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The phosphate transporter NaPi-IIa determines the rapid renal adaptation to dietary phosphate intake in mouse irrespective of persistently high FGF23 levels

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Abstract Renal reabsorption of inorganic phosphate (Pi) is mediated by the phosphate transporters NaPi-IIa, NaPi-IIc, and Pit-2 in the proximal tubule brush border membrane (BBM). Dietary Pi intake regulates these transporters; however, the contribution of the specific isoforms to the rapid and slow phase is not fully clarified. Moreover, the regulation of PTH and FGF23, two major phosphaturic hormones, during the adaptive phase has not been correlated. C57/BL6 and NaPi-IIa$^{-/-}$ mice received 5 days either 1.2 % (HPD) or 0.1 % (LPD) Pi-containing diets. Thereafter, some mice were acutely switched to LPD or HPD. Plasma Pi concentrations were similar under chronic diets, but lower when mice were acutely switched to LPD. Urinary Pi excretion was similar in C57/BL6 and NaPi-IIa$^{-/-}$ mice under HPD. During chronic LPD, NaPi-IIa$^{-/-}$ mice lost phosphate in urine compensated by higher intestinal Pi absorption. During the acute HPD-to-LPD switch, NaPi-IIa$^{-/-}$ mice exhibited a delayed decrease in urinary Pi excretion. PTH was acutely regulated by low dietary Pi intake. FGF23 did not respond to low Pi intake within 8 h whereas the phospho-adaptator protein FRS2α necessary for FGF-receptor cell signaling was downregulated. BBM Pi transport activity and NaPi-IIa but not NaPi-IIc and Pit-2 abundance acutely adapted to diets in C57/BL6 mice. In NaPi-IIa$^{-/-}$, Pi transport activity was low and did not adapt. Thus, NaPi-IIa mediates the fast adaptation to Pi intake and is upregulated during the adaptation to low Pi despite persistently high FGF23 levels. The sensitivity to FGF23 may be regulated by adapting FRS2α abundance and phosphorylation.

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Introduction

In mammals, including humans, plasma concentration of inorganic phosphate (Pi) is determined by intestinal intake, excretion via feces, distribution between bone and soft tissues, and renal excretion and reabsorption, respectively (for review: [12, 38, 40]). The latter mechanism is of major importance for whole-body Pi homeostasis and therefore tightly controlled. Reabsorption of filtered Pi by the kidneys occurs mostly in the proximal tubule [12, 38, 40]. In the brush border membrane (BBM), three transporters have been identified so far: NaPi-IIa (SLC34A1) and NaPi-IIc (SLC34A3), two transporters from the NaPi/SLC34 family, and, more recently, Pit-2 (SLC20A2) [12, 38, 49, 59]. However, these transporters have distinct transport properties with respect to ion coupling, preferred species of phosphate, voltage sensitivity, and pH dependence [12, 38, 46, 49, 60]. NaPi-IIa and Pit-2 mediate the electrogenic transport of inorganic phosphate coupled to three and two sodium ions, respectively, whereas NaPi-IIc transports inorganic phosphate together with two sodium ions in an electroneutral fashion. Moreover, NaPi-IIa and NaPi-IIc prefer divalent inorganic phosphate (HPO_4^{2-}). In contrast, Pit transporters preferentially transport monovalent phosphate ($H_2PO_4^-$) [12, 38, 46, 60].

Renal phosphate reabsorption is regulated by many factors including parathyroid hormone (PTH), dopamine, dietary phosphate intake, glucocorticoids, vitamin D_3, acid–base status, growth factors, insulin, sFRP4, and FGF23 [4–6, 11, 12, 17, 35, 39, 42, 44]. NaPi-IIa abundance in the brush border membrane is controlled by parathyroid hormone, FGF23, and dietary Pi intake [8, 12, 29]. In rat, mouse, and rabbit, NaPi-IIa is upregulated by low Pi diet and decreased by high Pi intake both

Table 1 Metabolic values in *C57BL6* and *NaPi-IIa*$^{-/-}$ during a 4-h period of feeding and collecting period with chronic and acute high and low phosphate diets (day 9)

	High Pi diet		High to Low Pi diet		Low Pi diet		Low to High Pi diet	
	C57/BL6 (n=5)	*NaPi-IIa*$^{-/-}$ (n=5)	*C57/BL6* (n=5)	*NaPi-IIa*$^{-/-}$ (n=5)	*C57/BL6* (n=5)	*NaPi-IIa*$^{-/-}$ (n=7)	*C57/BL6* (n=5)	*NaPi-IIa*$^{-/-}$ (n=7)
Mice weight (g)	21.9±1.0	19.1±0.2	22.5±1.4	21.1±0.6	25.8±0.9	19.5±0.4	23.4±0.9	20.8±0.8
Food intake (g/4 h/body weight)	0.13±0.01	0.10±0.02	0.08±0.01$^{\#}$	0.12±0.00	0.06±0.00$^{\#}$	0.09±0.01	0.11±0.00	0.09±0.01
Plasma								
Phosphate (mM)	2.42±0.20	1.95±0.44	1.09±0.18$^{\#}$	0.34±0.03$^{\#}$	0.50±0.06$^{\#}$	0.32±0.09$^{\#}$	2.20±0.07	1.45±0.17
Calcium (mM)	1.82±0.05 (9)	ND	1.91±0.08	ND	2.07±0.04 (6)$^{\#}$	ND	1.71±0.14	ND
Urine:								
Creatinine (μmol/4 h)	0.16±0.04	0.14±0.01	0.18±0.03	0.12±0.01	0.16±0.05	0.14±0.02	0.26±0.05	0.13±0.01*
Pi/creatinine (mM/mM)	42.3±2.8	53.6±5.4	4.9±2.7$^{\#}$	21.7±0.8*$^{\#}$	1.0±0.1$^{\#}$	7.7±2.0*$^{\#}$	28.7±2.1$^{\#}$	36.2±4.4$^{\#}$

*p<0.05 versus C57/BL6 under same diet; $^{\#}$ p<0.05 versus same genotype under High Pi diet

acutely and chronically [16, 17, 29, 57]. This regulation occurs essentially by trafficking of the protein, lysosomal degradation, recruitment of newly synthesized transporters [5, 26, 29, 30] and may also include transcriptional regulation [34]. Much less is known about the regulation of NaPi-IIc being regulated also by PTH, FGF23, and Pi intake with a slower change in brush border membrane abundance upon high Pi intake or PTH application [8, 28, 43, 44]. Similarly, Pit-2 is regulated by PTH and dietary Pi or potassium intake but no further regulators have been identified to date [15, 44, 59].

