Acute Adaption to Oral or Intravenous Phosphate Requires Parathyroid Hormone

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ABSTRACT

Phosphate (Pi) homeostasis is regulated by renal, intestinal, and endocrine mechanisms through which Pi intake stimulates parathyroid hormone (PTH) and fibroblast growth factor-23 secretion, increasing phosphaturia. Mechanisms underlying the early adaptive phase and the role of the intestine, however, remain ill defined. We investigated mineral, endocrine, and renal responses during the first 4 hours after intravenous and intragastric Pi loading in rats. Intravenous Pi loading (0.5 mmol) caused a transient rise in plasma Pi levels and creatinine clearance and an increase in phosphaturia within 10 minutes. Plasma calcium levels fell and PTH levels increased within 10 minutes and remained low or high, respectively. Fibroblast growth factor-23, 1,25-(OH)2-vitamin D3, and insulin concentrations did not respond, but plasma dopamine levels increased by 4 hours. In comparison, gastric Pi loading elicited similar but delayed phosphaturia and endocrine responses but did not affect plasma mineral levels. Either intravenous or gastric loading led to decreased expression and activity of renal Pi transporters after 4 hours. In parathyroidectomized rats, however, only intravenous Pi loading caused phosphaturia, which was blunted and transient compared with that in intact rats. Intravenous but not gastric Pi loading in parathyroidectomized rats also led to higher creatinine clearance and lower plasma calcium levels but did not reduce the expression or activity of Pi transporters. This evidence suggests that an intravenous or intestinal Pi bolus causes rapid phosphaturia through mechanisms requiring PTH and downregulation of renal Pi transporters but does not support a role of the intestine in stimulating renal clearance of Pi.

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The actions of FGF23, PTH, and 1,25-(OH)₂-vitamin D₃ are coupled through multiple negative and positive feedback loops. However, the exact roles of these hormones in the acute and chronic response to changes in Pi intake are not fully defined. Moreover, the existence and role of additional factors and mediators in the control of Pi homeostasis have been proposed. Klotho, a cofactor for FGF23 signaling, also exerts direct effects on renal Pi and calcium transporters independent from FGF23. MEPE and sFRP4 may act on renal and extrarenal targets to lower plasma Pi. Also, the existence of a putative intestinal phosphaturic factor has been postulated on the basis of the rapid phosphaturic effect of duodenal Pi infusion; the absence of changes in plasma Pi, PTH, and FGF23; its independence from renal innervation; and the phosphaturic effect of duodenal extracts from Pi-infused rats. However, in humans, acute enteral and parenteral Pi loads cause dose-dependent changes in phosphaturia only on changes in plasma Pi and PTH, and a similar phosphaturic response is observed with both routes of Pi administration. Thus, in contrast to rodents, the human data are not consistent with the presence of an intestinal Pi sensor. Local Pi sensing mechanisms in kidney and other organs may be involved in sensing changes in dietary Pi intake and may mediate or modulate some of the effects on renal Pi handling. Evidence for Pi sensing has been obtained from isolated parathyroid gland cells in vitro, the opossum kidney–derived OK cell line, bone, and vascular cells.

The aim of this study was to investigate the endocrine, mineral, and renal responses to acute Pi loading in rats and test for evidence for a role of the intestine in determining the adaptive response by administering Pi either intravenously or intragastrically.

**RESULTS**

**Pi Loading Rapidly Elicits Phosphaturia**

Intravenous infusion of a Pi bolus (500 μmol Pi) caused a rapid and transient increase in plasma Pi concentration in intact rats (Figure 1A), with a peak after 10 minutes. Two hours after infusion, plasma Pi had returned to baseline values. A significant, although blunted, increase in plasma Pi was observed after 10 minutes on infusion of 150 μmol Pi but not with 50 μmol Pi (Supplemental Figure 1A). In contrast, plasma Pi did not change in rats gavaged with 500 μmol Pi (Figure 1A). The concentration of plasma Pi remained normal after intravenous or intragastric administration of 500 or 150 μmol NaCl (Figure 1A, Supplemental Figure 2A).

