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Phosphatonin washout in *Hyp* mice proximal tubules: evidence for posttranscriptional regulation

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Abstract

X-linked hypophosphatemia is the most common inherited form of rickets. It is characterized by renal phosphate wasting, leading to hypophosphatemia and an inappropriately normal or low serum level of 1,25(OH)₂ vitamin D. Previous studies have pointed to a circulating factor or phosphatonin-inhibiting phosphate transport by decreasing mRNA of the proximal tubule NaP_i cotransporter NaPi-2A. The present study examined the hypothesis that there was also posttranscriptional regulation of the NaPi-2A cotransporter in *Hyp* mice proximal tubules and whether the phosphate transport defect in *Hyp* mice persisted when they were studied in vitro. We found that the rate of phosphate transport in *Hyp* mice was <50% that in C57/B6 control mice. While phosphate transport remained stable during incubation with time in C57/B6 mice proximal tubules, it increased from 0.46 ± 0.47 to 1.83 ± 0.40 pmol·mm⁻¹·min⁻¹ in *Hyp* proximal tubules ($P < 0.01$) consistent with phosphatonin washout in *Hyp* proximal tubules perfused in vitro. This time-dependent increase in phosphate transport was still observed in the presence of cycloheximide. There was also a reduction of proximal tubule apical NaPi-2A expression from *Hyp* mice compared with C57/B6 mice using single-tubule immunohistochemistry. Using immunohistochemistry, we demonstrate an increase in apical expression of the NaPi-2A transporter in proximal tubules perfused in vitro in *Hyp* mice even in the presence of bath cycloheximide. The increase in apical expression of the NaPi-2A transporter in proximal tubules perfused in vitro in *Hyp* mice was blocked by colchicine. These data are consistent with a rapidly reversible posttranscriptional defect in *Hyp* mice causing a reduction in phosphate transport.

Keywords

phosphate transport; proximal tubule transport; in vitro microperfusion; X-linked hypophosphatemia

X-linked hypophosphatemia is the most common inherited cause of rickets (44). Children with this disorder have marked short stature and rickets. This X-linked dominant disorder is characterized by hypophosphatemia, normocalcemia, and a relative deficiency of 1,25(OH)₂ vitamin D (44). The hypophosphatemia is due to impaired proximal tubule phosphate reabsorption (10, 14, 19, 22, 44, 48). The vitamin D deficiency is due to decreased 25(OH)D-1 α -hydroxylase activity (11, 13, 40, 45).

The *Hyp* mouse has a mutation in the same ortholog as that in patients with X-linked hypophosphatemia (7, 18, 44). The overwhelming evidence points to a humoral factor mediating the aberrant proximal tubule phosphate transport in this disorder. Parabiosis studies have demonstrated that this humoral factor can impair phosphate transport in normal mice and that the humoral factor is not parathyroid hormone (PTH) (29, 30). Transplantation of a *Hyp* mouse kidney into a control mouse leads to normal phosphate levels and rates of phosphate excretion, whereas transplantation of a normal kidney into a *Hyp* mouse results in hyperphosphaturia (33). The factor that inhibits proximal tubule transport may be liberated from bone (23, 39). Both serum from *Hyp* mice and conditioned media from *Hyp* mouse primary osteoblastlike cell cultures inhibit mouse proximal tubule phosphate transport (39). Thus there does not appear to be an intrinsic proximal tubule defect in X-linked hypophosphatemia.

While there is no intrinsic defect in the proximal tubule or the NaPi cotransporter, the $V_{\max}$ of the NaPi cotransporter (NaPi-2A) was 50% less than that in control mice (31, 46). Previous studies have also demonstrated that the decrease in phosphate transport was due, at least in part, to a reduction in NaPi-2A mRNA and brush-border membrane protein abundance compared with control mice (9, 48). The purpose of the present study was to examine the relative rates of phosphate and volume absorption in proximal tubules from *Hyp* mice and to determine whether the reduction in phosphate transport was acutely reversible in vitro. The present study provides evidence that the reduction in phosphate transport in *Hyp* mice is due, at least in part, to posttranscriptional regulation of the NaPi-2A cotransporter.