The relative contribution of NaPi-IIa to the adaptive response of the kidney to acute and chronic changes in dietary phosphate intake remains to be clarified. Therefore, we compared renal phosphate handling during acute and chronic adaptive changes in dietary phosphate intake between C57/BL6 mice and mice lacking NaPi-IIa. Our second aim was to correlate changes in renal phosphate handling in C57/BL6 mice with levels of the two major phosphaturic hormones PTH and FGF23 and their main cellular

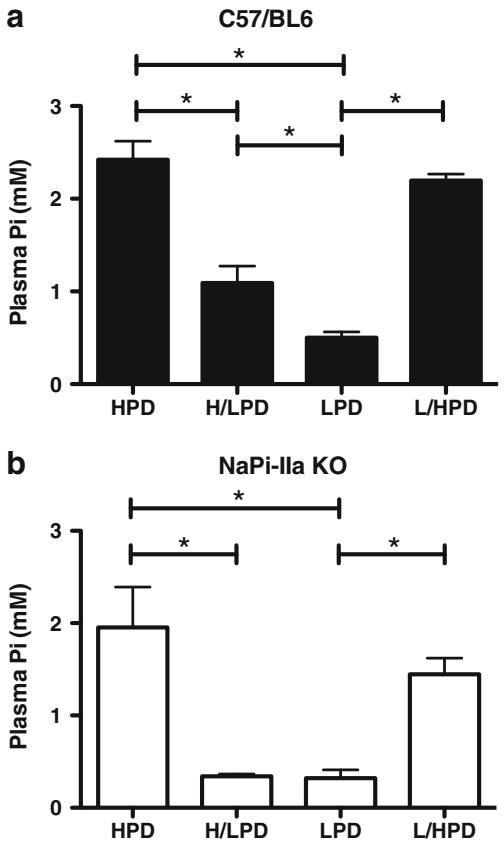

Fig. 1 Plasma phosphate levels during acute and chronic changes in dietary Pi. **a** Plasma Pi in C57/BL6 mice (n=5 mice/group). **b** Plasma Pi in NaPi-IIa$^{-/-}$ mice (n=5 to 7 mice/group). C57/BL6 and NaPi-IIa$^{-/-}$ mice were fed 4 h/day with high (HPD) or low Pi (LPD)-containing diets for 4 days. Some mice were switched on the last day from high to low (H/LPD) or from low to high (L/HPD) Pi-containing diets. Both strains of mice were able to modulate their blood Pi during these dietary conditions. However, NaPi-IIa$^{-/-}$ mice more drastically decreased their bood Pi level during the acute H/LPD switch. *p<0.05

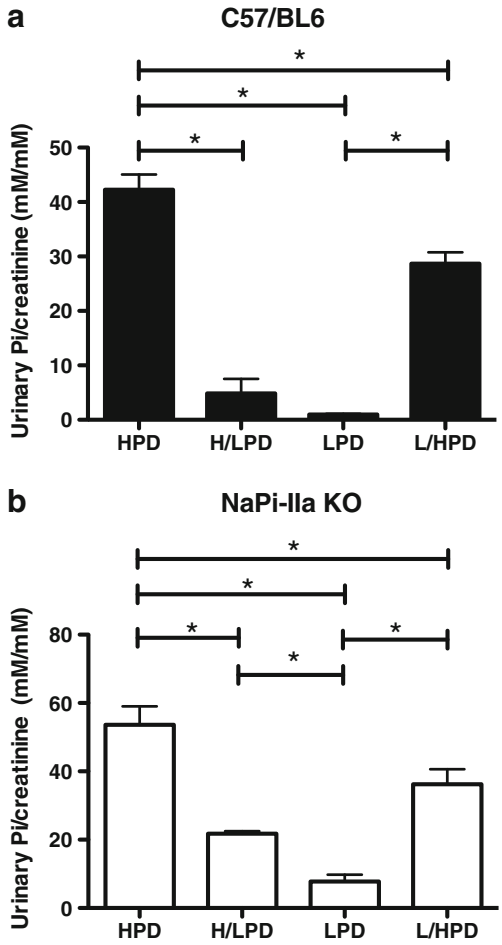

Fig. 2 Urine Pi excretion during chronic and acute changes in dietary Pi. **a** Urinary Pi in C57/BL6 mice (n=4 to 6 mice/group). **b** Urinary Pi in NaPi-IIa$^{-/-}$ mice (n=5 to 7 mice/group) . C57/BL6 mice and NaPi-IIa$^{-/-}$ mice were fed 4 h/day with high (HPD) or low Pi (LPD)-containing diets for 4 days. Some mice were switched on the last day from high to low (H/LPD) or from low to high (L/HPD) Pi-containing diets. Both strains of mice were able to modulate their urinary Pi excretion during these dietary conditions. However, NaPi-IIa$^{-/-}$ mice lost more Pi into urine compared to C57/BL6 mice whatever the diet. *p<0.05

downstream effectors PTH receptor 1 and the FGF receptor adaptor protein FRS2α.

Our results demonstrate a major role of NaPi-IIa during the acute phase of renal adaptation to low phosphate intake whereas during chronic low phosphate intake lack of NaPi-IIa is mostly compensated by enhanced intestinal phosphate absorption. PTH levels followed phosphate intake whereas FGF23 responded more slowly to switching from high to low phosphate intake and high FG23 levels persisted for more than 8 h. At the cellular level, low phosphate intake correlated with a reduction in the abundance of FRS2α, the major adaptor protein mediating the downstream signaling from the FGF1 receptor. NaPi-IIa membrane abundance increased during these 8 h despite high FGF23.