Intravenous infusion of 500 μmol Pi rapidly and strongly increased urinary Pi excretion within 10 minutes. Although phosphaturia tended to decrease after 30 minutes, it remained high over 4 hours after administration (Figure 1B). Infusions of 150 and 50 μmol Pi failed to induce any significant phosphaturic response (Supplemental Figure 1B). Intragastric administration of 500 μmol Pi increased urinary excretion of Pi to a similar extent as intravenous administration; however, the onset of phosphaturia was delayed compared with infusion, reaching significance only 60 minutes after the Pi bolus (Figure 1B). Phosphaturia remained high over 4 hours postgavage (Figure 1B). Changes in the fractional excretion of Pi...
after the Pi bolus paralleled urinary Pi concentration (Supplemental Figure 3A). Neither intravenous infusion nor gavage of 500 or 150 μmol NaCl affected the urinary excretion of Pi (Figure 1B, Supplemental Figure 2B).

Creatinine clearance significantly increased within 10 minutes after intravenous application of 500 μmol Pi and thereafter, rapidly normalized (Figure 1C). Intragastric loading with 500 μmol Pi did not alter creatinine clearance (Figure 1C). Similarly, administration of 500 or 150 μmol NaCl by infusion or gavage had no effect (Figure 1C) (data not shown).

The cumulative urinary excretion of Pi showed a similar level after 4 hours of the oral or intravenous bolus (Figure 1D). Although a delay was observed in the gavage group, both groups had excreted comparable amounts of Pi 4 hours post-application, with mean values of around 230 and 280 μmol Pi, respectively, representing about 50%–60% of the initial Pi load (500 μmol).

To examine possible organ storage of the nonexcreted Pi, we assessed the Pi content in femurs, liver, and skeletal muscle in tissues from rats infused and gavaged with Pi or NaCl. As expected, the concentration of Pi in femurs was higher (10–60 times) than in the other two organs (Supplemental Figure 4). However, in all three tissues, similar levels of Pi were measured in samples from control and Pi-loaded rats.

The concentration of total Ca²⁺ in plasma slightly but significantly decreased on intravenous infusion of 500 μmol Pi (Figure 2A). The reduction was already detectable 10 minutes after the Pi bolus, and although it tended to normalize at the latest time points, plasma Ca²⁺ remained low until the end of the experiment (4 hours). Similar changes were observed on Pi infusion with 150 μmol Pi but not observed with 50 μmol Pi (Supplemental Figure 1C). In contrast, total plasma Ca²⁺ did not change significantly after intragastric administration of 500 μmol Pi (Figure 2A). Infusion or gavage with either 500 or 150 μmol NaCl did not alter total plasma Ca²⁺ (Figure 2A, Supplemental Figure 2C).

The urinary excretion of Ca²⁺ remained unchanged after intravenous or intragastric Pi or NaCl application (Figure 2B, Supplemental Figures 1D and 2D). However, infusion with Pi or NaCl caused a small, nonsignificant, and transient increase in urinary Ca²⁺ excretion, possibly reflecting an acute volume load (Figure 2B).

Figure 2. Effect of intravenous and intragastric administration of Pi on Ca²⁺ plasma levels and urinary excretion in intact rats. Rats were loaded with 500 μmol Pi or saline intravenously or orally. (A) Plasma Ca²⁺ and (B) urinary Ca²⁺/creatinine. Four profiles are shown: saline intravenous infusion (black), Pi intravenous infusion (red), saline intragastric gavage (green), and Pi intragastric gavage (blue). Data are presented as the means±SEM. ANOVA test; n=5–9 per group and time point. *P<0.05 versus time 0.
with 500 μmol Pi (Figure 5C), whereas the expression of NaPi-IIa but not NaPi-IIc was diminished 4 hours after intragastric loading (Figure 5D).

