal of flux coupling in biology. Fructose is transported by a mechanism that neither needs sodium nor the glucose carrier (Guy and Deren,1971, Boyd and Parsons 1979).The majority of research supports the claim that fructose absorption occurs on the mucosal membrane via facilitated transport using GLUT-5 transport proteins.

Since the concentration of fructose is higher in the lumen, fructose is able to flow down a concentration gradient into the enterocyte, assisted by this transport proteins, GLUT - 5 located at the apical pole of the enterocyte. Contrary to glucose, this process does not require ATP hydrolysis and is independent of sodium absorption. (Corpe et al., 1999; Dourd and Ferraris; 2008). The small intestine regulates fructose absorption from dietary sources and therefore the availability of fructose to other tissues. But most cells have only low amounts of the GLUT-5 transporter, which transports fructose into cells. Even though active transports of glucose and phosphate by the intestine were shown to be sodium dependent (Fuchs and Peterlik,1979) and to be stimulated by vitamin D (Wasserman & Taylor, 1973; Peterlik et al,1981b) it should not be assumed that a common carrier is involved in both the transports. Evidence employing competitive inhibitors indicates that arsenate and phlorizine specifically block only phosphate and glucose transport respectively (Peterlik et al, 1981a).

Inhibitory mechanism operating on sodium dependent intestinal transports

Different explanations have been offered to explain the mutual inhibition between sodium dependent transport systems across the brush border membrane. Barret and Aronson (1982) while studying the inhibitory interaction between glucose, alanine and phosphate opined that the inhibition is due to an alteration in the transmembrane electro-chemical gradient. Bingham et al, (1966) hypothesized that inhibition between galactose and aminoacid transport in rat is due to a competition for energy between the two substrates. Browne and Smythe (1975) found that augmentation of energy by supplying citrate led to a rise in α methyl glucoside transport by 25%. L-proline which normally inhibits the transport of sugar , failed to do so in presence of citrate .It is possible that these two substances , α methyl glucoside and proline compete for a common source of energy and if the provision of energy is augmented by supply of citrate the mutual inhibition seems to disappear.

Alvarado & Robinson (1979) classified the mechanisms of inhibition available into two categories: cis -hypothesis and trans- hypothesis. According to cis-hypothesis, there is an allosteric interaction between substrates- binding to separate but related sites at the outer face of the brush border matrix. In contrast trans-hypothesis envisages the interaction to result from a partial dissipation of the electro-chemical sodium gradient due to the co-transport of each substrate with sodium ions. The results obtained in the present experiments indicate that galactose even though it carries less sodium than glucose (Taylor et al, 1968) was less potent than glucose in inhibiting Pi uptake. Such an evidence lends support to trans-hypothesesis.

Inhibitory mechanism involved in fructose inhibition on Pi release

The observation that fructose does not interfere with uptake of phosphate, coupled with the fact that fructose undergoes phosphorylation after absorption (Crane, 1960), strongly suggests that the significant decrease in phosphate release may be due to sequestration of phosphate intracellularly. Small intestine is the organ system expressing the greatest amount of GLUT-5 in humans (Blackmore et al., 1995, Dyer eta al., 2002) and therefore accumulation of fructose is very high in intestinal tissue.

Fructose is known to be phosphorylated upon entry into the cell. Fructose phosphorylation has been shown to cause a fall in cellular phosphate and ATP content inside the liver (Maenppa et al, 1968) and in the renal cortex (Morris et al., 1978). It is possible that fall in release of phosphate into the serosal compartment may be due to phosphorylation of fructose in the intestinal wall leading to a decrease in the cellular Pi available for efflux. ATP is needed for the further breakdown of phosphorylated fructose derivatives (Harper, ed.1979). Fumarate and succinate are known to increase cell respiration and ATP generation (Baldwin, 1957).

These compounds are partly able to reverse the inhibition exerted by fructose on Pi efflux, probably by increasing the breakdown of fructose as evidenced by increase in lactate release, the end product of fructose metabolism, thereby elevating the free phosphate level of the cell. Kirchner et al. (2008) attributes inhibitory action of fructose in their study on intestinal sodium-phosphate co-transporter gene expression and on phosphate uptake in rats. Similar study of Armbrecht et al. (2003) showed a strong correlation between Ca intestinal absorption and CaBP-9k expression levels. But studies of Aeberli et al. (2007) suggest that the relation of fructose to health needs re-evaluation.

Effect of preloading the tissue with mannoheptulose on fructose induced inhibition

Preloading of intestinal tissue with mannoheptulose, a known inhibitor of the enzyme hexokinase (Coore & Randle, 1964) also decreased the inhibition exerted by fructose on Pi release. Mannoheptulose is a heptose that inhibits glucokinases and hexokinases in diverse organisms. Naftalin and Rist (1989) have demonstrated a decrease in phosphorylation of 2-deoxy-glucose by treatment with mannoheptulose. But how mannoheptulose is responsible for its effectiveness in reversing fructose induced inhibition on phosphate release remains inexplicable.

Uses of mannoheptulose: Mannoheptulose being a non-toxic glycolytic inhibitor with negligible effects on food intake and body water is currently used as an effective caloric restriction mimetic (CRM) in anti-aging and health promoting (George Roth et al, 2009).This sugar found in avocados got the ability to alter energy intake, to lower daily energy expenditure and also to promote satiation in dogs (Leslie et al. 2015) . With furthur research this interesting botanical extract could be used as a new cancer adjunct as it was shown to inhibit the growth rate of cultured tumour cell lines (Patrick Quillin, 2000).

Importance of this study

If fructose inhibits the intestine's absorption of phosphate it can result in fragile bones, leading to osteoporosis in adults or rickets in children. Excessive consumption of carbonated beverages with high fructose have been strongly associated with increased incidence of bone fractures in the adolescent population (Wysshak, 2000).

This adverse effect of fructose should be kept in mind as fructose is often recommended for diabetics because it does not trigger the production of insulin by pancreatic β cells probably because β cells have low levels of GLUT5 (Curry,1989; Grant et al, 1980).

As fructose is obviously causing an inhibition on Pi transport in this study further investigation is required as the consumption of fructose has gone very high nowadays. Not only fructose but the usageof other sugars also raise the same question since other sugars too affect the Pi transport acting on the brush border mechanism in our study. In fact Tsanzi et al (2008) came across very deleterious adverse effects of glucose in rats . They observed rats provided with the glucose-sweetened beverages had reduced femur and tibia total phosphate, reduced phosphate and calcium intake, and increased urinary calcium excretion compared with the rats provided with the fructose-sweetened beverage. So further research is required as there is controversy in the reports regarding the adverse effects of sugars on skeletal system.

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