METHODS

In vitro microperfusion flux studies

Male C57/B6 and *Hyp* mice were of the same genetic background. They were allowed free access to food and water until the time of the study. This study conformed to the American Physiological Society's *Guiding Principles in the Care and Use of Laboratory Animals* and was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Isolated segments of superficial proximal straight tubules (PST) were perfused as previously described (3, 36). Briefly, tubules were dissected in Hanks' balanced salt solution containing (in mM) 137 NaCl, 5 KCl, 0.8 MgSO₄, 0.33 Na₂HPO₄, 0.44 KH₂PO₄, 1 MgCl₂, 10 Tris, 0.25 CaCl₂, 2 glutamine, and 2 L-lactate at 4°C. Dissected tubules were then transferred to a 1.2-ml temperature-controlled bath chamber and perfused using concentric glass pipettes at 38°C.

PST were perfused at ~10 nl/min. The perfusion solution was an ultrafiltrate-like solution containing (in mM) 115 NaCl, 25 NaHCO₃, 4.0 Na₂HPO₄, 10 Na acetate, 1.8 mM CaCl₂, 1 MgSO₄, 5 KCl, 8.3 glucose, and 5 alanine. The bathing solution contained 6 g/dl BSA. The osmolality of all solutions were adjusted to 295 mosmol/kgH₂O. The pH and osmolality of the bathing solution were maintained constant by continuously changing the bath at a rate of at least 0.5 ml/min. Net volume absorption (J_V ; in nl·mm⁻¹·min⁻¹) was measured as the difference between the perfusion (V_o) and collection (V_L) rates (nl/min) normalized per millimeter of tubular length (L). Freshly dialyzed [*methoxy*-³H]inulin was added to the perfusate at a concentration of 75 µCi/ml so that the perfusion rate could be calculated. The collection rate was measured with a 50-nl constant-volume pipette. The length (in mm) was measured with an eyepiece micrometer. Tubules were incubated for 15–20 min before initiation of the control period. Phosphate transport was determined using the following equation

$$J_{\text{PO}_4} = [(V_o C_o^* - V_L C_L^*) / L] P_o / C_o^*$$

where P_o is the phosphate concentration in the perfusate, and C_o^* and C_L^* are the phosphate concentrations in the perfusate and collected fluids, respectively, in counts per minute per nanoliter.

Brush-border membrane vesicle isolation

C57/B6 and *Hyp* mouse kidneys were removed and placed in ice-cold isolation buffer containing (in mM) 300 mannitol, 16 HEPES, and 5 EGTA titrated to pH 7.4 with Tris. The isolation buffer contained aprotinin (2 µg/ml), leupeptin (2 µg/ml), and phenylmethylsulfonyl fluoride (100 µg/ml). The cortex was homogenized with 20 strokes of a Potter Elvehjem homogenizer at 4°C. Brush-border membrane vesicles (BBMV) were then isolated by differential centrifugation and magnesium precipitation as described previously (17). The final BBMV fraction was resuspended in isolation buffer. Protein was assayed using the Lowry method with crystalline BSA as the standard (26).

SDS-PAGE and immunoblotting

Brush-border membrane proteins and proteins from the cortical homogenate (25 µg/lane) were denatured and then separated on a 7.5% polyacrylamide gel using SDS-PAGE as previously described (4). The proteins were transferred overnight to a polyvinylidene difluoride membrane at 120–140 mA at 4°C. The blot was blocked with fresh Blotto (5% nonfat milk and 0.1% Tween 20 in PBS, pH 7.4) for 1 h followed by incubation with primary antibody to NaPi-2A. NaPi-2A antibody, a generous gift from Dr. Jürg Biber (University of Zürich, Zürich, Switzerland), was added at 1:1,000 dilution overnight at 4°C. The blot was then washed extensively with Blotto. The secondary antibody, horseradish peroxidase-conjugated donkey anti-rabbit Ig was added at 1:10,000 dilution and incubated in room temperature for 1 h. The blot was again washed with Blotto, and enhanced chemiluminescence was used to detect bound antibody (Amersham Life Science). The NaPi-2A protein abundance was quantitated using densitometry. Equal loading of the

samples was confirmed using an antibody to β -actin at a 1:5,000 dilution (Sigma, St. Louis, MO).