**PTH Is Required to Clear the Acute Phosphate Load**

Because PTH increased very rapidly on Pi loading and paralleled or even preceded phosphaturia, we tested the role of PTH in the adaptive response in parathyroidectomized (PTX) rats. Infusion of 500 μmol Pi caused a rapid increase in plasma Pi in PTX rats (Figure 6A), peaking after 10 minutes and decreasing thereafter. However, whereas the levels of Pi fully normalized within 2 hours postinfusion in intact rats, Pi levels remained elevated until the end of the experiment in PTX animals (Figure 6A). Gavage of 500 μmol Pi in PTX rats induced a slow rise in plasma Pi (Figure 6A) in contrast with the lack of effect of the Pi gavage in intact animals (Figure 1A). Administration of saline to PTX rats by infusion or gavage did not alter plasma Pi (Figure 6A).

Infusion with 500 μmol Pi induced a fast but small increase in urinary excretion of Pi in PTX rats (Figure 6B). Maximal phosphaturia was detected 10 minutes postinfusion, similar to in intact animals (Figure 1B). However, phosphaturia returned to baseline within 2 hours after infusion in PTX rats, despite elevated plasma Pi levels (Figure 6B). Moreover, gavage of Pi in PTX rats failed to elicit any significant phosphaturia (Figure 6B). Neither infusion nor gavage with saline affected urinary Pi (Figure 6B).

In PTX rats, creatinine clearance increased within 10 minutes of 500 μmol Pi infusion and rapidly normalized thereafter, whereas it was not altered with either Pi gavage or saline administration (Figure 6C).

The cumulative urinary excretion of Pi over 4 hours in PTX rats infused with 500 μmol Pi was of about 30 μmol (Figure 6D), equivalent to about 6% of the Pi load. The cumulative urinary excretion of Pi over 4 hours in PTX rats receiving Pi by gavage was about 3 μmol (Figure 6D), representing <1% of the Pi load.

The content of Pi was higher in femurs than liver and muscle in PTX animals, and no differences were found between organs extracted from saline-treated and Pi-loaded rats (Supplemental Figure 5).

Plasma total Ca$^{2+}$ decreased slightly within 10 minutes on 500 μmol Pi infusion in PTX rats (Figure 7A) and remained low until the end of the experiment. In contrast, plasma total Ca$^{2+}$ did not change significantly in Pi-gavaged PTX rats (Figure 7A). Administration of 150 μmol NaCl by infusion or gavage did not alter plasma Ca$^{2+}$.

Urinary excretion of Ca$^{2+}$ in PTX showed no significant changes after administration of Pi or saline (Figure 7B).

As expected, plasma PTH was undetectable in PTX rats under all conditions (Figure 8A). In contrast to intact rats, FGF23 was elevated in PTX animals 4 hours after oral or intravenous Pi loading (Figure 8B). The plasma concentrations of 1,25-(OH)$_2$-vitamin D$_3$ (Figure 8C), insulin (Figure 8D), and
dopamine (Figure 8E) as well as urine dopamine (Figure 8F) remained unchanged under all conditions.

**DISCUSSION**

Acute and chronic changes in plasma Pi elicit adaptive responses in several hormones that regulate renal and intestinal epithelia—(re)absorbing Pi.1–3 High dietary Pi increases the phosphaturic hormones PTH, FGF23, and dopamine while decreasing the levels of 1,25-(OH)₂-vitamin D₃,6–7,31 resulting in reduced expression and activity of NaPi cotransporters in renal and intestinal epithelia, blunting intestinal Pi absorption, and increasing urinary excretion. However, there are conflicting data regarding the sequence of events triggered by high dietary Pi as well as the nature of the trigger itself. PTH may have a key role in the acute renal response, with other hormones coming into play only later on,23,32–34 but the presence of yet-unidentified intestinal factor(s) stimulating renal Pi excretion independent from PTH has been proposed.22 Here, we administered a Pi load to rats both intravenously (to bypass the gastrointestinal tract) and intragastrically and compared the acute responses in intact and PTX animals.