Methods

Animals

All experiments were performed with 10–14-week-old C57BL/6J and homozygous NaPi-IIa-deficient (Slc34a1$^{-/-}$) male mice bred in a pure C57/BL6 background [7]. The generation, breeding, and genotyping of NaPi-IIa KO mice have been described previously [7]. All experiments were performed according to the Swiss Animal Welfare laws and approved by the local veterinary authority (Veterinäramt Zürich, autorisation no. 32/2009).

Metabolic studies

Mice were kept in standard cages for the first 4 days (days 1-4) and adapted to metabolic cages (Tecniplast, Buguggiate, Italy) by placing them in metabolic cages during the day and in standard cages during the night. Days 5–9, mice were housed in metabolic cages. Mice received standard rodent chow (0.8 % P_i, 1.2 % Ca^{2+}; Kliba NAFAG, Kaiseraugst, Switzerland) during days 1–4 but were trained to receive food only during 4 h in the morning (between 7 AM to 11 AM). On day 5, animals were subdivided into two experimental groups. The first group received 0.1 % P_i and 1.0 % Ca^{2+} diet and the second 1.2 % P_i and 1.0 % Ca^{2+} diet (Kliba; NAFAG, Kaiseraugst, Switzerland). Both diets contained equal amounts of vitamin D (1,000 IU/kg). On day 7, 24-h urine and feces collections were performed in metabolic cages to assess chronic adaptation.

On day 9, half of each group was switched for 4 h in the morning to low or high Pi diets, respectively, and the following experimental groups were established: (1) chronic low Pi diet group (LPD) receiving only 0.1 % Pi diet; (2) acute high Pi diet (L/HPD): mice were fed 0.1 % Pi diet for 4 days and acutely switched to 1.1 % Pi diet for 4 h on the last day; (3) chronic high Pi diet group (HPD) receiving only 1.1 % Pi diet; (4) acute low Pi diet (H/LPD) mice were fed for 4 days 1.1 % Pi diet and were acutely switched on the last day to 0.1 % Pi diet for 4 h. Some mice received also only high or low Pi diets for 5 days with access to food over 24 h to assess the effects of timed feeding on overall adaption. Mice were sacrificed on day 9 and urine, blood, and kidneys were collected for acute studies and stored at −80 °C for further analyses.

Urine, plasma, and feces analysis

Twenty-four-hour feces were solubilized overnight at 95 °C in 2 ml of 1.2 M nitric acid in a luer lock glass tube. Volumes were equalized with water to 4 ml in each sample and ions were measured in the supernatant. Plasma, urine, and feces P_i concentration was determined by the phosphomolybdate method [23] and urinary creatinine by a modified kinetic

Table 2 Metabolic values in C57*BL6* and *NaPi-IIa*$^{-/-}$ during 4-h feeding/day and 24-h collecting period with chronic high and low phosphate diets (day 8)

	High Pi diet		Low Pi diet	
	C57/BL6 (n=10)	*NaPi-IIa*$^{-/-}$ (n=10)	*C57/BL6* (n=10)	*NaPi-IIa*$^{-/-}$ (n=14)
Mice weight (g)	23.2±0.9	20.5±0.5	25.5±0.7	20.5±0.6
Food intake (g/24 h/body weight)	0.10±0.00	0.09±0.1	0.06±0.00$^{\#}$	0.08±0.00
Pi intake (mg/24 h)	26.4±1.4	21.4±1.9	1.6±0.1	1.5±0.1
Urine:				
Volume (ml/24 h)	1.39±0.15	1.47±0.16	1.47±0.16	1.70±0.20
Creatinine excretion (μmol/24 h/g BW)	0.13±0.01	0.09±0.03*	0.14±0.02	0.11±0.01
Pi/creatinine (mM/mM)	36.6±2.6	67.7±2.2*	0.6±0.1$^{\#}$	7.6±1.2*$^{\#}$
Feces				
Dry weight (mg/24 h)	30.2±1.9	26.1±2.1	19.6±1.5$^{\#}$	24.8±4.7
Pi excretion (mg/24 h)	7.05±0.30	6.15±0.46	0.80±0.04$^{\#}$	0.47±0.05*$^{\#}$
Pi absorption (mg/24 h)	19.3±1.3	16.4±1.5	0.8±0.1$^{\#}$	1.0±0.2$^{\#}$

*p<0.05 versus C57/BL6 under same diet, $^{\#}$$p$<0.05 versus same genotype under high Pi diet

Jaffé colorimetric method [52]. PTH 1–84 and FGF23 plasma levels were measured with ELISA assays specific for mouse PTH 1–84 and mouse C-terminal FGF23 (Immunotopics, San Clemente, CA).

RNA extraction, reverse transcription, and semi-quantitative real-time PCR

Kidneys were harvested and rapidly frozen in liquid nitrogen. Snap-frozen kidneys (five to seven kidneys for each condition) were homogenized in RLT-Buffer (Qiagen, Basel, Switzerland) supplemented with β-mercaptoethanol to a final concentration of 1 %. RNA extraction, reverse