In vitro microperfusion and single-tubule immunohistochemistry

Mouse PST were dissected and perfused as described above with slight modifications. The perfusion and bathing solutions were an ultrafiltrate-like solution, which both contained 2 mM glutamine and lactate. *Hyp* and C57/B6 mouse tubules were perfused for either 5 min and maintained at 4°C or were heated to 38°C for 60 min. They were then fixed while still being perfused for 5–10 min in the bathing chamber. The fixative consisted of 3% paraformaldehyde and 0.05% picric acid, which was dissolved in a 3:2 mixture of 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mosmol/kgH₂O with sucrose) and 10% hydroxyethyl starch in saline (HAES-sterile, Fresenius, Stans, Switzerland) as described by Traebert et al. and our laboratory (2, 51). The tubule was then released near the bottom of the bathing chamber, and the bathing chamber was removed to a dissection microscope for better visualization of the tubule. Immediately before the tubule was transferred, a drop of warm (38°C) 10% gelatin (Gelatin Type A 175 Bloom, Sigma) in PBS was placed on a microscope slide cooled to 4°C. After the gelatin hardened slightly, the tubule was transferred with an Eppendorf pipette from the bathing chamber to the gelatin. The excess fixative was removed from the drop of gelatin, and a second drop of cooled but still molten gelatin was placed on top of the tubule. The slide was placed at 4°C for 1 h, and then a cube of gelatin containing the tubule oriented in the long axis was cut and stored in fixative at 4°C.

For immunodetection of NaPi-2A in single tubules, the gelatin droplets containing the tubules were washed for 2 h in PBS and cut into cubes before being frozen in liquid propane. Cryosections of 2- to 3- μ m thickness were mounted on chromalum/gelatin-coated glass slides, thawed, and stored in PBS until labeling use. For immunofluorescence, sections were pretreated with 3% milk powder in PBS for 10 min and incubated overnight at 4°C with a rabbit anti-rat polyclonal antiserum against NaPi-2A. For type 3 Na⁺/H⁺ exchanger (NHE3), we used a polyclonal anti-rat NHE3 antibody diluted 1:300 in 5% milk powder in PBS containing 0.05% Tween 20. Sections were then rinsed three times with PBS and incubated for 45 min at 4°C with the secondary antibody (swine anti-rabbit IgG conjugated to fluorescein isothiocyanate, Dakopatts, Glostrup, Denmark) diluted 1:50 in PBS/milk powder. Finally, the sections were rinsed three times with distilled water, plated on coverslips by using DAKO-Glycergel (Dakopatts) containing 2.5% 1,4-diazabicyclo (2.2.2.) octane (DABCO; Sigma) as a fading retardant, and studied by an epifluorescence microscope (Polyvar, Reichert-Jung). Controls performed with omission of the primary antiserum or with nonimmune sera were all negative.

RESULTS

In the first series of experiments, we compared the rate of volume absorption and phosphate transport in C57/B6 and *Hyp* mice PST perfused in vitro. The rates of volume absorption in proximal tubules from C57/B6 and *Hyp* mice were 0.32 ± 0.07 and 0.23 ± 0.06 nl·mm⁻¹·min⁻¹, respectively. This difference was not statistically significant. However, as