Our data show that, compared with equimolar NaCl infusions and the time point 0, the acute infusion of Pi elicited a dose-dependent response that consisted at the highest dose of (1) an immediate but transient rise in plasma Pi, (2) a nearly and transient increase in creatinine clearance, (3) a rapid phosphaturic response, (4) a fall in plasma total calcium, (5) a rapid and sustained phosphaturia, and (6) a reduced expression and activity of renal Pi transporters. Intragastric application of Pi caused a qualitatively similar response in the cumulative Pi excretion without a significant change in creatinine clearance and with no obvious hyperphosphatemia, a lower and delayed rise in PTH, a nonsignificant reduction in renal Pi transporter activity and lower expression of only NaPi-IIa but not NaPi-IIc, and a slower onset in phosphaturia. Infusion or gavage of NaCl had no effects.

Several points are of major interest: the onset of phosphaturia is paralleled by a rise in PTH in infused and gavaged abundances of NaPi-IIa and NaPi-IIc in PTX rats were similar in the Pi-loaded and saline-treated animals 4 hours postadministration (Figure 10).
animals and precedes changes in plasma dopamine, whereas levels of 1,25-(OH)2-vitamin D3, FGF23, insulin, and urine dopamine were not altered, suggesting an important role of PTH. Early phosphaturia (40 minutes) occurred in infused animals without obvious changes in the activity or abundance of renal Pi transporters expressed in the brush border membrane of the proximal tubule, which may be explained at least in part by a higher tubular load in the infused animals, because creatinine clearance had more than doubled and plasma Pi levels were elevated. However, we noted also a partial dissociation between the degree of phosphaturia, Pi transport activities in BBMVs, and the abundance of the NaPi-IIa and NaPi-IIc transporters as most evident in the Pi-gavaged group after 4 hours. Activity of NaPi-IIa transporters in the brush border membrane is influenced by lipid composition of the plasma membrane and in situ cleavage by klotho and possibly, regulated association with and dissociation from NHERF1 through phosphorylation of NHERF1 stimulated by PTH or dopamine.17,35–37 We did not obtain evidence for cleavage of NaPi-IIa as evident from immunoblotting, but changes in lipid composition or NHERF1 phosphorylation were not tested and may contribute to the dissociation of transport activity and NaPi-IIa abundance.

Plasma PTH rises in vivo and in vitro in response to a fall in ionized calcium or an increase in Pi concentrations. Whether the response to Pi is independent from calcium has remained unclear, because Pi retains its ability to stimulate PTH secretion, even in the absence of a measurable fall in ionized or total calcium.23–25,33,38 Along the same line, in vitro incubation of human parathyroid glands with escalating concentrations of Pi while keeping ionized calcium constant stimulated PTH secretion.24,38 However, inhibitors of calcium-stimulated receptor-mediated PTH secretion (calcimimetics) have been shown to suppress Pi-induced PTH secretion in vivo, suggesting a role of the calcium-stimulated receptor, even in the absence of changes of ionized calcium levels.25 Here, plasma total calcium decreased in parallel with the rise in PTH, allowing no clear distinction between a calcium-dependent or -independent mechanism. However, infusion of 150 μmol Pi caused a similar fall in plasma total calcium as 500 μmol Pi, but the rise in PTH was blunted and detected only 10 minutes postadministration, suggesting that Pi may stimulate PTH release synergistically or independently. The early response of PTH found in our animal model is consistent with experiments in humans and other rodent models.23,32,33

Figure 5. Effect of intravenous and intragastric administration of Pi on the expression of renal NaPi cotransporters in intact rats. Rats were loaded with 500 μmol Pi or saline intravenously or orally. Kidneys were extracted 40 minutes or 4 hours postadministration, and the abundance of NaPi-IIa and NaPi-IIc in brush border membranes was determined by Western blot. Expression of cotransporters (A) 40 minutes after intravenous infusion of saline (gray bars) and Pi (black bars), (B) 40 minutes after intragastric administration of saline (gray bars) and Pi (black bars), (C) 4 hours after intravenous infusion of saline (gray bars) and Pi (black bars), and (D) 4 hours after intragastric administration of saline (gray bars) and Pi (black bars). Unpaired t test; n=5–9 per group and time point. *P<0.05 versus saline group.
combination of hyperphosphatemia and elevated GFR (as indicated by higher creatinine clearance). Phosphaturia ceased after normalization of creatinine clearance and the fall of plasma Pi below about 5 mM. The transient increase in creatinine clearance in the Pi-infused animals is independent from PTH and may involve other Pi-sensitive mechanisms. The rise in plasma Pi and/or the fall in plasma calcium may be (in)direct triggers affecting factors controlling glomerular filtration, such as vascular tone of afferent and/or efferent arterioles, where calcium channels play an important role. PTH is also required for the downregulation of renal Pi transporters after 4 hours, because this response was also blunted in PTX rats. Thus, our results show that PTH is required for the early response to high Pi intake.