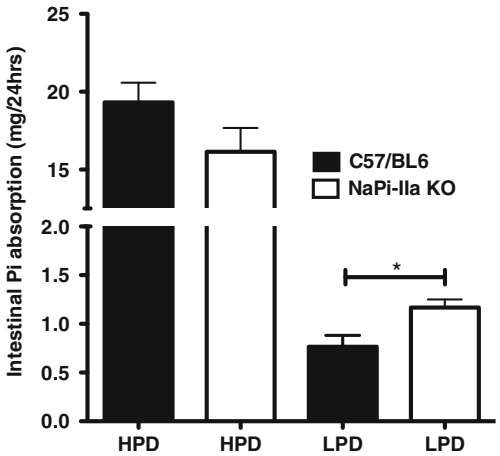

Fig. 3 Intestinal Pi absorption in C57/BL6 and NaPi-IIa$^{-/-}$ mice during high and low Pi diets. Pi excretion was determined in feces from 4 h/day feeding mice treated with chronic high (HPD) or low (LPD) Pi diets for 4 days. Pi absorption was estimated from the total daily amount of Pi consumption minus the daily Pi excretion in 24-h fecal collections on the last day. P<0.05, n=10 to 13 mice/group. NaPi-IIa$^{-/-}$ mice increased Pi absorption compared to C57/B6 mice during LPD

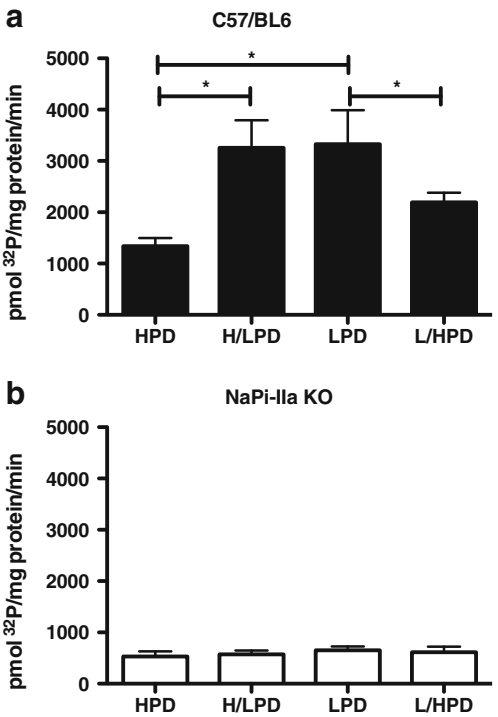

Fig. 4 Brush border membrane (BBM) sodium-dependent phosphate (NaPi) transport activity in C57/BL6 and NaPi-IIa$^{-/-}$ mice. Sodium-dependent ^{32}P-uptake was determined in BBMV from C57/BL6 and NaPi-IIa$^{-/-}$ mice during acute and chronic phosphate diets. **a** BBMV prepared from C57/BL6 mice demonstrated that dietary phosphate intake modulates Na^+-dependent Pi cotransport activity acutely and chronically. **b** In contrast, in BBMV prepared from NaPi-IIa$^{-/-}$ mice Na^+-dependent Pi cotransport activity was always lower than in C57/BL6 mice and did not show any regulation. n=5 to 7 mice/group, *p<0.05

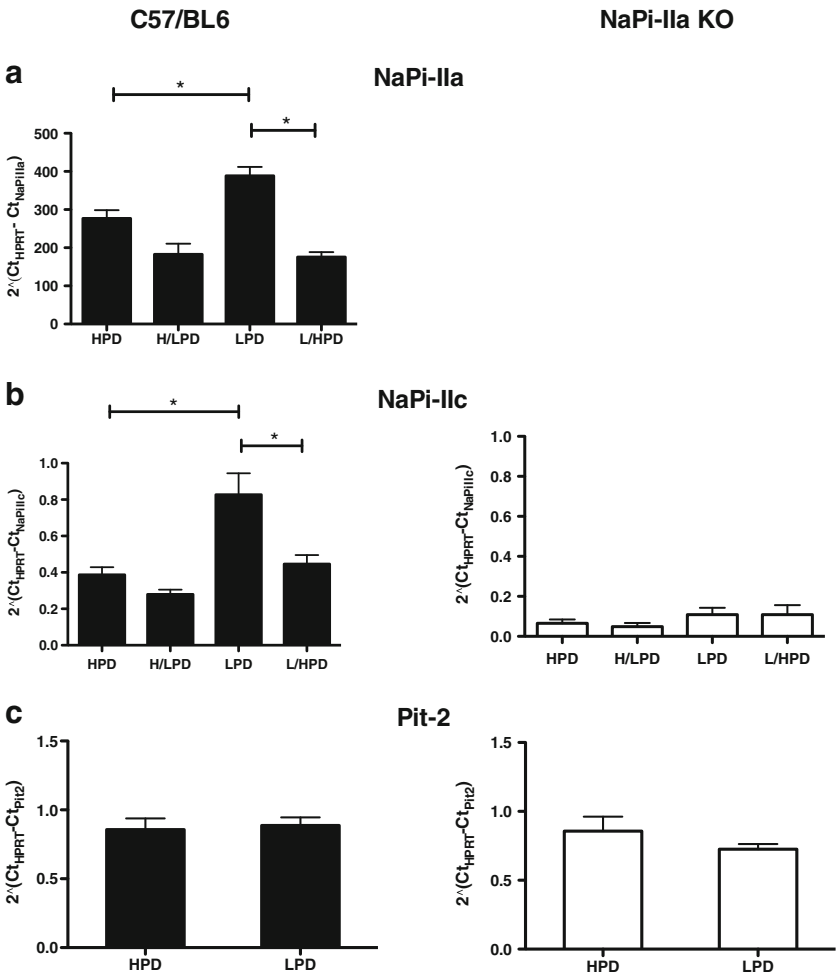

Fig. 5 Dietary phosphate intake regulates NaPi-IIa and NaPi-IIc transcripts but not Pit-2 mRNA. Real-time PCR was used to assess NaPi-IIa, NaPi-IIc, and Pit-2 mRNA levels in total kidneys from C57/BL6 and NaPi-IIa$^{-/-}$ mice. **a** NaPi-IIa mRNA levels were modulated by dietary Pi intake. **b** NaPi-IIc mRNA level was modulated in C57/BL6 kidneys except during H/LPD but was very low and did not adapt to dietary phosphate intake in NaPi-IIa$^{-/-}$ kidneys. **c** Pit-2 mRNA level was not regulated by chronic dietary phosphate intake in both C57/BL6 and NaPi-IIa$^{-/-}$ mice. n=4 to 8 mice/group. *p<0.05

transcription, and semi-quantitative real-time PCR were performed as described previously [42].