shown in Fig. 1, the rate of phosphate transport in C57/B6 male was over twofold greater than that of *Hyp* male mice. Thus there is reduced phosphate transport in intact *Hyp* mice tubules perfused in vitro. We next examined whether the reduction in phosphate transport was reflected by a reduction in BBMV NaPi-2A cotransporter abundance. The results are shown in Fig. 2. While BBMV in *Hyp* mice had significantly less NaPi-2A protein abundance than C57/B6 mice, total cellular NaPi-2A protein abundance was not different between these mice. This result suggested to us that there may be posttranscriptional regulation of the NaPi-2A cotransporter. In the next series of experiments, we examined whether the time of incubation affected the rate of volume and phosphate absorption in C57/B6 and *Hyp* mice. Proximal tubules from both groups were perfused in vitro at 38°C for 15–20 min, after which determinations were made of phosphate and volume absorption. The tubules were then incubated again for a total of ~1 h, and measurements of transport rates were repeated. The rate of volume absorption in proximal tubules from C57/B6 mice was 0.39 ± 0.09 in the first period and 0.35 ± 0.11 nl·mm⁻¹·min⁻¹ in the second period. For in vitro perfused proximal tubules from *Hyp* mice, the rate of volume absorption was 0.32 ± 0.09 in the first period and 0.27 ± 0.11 nl·mm⁻¹·min⁻¹ in the second period. Thus the rate of volume absorption did not change significantly in either strain. As shown in Fig. 3, while the rate of phosphate transport did not change in proximal tubules from C57/B6 mice, there was a significant increase in the rate of phosphate transport with time in the *Hyp* mouse proximal tubules consistent with phosphatonin washout.

We next examined whether the time-dependent increase in phosphate transport that occurred with time of incubation occurred in the presence of 5 µg/ml cycloheximide. This dose of cycloheximide has been shown to inhibit >95% [³H]leucine incorporation in MCT and NIH cells as well as inhibit the glucocorticoid-induced increase in bicarbonate absorption in rabbit proximal tubules perfused in vitro (6, 53). As shown in Fig. 4, there was an increase in the rate of phosphate transport even with cycloheximide in the bathing solution. Thus the proposed phosphatonin washout occurs in the absence of new protein synthesis.

In Fig. 5, we compared basal NaPi-2A expression in C57/B6 and *Hyp* mice proximal tubules perfused in vitro. In this experiment proximal tubules were dissected at 4°C, transferred to a microperfusion chamber, and perfused in vitro for 5 min at 4°C. As can be seen, there is a paucity of apical membrane NaPi-2A expression in *Hyp* mouse proximal tubules, whereas there was intense staining in proximal tubules from C57/B6 mice. Thus C57/B6 proximal tubules had more apical NaPi-2A expression than *Hyp* mouse tubules as expected from the immunoblot of BBMV from the two strains.

We next examined whether the apical expression of NaPi-2A would mirror the increase in phosphate transport with incubation for 1 h at 38°C. Apical NaPi-2A expression remained intense in C57/B6 mice (Fig. 6). In *Hyp* mouse proximal tubules, the staining for NaPi-2A increased after 1 h of incubation, consistent with the increase noted in our phosphate absorption data. This was also true if the bathing solution contained cycloheximide. In the final series of experiments, we examined whether there was an increase in apical NaPi-2A expression in *Hyp* mouse proximal tubules in the presence of 10⁻⁴ M luminal and bath colchicine, which disrupts microtubules. As shown in Fig. 7, the increase in NaPi-2A after 1 h of incubation at 38°C was prevented by colchicine.

DISCUSSION

The present study used in vitro microperfusion of proximal tubules to compare the rates of phosphate transport in *Hyp* and C57/B6 mice. We demonstrate that while the rate of total volume absorption was not significantly lower than that in C57/B6 mouse tubules, the rate of phosphate transport in *Hyp* mice proximal tubules was far lower than that of C57/B6 mouse tubules. These data are consistent with a selective decrease in proximal tubule phosphate transport in these mice.

Previous studies have examined phosphate transport in *Hyp* mice compared with C57/B6 mice. BBMVs from *Hyp* mice have a lower rate of phosphate transport than those in control mice due to a lower $V_{\max}$ of NaPi cotransport (31, 46). Several studies point to the fact that the phosphate transport defect in the *Hyp* mouse is not due to an intrinsic abnormality in the *Hyp* proximal tubule. First, the NaPi-2A cotransporter is not mutated in the *Hyp* mouse (9). Furthermore, immortalized proximal tubule cells from *Hyp* mice have normal rates of phosphate transport when studied days to weeks after removal from the *Hyp* mouse (32, 34). The present study demonstrates that while the intrinsic rate of phosphate transport is lower in *Hyp* proximal tubules, the rate of phosphate transport normalizes rapidly after perfusion in vitro. This effect was not due to a nonspecific effect of in vitro microperfusion since the rate of phosphate transport was stable in proximal tubules from C57/B6 mice.