We detected only small or no changes in FGF23, 1,25-(OH)\textsubscript{2}-vitamin D\textsubscript{3}, insulin, and dopamine. The increase in dopamine or FGF23 was found only at the latest time point and is probably not responsible for the massive phosphaturia at earlier time points. In Pi-infused intact rats, the late rise in plasma dopamine (but not in urine) may enhance PTH-induced phosphaturia. Dopamine acts via D\textsubscript{1} receptors to down-regulate NaPi-IIa in proximal tubules.\textsuperscript{7–9} The increase in FGF23 in PTX rats occurs between 50 and 240 minutes after the Pi bolus (because of the small volumes of blood that could be collected, only a few FGF23 determinations were possible). This finding suggests first that, in the absence of PTH, FGF23 levels adapt more acutely to the Pi overload and second, that systemic Pi can regulate FGF23 production and/or stability independently of PTH. Interestingly, FGF23 production by osteocytes is not directly regulated by Pi\textsuperscript{40} and instead, requires previous production of PTH and activation of protein kinase A and Wnt pathways by PTHR1.\textsuperscript{23,41,42}

Intestinal Pi sensors and an intestine-derived phosphaturic factor had been postulated on the basis of experiments with rats infused with Pi into duodenum. This mechanism would allow for a rapid crosstalk between intestine and kidney and provide a feedforward mechanism preventing a potentially detrimental hyperphosphatemia.\textsuperscript{22} The existence of such feedforward mechanisms has been shown for potassium and salt.\textsuperscript{43–46} However, Scanni et al.\textsuperscript{23} had found, in healthy humans, that the rate of elimination and overall quantity of Pi excretion did not depend...
on the route of application (intravenous versus intraduodenal infusion). Consistently, our data do not provide any evidence for a role of the intestine in promoting phosphaturia. Moreover, in the absence of PTH, no phosphaturia could be elicited by intragastric Pi infusions.

The cumulative urinary elimination of the Pi bolus reached only about 50%–60% after 4 hours in intact animals, whereas normophosphatemia was achieved much faster, suggesting that a large amount of Pi had been eliminated by other routes (i.e., intestinal tract), extravasated and accumulated in tissues, or complexed in blood into a pool that is not measurable by the Fiske–Subbarow method. Scanni et al.23 showed that urinary excretion of Pi accounted for 100% of the intravenous Pi overload in humans, but full elimination required 120 hours; the authors rule out a contribution of the gastrointestinal tract in the elimination of Pi. We quantified the Pi content in femurs as well as liver and skeletal muscle. Pi content in bones is much higher than in the other two organs, and the measured values were so high that a rough estimation of the total amount of Pi stored in the skeleton (assuming a 10% contribution to body weight and a comparable composition of all bones) suggests that, even if all of the nonexcreted Pi would have been accumulated in bones, this would only result in a small change of content (approximately 0.5%), nondetectable with the Fiske–Subbarow method. Although similar estimations predicted that partial accumulation of Pi in liver and skeletal muscle could be detectable, we failed to observe any changes. Thus, other approaches should be used to identify the organs responsible for a transient accumulation of a large excess of Pi.

In summary, our data indicate that (1) normophosphatemia is rapidly re-established after intravenous and intragastric Pi loading by mechanisms largely depending on the ability of the kidneys to excrete Pi; (2) an efficient phosphaturic response requires increased levels of PTH and reduced expression of renal Pi transporters; (3) these compensatory responses are, to a major extent, similar, regardless of whether Pi bypasses the gastrointestinal tract; and (4) reduced plasma Ca2+ together with elevated Pi may trigger the secretion of PTH in intravenously loaded animals. Our findings leave two major issues unresolved, namely the identity of the compartment responsible for the rapid quenching of a large fraction of the loaded Pi and the nature of the signal that triggers the stimulation of PTH release.