Brush border membrane vesicle preparation and phosphate uptake experiments

Brush border membranes vesicles (BBMV) were prepared from total frozen kidneys using the Mg^{2+} precipitation technique as described previously [13, 14]. The phosphate transport rate into BBMV was measured in freshly prepared BBMV at 25 °C in the presence of inward gradients of 100 mM NaCl or 100 mM KCl and 0.1 mM K-phosphate. The substrate P_i was made with 0.125 mM K_2HPO_4 and ^{32}P (1 μCi/ml) to give a final concentration 0.1 mM close to the expected apparent Km_{Pi} for Na^+-dependent transport in renal BBMV. The stop solution contained 100 mM Mannitol, 5 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM Pi. Na^+ dependence was established by incubating BBMV in solutions in which KCl replaced NaCl equimolarly. Phosphate uptake was determined after 60 s, representing initial linear conditions, and after 120 min, to determine the equilibrium values. Total protein concentration was measured using the Bio-Rad Protein Assay kit, Bio-Rad, Hercules, CA, USA. Remaining BBMV were stored at −80 °C until further use.

Immunoblotting

Fifteen to twenty micrograms of renal brush border membrane proteins or 50 μg of kidney homogenates were solubilized in Laemmli loading buffer containing DTT (2 mM) and separated on 8 % polyacrylamide gels. Coomassie blue-stained polyacrylamide gels were used to control equality of protein loading for each series [45]. For immunoblotting, the proteins were transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA). After blocking with 5 % milk powder in Tris-buffered saline for 60 min, the blots were incubated with the primary antibodies: rabbit polyclonal anti-NaPi-IIa (1:4,000) [19], rabbit polyclonal anti-NaPi-IIc (1:5,000) [42], rabbit polyclonal anti-Pit-2 (1:3,000; kind gift from V. Sorribas, University of Zaragossa, Spain) [59], rabbit polyclonal anti-parathyroid hormone receptor (1:1,000; Covance, NJ, USA), rabbit polyclonal anti-phospho-FRS2α (1:2,000; Cell Signaling, MA, USA), mouse monoclonal

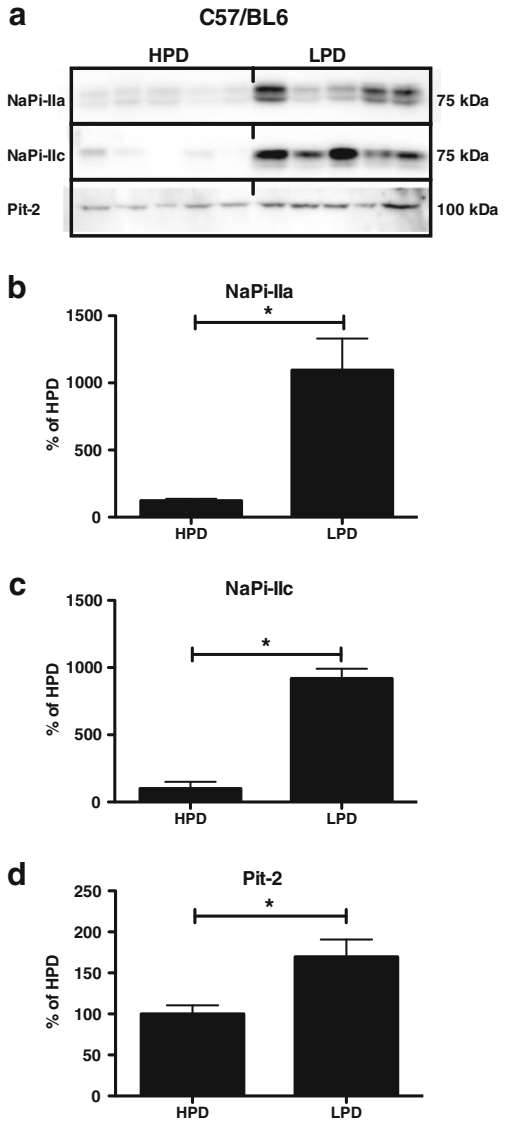

Fig. 6 Brush border membrane abundance of NaPi-IIa, NaPi-IIc, and Pit-2 adapts to continuous chronic dietary phosphate intake in C57/BL6 mice. Brush border membranes were prepared from mice kept over 24 h on high or low phosphate diets for 5 days. **a** Representative immunoblottings for NaPi-IIa, NaPi-IIc, and Pit-2. All blots were normalized against total protein amount stained on parallel Coomassie blue gels (see Methods sections). **b**–**d** *Bar graphs* summarizing data from immunoblottings showing mean±SEM (n=5 mice/group). All data were normalized to the HPD (set as 100 %). BBM NaPi-IIa, NaPi-IIc, and Pit-2 protein abundance was significantly higher during chronic LPD. *$p<0.05$

anti-FRS2α (1:1,500; R&D Systems, MI, USA), and mouse monoclonal anti β-actin 1:25,000 (Sigma Aldrich, Buchs, Switzerland) overnight at 4 °C. After washing, membranes were then incubated for 1 h at room temperature with secondary goat anti-rabbit antibodies 1:5,000 linked to alkaline phosphatase (Promega, Madison, WI, USA) or HRP 1:10,000 (Amersham Life Science, Little Chalfont Buckinghamshire, UK). The protein signal was detected with the appropriate substrates (Millipore Corp, Bedford, MA, USA) using the DIANA III-chemiluminescence detection system (Raytest, Straubenhardt, Germany). All images were analyzed using the software Advanced Image Data Analyser AIDA (Raytest, Straubenhardt, Germany).