The fact that *Hyp* proximal tubules maintain a reduced rate of phosphate transport when perfused in vitro, at least transiently, indicates that the proximal tubule from the *Hyp* mouse has a memory for its in vivo environment when the tubules are perfused in vitro. This memory effect has been described previously in other in vivo microperfusion studies of both the proximal and distal tubule where the in vivo transport properties are maintained when the tubule is studied in vitro (1, 27, 28, 41). *Hyp* mice and patients with X-linked hypophosphatemia have a mutation in the PHEX gene (phosphateregulating gene with endopeptidase activity, which is located on the X chromosome) (12, 16, 38, 49). One laboratory has demonstrated that FGF-23 is a substrate for PHEX but not the mutant form of FGF-23 produced by patients with autosomal dominant hypophosphatemic rickets (8). However, others have found that FGF-23 is not a PHEX substrate (15, 24) and that in some way the absence of PHEX results in overexpression of FGF-23 (24). In addition, there are likely to be other phosphatonins. Most patients with X-linked hypophosphatemia have inappropriately high levels of FGF-23 (20, 52, 55).

There is also direct evidence that FGF-23 inhibits phosphate transport in vivo and in vitro. Injection of recombinant FGF-23 into mice resulted in a reduction in serum phosphate levels and an increase in urinary excretion of phosphate (42). FGF-23 did not cause an increase in glucose or amino acid excretion (42). In addition, implantation of FGF-23-secreting tumor cells into nude mice resulted in the development of hypophosphatemia, hyperphosphaturia, an increase in alkaline phosphatase, and a reduction in 1α -vitamin D hydroxylase (42). Similar studies have been performed using mutant FGF-23 proteins found in patients with autosomal dominant hypophosphatemic rickets (43). However, this group failed to find an effect of recombinant native FGF-23 on phosphate transport in opossum kidney (OK) cells (42). On the other hand, others have demonstrated a reduction in phosphate transport in OK

cells incubated with conditioned media from COS-7 cells that have been transfected with V5-tagged FGF-23 (8). There is evidence from the crystal structure of the FGF receptor that heparin is necessary for the complete assembly of FGF and the FGF receptor (35, 37). While one group has found that FGF-23 inhibits phosphate transport in OK cells (8), a cell line with characteristics of proximal tubule cells, others have not seen an effect of FGF-23 in the absence of 10 μ g/ml heparin (42, 54).

The normalization of phosphate transport acutely after removal from the in vivo environment is consistent with phosphatonin washout. Previous studies have examined the mechanism for the reduction in phosphate transport in *Hyp* mice compared with C57/B6 mice and found a reduction in not only BBMV NaPi-2A protein abundance but also mRNA abundance (31, 48). The finding in the current study demonstrating that total cellular NaPi-2A protein abundance is comparable in *Hyp* and C57/B6 mice despite the fact that there is a reduction in NaPi-2A mRNA suggests that there may be posttranscriptional regulation of NaPi-2A in *Hyp* mice. A dissociation of BBMV NaPi-2C protein and NaPi-2C mRNA abundance has previously been found in NaPi-2A $-/-$ mice (47).

The finding of a reduction in NaPi-2A mRNA abundance in *Hyp* mice by others and our findings are consistent with posttranscriptional regulation of NaPi-2A comparable to the regulation of NaPi-2A by PTH (21, 25, 50). PTH infusion into parathyroidectomized rats can reduce NaPi-2A mRNA abundance in as little as 2 h (21). PTH also affects phosphate transport by regulating NaPi-2A cotransporter trafficking (25, 50). The circulating phosphatonin in *Hyp* mice might similarly have an effect on posttranscriptional regulation of the NaPi-2A transporter. The increase in phosphate transport and apical membrane NaPi-2A protein abundance in *Hyp* mice with time when the tubules are perfused in vitro was still present in the presence of cycloheximide. Thus the acute recovery in phosphate transport in *Hyp* mice is not dependent on NaPi-2A protein synthesis. The increase in apical membrane NaPi-2A protein abundance in *Hyp* mice proximal tubules with time when the tubules are perfused in vitro was not seen in the presence of colchicine.