CONCISE METHODS

Animal Experimental Protocol
Male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 250–350 g were adapted to a low-Pi diet (0.1%) for 5 days. After an overnight fast in metabolic cages with free access to water, animals were anesthetized with 3% isoflurane/air and placed on a heated pad to maintain body temperature at 37°C–38°C. Rats inhaled
constantly a low dose of anesthesia (1%–2% isoflurane/air) until the end of the experiment. Catheters (BPE-T 50; Instech) were placed into the femoral vein, femoral artery, and urinary bladder (for infusion of solutions and collection of blood and urine, respectively). A Ringer solution (116 mM NaCl, 1.2 mM KCl, 1 mM CaCl₂, and 2.7 mM NaHCO₃) containing 5% glucose was infused continuously into the femoral vein, femoral artery, and urinary bladder (for intermediate time points and at termination) were centrifuged immediately after intragastric administration of saline (gray bars) and Pi (black bars), and (C) ³H-glucose uptakes 4 hours after intragastric administration of saline (gray bars) and Pi (black bars). Data are presented as the means±SEM. Unpaired test; n=4–6 per group and time point.

General Analytic Measurements

The concentration of Pi in plasma and urine was determined according to the Fiske–Subbarow method (Randox). Pi and glucose transport activities in renal BBMVs from PTX rats. PTX rats were loaded with 500 μmol Pi or saline intravenously or orally. Kidneys were extracted 4 hours after infusion or gavage, and Na⁺–dependent and -independent Pi and glucose transport activities in isolated BBMVs were measured. Pi transport was assayed in the presence or absence of the SLC34 transport inhibitor phosphonoformic acid. (A) ³²P uptakes 4 hours after intravenous infusion of saline (gray bars) and Pi (black bars), (B) ³²P uptakes 4 hours after intragastric administration of saline (gray bars) and Pi (black bars), and (C) ³H-glucose uptakes 4 hours after intravenous and intragastric administration of saline (gray bars) and Pi (black bars). Data are presented as the means±SEM. Unpaired test; n=4–6 per group and time point.

Figure 9. Effect of intravenous and intragastric administration of Pi on Pi and glucose transport activities in renal BBMVs from PTX rats. PTX rats were loaded with 500 μmol Pi or saline intravenously or orally. Kidneys were extracted 4 hours after infusion or gavage, and Na⁺–dependent and -independent Pi and glucose transport activities in isolated BBMVs were measured. Pi transport was assayed in the presence or absence of the SLC34 transport inhibitor phosphonoformic acid. (A) ³²P uptakes 4 hours after intravenous infusion of saline (gray bars) and Pi (black bars), (B) ³²P uptakes 4 hours after intragastric administration of saline (gray bars) and Pi (black bars), and (C) ³H-glucose uptakes 4 hours after intravenous and intragastric administration of saline (gray bars) and Pi (black bars). Data are presented as the means±SEM. Unpaired test; n=4–6 per group and time point.

on collection, and plasma was aliquoted. Plasma and organs were store at −80°C until further used.

In addition, an identical experimental protocol to the one described above was performed in 350–450 g PTX male rats (Charles River Laboratories) receiving 1% calcium gluconate in drinking water.

All animal experiments were according to Swiss and international laws of animal protection, and all protocols were approved by the appropriate local veterinary authority (Kantonales Veterinäramt Zürich).