Immunohistochemistry

Mouse kidneys were perfusion fixed through the right ventricle with a 4 % paraformaldehyde fixative/PBS solution and the subsequent immunohistochemistry was performed as described previously [5]. Slices of fixed kidneys were frozen in liquid propane and cooled with liquid nitrogen. Serial sections, 5 μm thick, were cut at −22 °C on a cryomicrotome (Leica, Solms, Germany), mounted on superfrost slides, thawed, and kept in cold PBS until use. Before immunofluorescence staining, sections were pretreated with blocking solution (bovine serum albumin 1 % in PBS) for 60 min. After blocking, serial sections were incubated overnight at 4 °C either with a rabbit anti-rat NaPi-IIa [19] diluted 1:1,000 or with an immunopurified rabbit anti-mouse NaPi-IIc [42] diluted 1:1,500 or a rabbit anti-rat Pit-2 [59] diluted 1:300. All primary antibodies were diluted in PBS with 1 % bovine serum albumin. Sections were then rinsed three times with PBS and covered for 1 h at 4 °C with the secondary antibodies: Alexa Fluor 555 goat–anti-rabbit IgG (1:500, Invitrogen), FITC–phalloidin (1:100, Invitrogen), and 4,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO; dilution 1:1,000). Finally, the sections were rinsed three times with PBS, coverslipped using Glycergel Mounting Medium (Dako AG, Baar, Schweiz).

Statistical analysis

Results are expressed as mean ± SEM. All data were tested for significance using ANOVA and unpaired Student's test where appropriate. Only values with $p<0.05$ were considered as significant.

Results

Adaption of plasma Pi concentration, urinary Pi excretion, and intestinal Pi reabsorption during chronic low and high phosphate diets

Results from plasma Pi determinations in C57/BL6 and NaPi-IIa$^{-/-}$ mice after 5-day chronic low or high phosphate diet are shown in Table 1 and Fig. 1. Both mouse strains exhibited similar plasma Pi concentrations depending on diets; high plasma Pi concentrations during HPD (2.42±0.20 and 1.95±0.44 mM) and lower plasma Pi concentrations on LPD (0.50±0.06 and 0.32±0.09 mM). Nevertheless, after 4 days of chronic diets, C57/BL6 and NaPi-IIa$^{-/-}$ mice had different 24-h Pi