The finding that there is not an intrinsic defect in the proximal tubule cell and the NaPi-2A cotransporter may be therapeutically important for X-linked hypophosphatemia. We have recently found that there is dysregulation of renal prostaglandin metabolism in *Hyp* mice (5). *Hyp* mice treated with indomethacin had a normalization in their rate of phosphate excretion due entirely to a tubular increase in phosphate transport. While the present study demonstrates that there was an increase in phosphate transport, it should be stated that while this increase was dramatic, it did not normalize the rate of phosphate transport to that measured in C57/B6 mice. Studies done in the presence of 10^{-6} M bath indomethacin, however, resulted in a complete normalization in the rate of phosphate transport.

In conclusion, this study readdresses the mechanism for the reduction in phosphate transport in *Hyp* mice compared with C57/B6 mice. We demonstrate that *Hyp* proximal tubules perfused in vitro have a reduction in the rate of phosphate transport, which normalizes when the tubules are perfused in vitro. This normalization in the rate of phosphate transport is paralleled by an increase in the expression of the NaPi-2A transporter to the apical membrane. Both the recovery in transport and the increased apical expression were not

dependent on new protein synthesis. This study suggests that the acute recovery in phosphate transport may be the result of posttranscriptional regulation.

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Fig. 1.

Proximal tubules from C57/B6 male and *Hyp* male mice were perfused in vitro. The rate of volume absorption (J_v) was similar in C57/B6 male and *Hyp* male mice proximal straight tubules (A). However, as shown in B, the rate of phosphate absorption (J_{phos}) was lower in *Hyp* male mice proximal straight tubules than in those of C57/B6 male mice. * $P < 0.01$

A **B** **Fig. 2.**

A: top immunoblot compares NaP_i cotransporter NaPi-2A brush-border membrane abundance in C57/B6 male and *Hyp* male mice. The *bottom* immunoblot compares total cellular NaPi-2A abundance in C57/B6 male and *Hyp* male mice. Cont, control. As shown in *B*, C57/B6 male mice had significantly greater brush-border membrane vesicle (BBMV) NaPi-2A/ β -actin abundance than *Hyp* mice, whereas there is no difference in C57/B6 male and *Hyp* mice total cellular NaPi-2A/ β -actin abundance.

Fig. 3. Effect of time of incubation on the rate of phosphate transport in C57/B6 (A) and *Hyp* mice (B). Tubules were initially perfused for 15–20 min before collections for phosphate transport were performed and again after 1-h incubation. There was no difference in the rate of phosphate transport with time in C57/B6 mice, but *Hyp* mice proximal tubules demonstrated a consistent increase in volume absorption with time.

Fig. 4.

Effect of time of incubation on the rate of phosphate transport in *Hyp* mice in the presence of cycloheximide. Tubules were initially perfused for 15–20 min before collections for phosphate transport were performed and again after 1-h incubation.

Fig. 5.

NaPi-2A and NHE3 expression in in vitro perfused proximal straight tubules obtained from *Hyp* mice and C57/B6 mice. In these studies, tubules were dissected at 4°C, perfused in vitro for 5 min at the same temperature, fixed, and then stained for NaPi-2A or NHE3. As can be seen, there is a paucity of NaPi-2A staining on the brushborder membrane in *Hyp* mice (A) compared with C57/B6 mice proximal tubules (B). The staining for NHE3 in *Hyp* mice (C) is comparable to that in C57/B6 mice (D).

Fig. 6. NaPi-2A expression in proximal straight tubules obtained from C57/B6 mice (A), *Hyp* mice (B), and *Hyp* mice in the presence of bath cycloheximide (C) that have been perfused in vitro for 60 min at 37°C. As can be seen, apical staining is intense in all 3 groups.

Fig. 7.

NaPi-2A expression in in vitro perfused (60 min at 37°C) proximal straight tubules obtained from C57/B6 mice (A) and *Hyp* mice (B) in the presence of bath and luminal 10^{-4} M colchicine. As can be seen, apical staining is still present in C57/B6 mouse tubules but not in *Hyp* mice tubules. Similar findings were found with luminal colchicine alone.