Isolation of Renal BBMVs and Flux Measurements of ³² Pi and ³H-Glucose

Kidney cortex and medulla were dissected from frozen kidneys and homogenized in a buffer containing 300 mM mannitol, 5 EGTA, and 12 Tris–HCl, pH 7.1; BBMVs were isolated according to the Mg²⁺ precipitation method as described in detail.⁴⁷ Uptake of ³²Pi and ³H-glucose was measured in three different solutions, all three containing 300 mM mannitol plus 20 mM HEPES-Tris, pH 7.4 and 125 mM NaCl, 125 mM KCl, or 125 mM NaCl. The uptake solutions contained either 0.125 μM K₂HPO₄/KH₂PO₄, pH 7.4 as cold substrate and ³²Pi as a tracer or 0.125 μM ³H-glucose as cold substrate and ³H-glucose as tracer. To measure the incorporation of ³²Pi/³H-glucose, 10 μl freshly prepared BBMVs were incubated for 1 minute or 2 hours with 40 μl different uptake solutions. The 1-minute time point was chosen, because ³²Pi/³H-glucose uptake was in the linear phase of
A Infusion

![Diagram](image)

**Figure 10.** Effect of intravenous and intragastric administration of Pi on the expression of renal NaPi cotransporters in PTX rats. PTX rats were loaded with 500 μmol Pi or saline intravenously or orally. Kidneys were extracted 4 hours postadministration, and the abundance of NaPi-IIa and NaPi-IIc in brush border membranes was determined by Western blot. Expression of cotransporters (A) 4 hours after intravenous infusion of saline (gray bars) and Pi (black bars) and (B) 4 hours after intragastric administration of saline (gray bars) and Pi (black bars). Unpaired t test; n=4–6 per group and time point.

the maximal transport rate as determined earlier. After the indicated incubation time, uptakes were stopped by transferring 20 μl sample to 1 ml ice cold stop solution (100 mM mannitol, 5 mM Tris-HCl, 150 mM NaCl, 5 mM Pi, and 5 mM glucose). The resulting suspension was then spotted onto a filter and vacuum washed with 10 ml ice cold stop solution. Filters were finally transferred into plastic vials, and on addition of 3 ml scintillation medium (PerkinElmer, Waltham, MA), the retained radioactivity was measured on a counter (TRI-CARB 2900TR; Packard). All measurements were carried out in triplicate. The Na"+-dependent uptakes were calculated by subtracting the uptake values obtained in the K"+ medium from those measured in the Na"+ medium (total uptake). Because phosphonoformic acid is a competitive inhibitor of SLC34 cotransporters (NaPi-IIa and NaPi-IIc), SLC34-mediated uptake was determined by subtracting the uptake values obtained in the presence of phosphonoformic acid from the Na"+-dependent values. The remaining BBMVs that were not used in the uptake experiments were stored immediately at –80°C for further experiments.

**B Gavage**

![Diagram](image)

**Immunoblotting**

The protein expression levels of NaPi-IIa and NaPi-IIc in renal brush border membranes were quantified by immunoblotting. To this end, 20 μg brush border membranes were solubilized in Laemmli buffer and separated on 10% SDS-PAGE, and then, they were transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA). After blocking nonspecific binding with 5% milk powder in Tris-buffered saline containing 0.1% Tween-20 for 40 minutes, the blots were incubated overnight at 4°C with primary antibodies against NaPi-IIa (1:4000),48 NaPi-IIc (1:2500),49,50 and β-Actin (1:10,000; Sigma-Aldrich, St. Louis, MO). After washing and further blocking, blots were incubated with appropriate secondary antibodies for 2 hours at room temperature. Finally, membranes were exposed to chemiluminescent substrate for 5 minutes, and protein signals were detected on an LAS-4000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan). All of the images were quantified with Advanced Image Data Analyzer (Raytest). The expression of both cotransporters was normalized to the abundance of β-Actin.

**Determination of Pi in Tissues**

Tissues (liver, skeletal muscle, and femur) collected from intact and PTX rats 4 hours after infusion or gavage were dried in an oven at 70°C for 24 hours. Samples were weighed, transferred into a silica crucible, and burned to ashes in an electric furnace at 700°C for 12 hours; 1 N HCl was used to dissolve the Pi present in ashes, and after centrifugation, supernatants were collected for Pi determination by the above-mentioned Fiske–Subbarow method.

**Statistical Analyses**

Statistical significances were calculated by t test or one-way ANOVA (Bonferroni) as indicated. P<0.05 was considered significant. Results are presented as means±SEM.

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**DISCLOSURES**

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