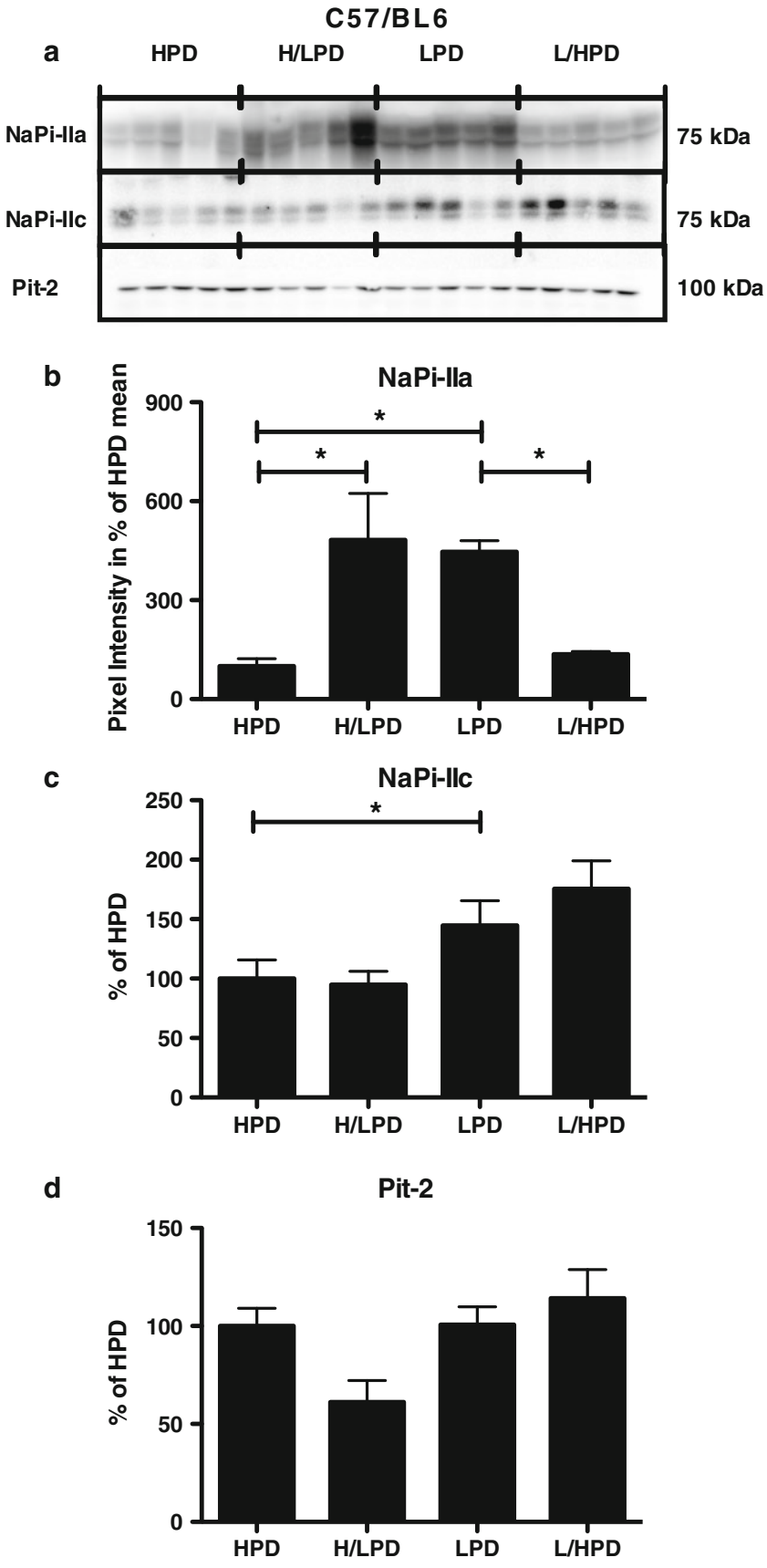

Fig. 7 Rapid adaptation of NaPi-IIa brush border membrane abundance during 4-h feeding acute and chronic phosphate in C57/BL6 mice. Brush border membranes were prepared from mice receiving only for 4 h/day high (HPD) or low (LPD) phosphate diets for 4 days. Some mice were switched on the last day from high to low phosphate (H/LPD) or from low to high phosphate diet (L/HPD). **a** Representative immunoblottings for NaPi-IIa, NaPi-IIc, and Pit-2 during acute phosphate diets. All blots were normalized against total protein amount stained on Coomassie blue gels (see Methods sections). **b**–**d** *Bar graphs* summarizing data from immunoblotting showing mean±SEM (n=5 mice/group). All data were normalized to the HPD (set as 100 %). Only NaPi-IIa BBM expression was modulated by acute phosphate diets. NaPi-IIc protein abundance exhibited only a change between HPD and LPD and Pit-2 protein abundance, no significant change. *p<0.05

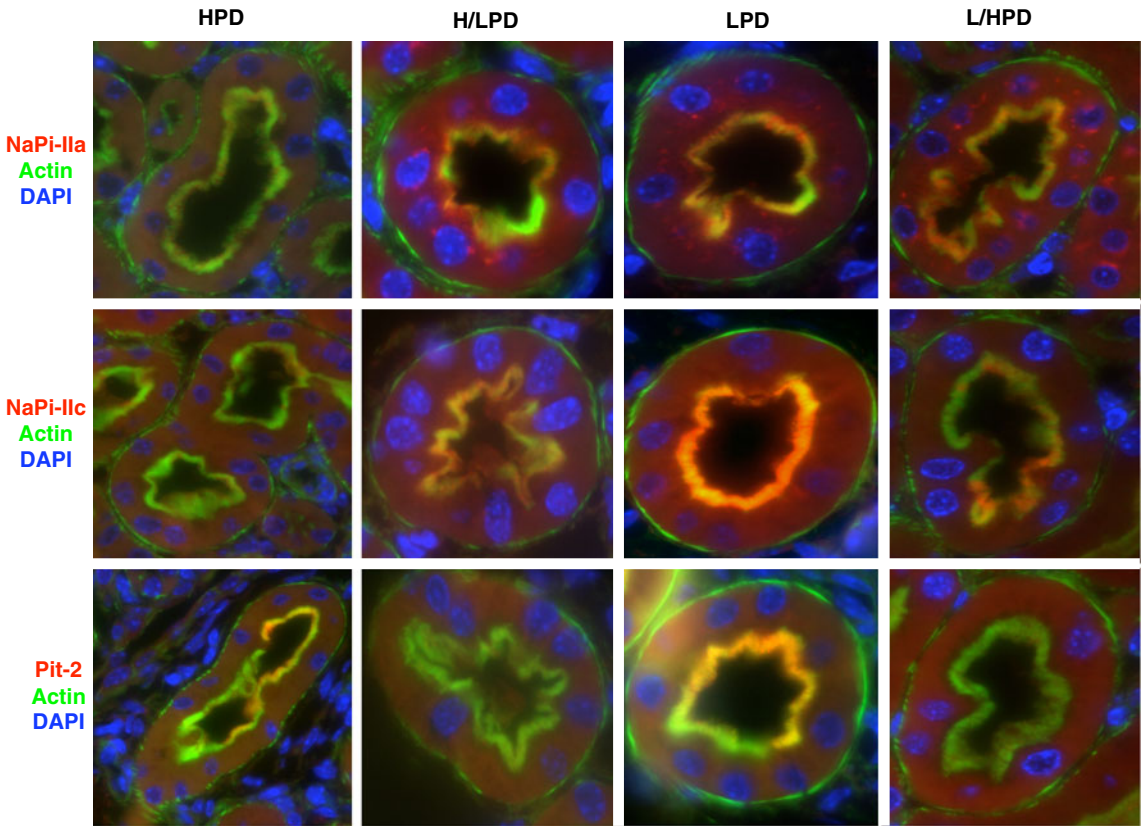

Fig. 8 Immunolocalization of NaPi-IIa, NaPi-IIc, and Pit-2 cotransporters in kidneys from C57/BL6 mice during acute and chronic changes in dietary phosphate intake. Kidney sections were prepared from mice receiving only for 4 h/day high (HPD) or low (LPD) phosphate diets for 4 days. Some mice were switched on the last day from high to low phosphate (H/LPD) or from low to high phosphate diet (L/HPD). All animals were fixed by perfusion 4 h after receiving the last diet. NaPi-IIa (**a**), NaPi-IIc (**b**), and Pit-2 (**c**) stainings are shown in red, the brush border membrane marked by labeling fo actin (green), nuclei are stained with DAPI (blue). Pictures were taken from juxtamedullary S1 proximal tubule segments. Only NaPi-IIa staining was observed both at the plasma membrane and in intracellular organelles possibly representing Golgi apparatus. NaPi-IIc and Pit-2 were detected only at the plasma membrane. During LPD all three transporters were detected abundantly in the BBM (yellow overlay), whereas during HPD only weak staining was observed in the BBM. The H/LPD switch induced NaPi-IIa and NaPi-IIc staining in the BBM whereas Pit-2 was not detected. Similarly, the L/HPD showed reduced BBM localization of NaPi-IIa and NaPi-IIc and no detectable signal for Pit-2. Original magnification ×40

urinary excretion rates. NaPi-IIa$^{-/-}$ mice excreted more Pi with the acute and chronic low Pi diets (Fig. 2, Table 1). This urinary leak was partially compensated by an increase in intestinal Pi absorption during LPD (Table 2, Fig. 3). We determined also total plasma calcium levels and found significantly elevated plasma calcium in the group of mice receiving a chronic low Pi diet (Table 1). In the groups with acute changes of Pi intake, no significant difference could be detected.

Plasma Pi concentration and urinary Pi excretion during acute switches in dietary Pi intake

Plasma and urine Pi concentrations were determined 4 h after switching diets. In C57/BL6 mice, switching from high to low Pi diet decreased plasma Pi concentration by 55 % whereas NaPi-IIa$^{-/-}$ mice drastically reduced their plasma Pi concentration by 83 % (Fig. 1, Table 1). In contrast, no significant difference between C57/BL6 and NaPi-IIa$^{-/-}$ was observed when mice were switched from low to high Pi intake.

NaPi-IIa$^{-/-}$ mice excreted inappropriately high amounts of phosphate not only during chronic dietary treatments but also during the acute switch. C57/BL6 mice rapidly adapted their Pi excretion when switched from high to low Pi intake, whereas NaPi-IIa$^{-/-}$ mice failed reducing their Pi excretion to the same level (89 % versus 59 % in NaPi-IIa$^{-/-}$ mice). This discrepancy was not observed to the same extent when mice were acutely adapted to high Pi diet (Fig. 2, Table 1).

Phosphate transport, NaPi-IIa, NaPi-IIc, and Pit-2 expression and trafficking during chronic and acute changes in dietary phosphate

In C57/BL6 mice, where NaPi-IIa, NaPi-IIc, and Pit-2 are all expressed, Na^+-dependent ^{32}P uptake into BBMV

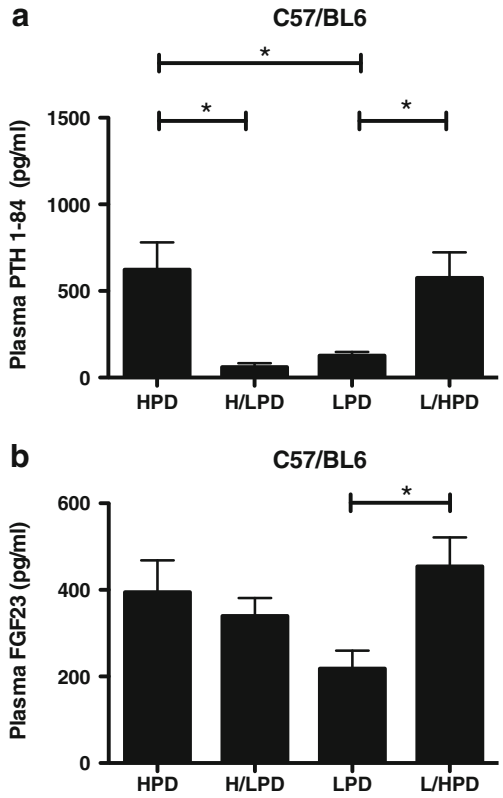

Fig. 9 Plasma PTH and FGF23 in C57/BL6 mice during acute and chronic high and low phosphate diets. Plasma PTH level was rapidly regulated by dietary phosphate within 4 h of switching while plasma FGF23 was significantly modulated only when mice were switched from low to high phosphate diet. *$p<0.05$ versus chronic diets ($n=8$ to 10 mice/group)

was enhanced with LPD as expected. Na^+-dependent ^{32}P uptake was also acutely regulated following Pi intake (Fig. 4a). In BBMV from NaPi-IIa$^{-/-}$ mice, uptake was significantly lower in all four groups and not altered by diet (Fig. 4b).

We performed real-time RT-PCR experiments on C57/BL6 tissue (Fig. 5). NaPi-IIa and NaPi-IIc mRNAs abundance were modulated chronically and acutely only under LPD to HPD. In contrast, we could not detect any regulation at the mRNA level for Pit-2 (Fig. 5). Moreover, in NaPi-IIa$^{-/-}$ tissue, no change in NaPi-IIc or Pit-2 mRNA was observed whatever the diets (Fig. 5).

Next, we determined the abundance of NaPi-IIa, NaPi-IIc, and Pit-2 proteins in the brush border membrane from C57/BL6 kidneys after 4- and 24-h feeding of high and low dietary phosphate. The expression of all three transporters was significantly increased in kidney BBM from mice fed 24 h with low phosphate (Fig. 6). However, when the mice were acutely challenged with 4-h feeding dietary phosphate, only the abundance of NaPi-IIa was significantly modulated by acute dietary phosphate. NaPi-IIc and Pit-2 expression remained unchanged (Fig. 7).

The subcellular distribution of NaPi-IIa, NaPi-IIc, and Pit-2 proteins was further investigated by immunohistochemistry on kidney sections from C57/BL6 mice fed 4 h with the different regimens. As already demonstrated previously in rat kidney [48], NaPi-IIa can be found both at the plasma membrane and in intracellular compartments during all diets, however, with different distribution along the nephron axis (data not shown). During HPD, NaPi-IIa-related staining was weak in the brush border membrane but rather diffusively expressed in the cytoplasm. Some intracellular organelles were highly stained that may represent the Golgi apparatus. In contrast, during LPD, NaPi-IIa was highly expressed at the brush border membrane but was only weakly detected in the cytoplasm and remained visible in intracellular organelles. NaPi-IIc and Pit-2 proteins were exclusively detected at the brush border membrane but their intensity of staining changed with dietary phosphate intake being low during HPD and high during LPD (Fig. 8).

PTH and FGF23 change with different kinetics to dietary phosphate intake

Plasma PTH and FGF23 are both modulated by dietary phosphate [8, 47]. Here, we determined plasma PTH and FGF23 levels in C57/BL6 mice under the various dietary conditions in order to correlate hormone levels with urinary Pi excretion and renal expression of phosphate transporters. PTH followed dietary phosphate intake increasing with high phosphate and decreasing with low phosphate intake and these changes were achieved within the 4 h after switching diets (Fig. 9 and Table 3). In contrast, the abundance of the PTH receptor 1 in kidney BBM remained unaltered during the acute and chronic switches (Fig. 10). In contrast, FGF23 showed a tendency to be lower with chronic low Pi intake compared to chronic high Pi intake and responded only to the acute switch from low to high phosphate but not to the high to low switch (Fig. 9 and Table 3). In order to test whether circulating FGF23 acted in the kidney, we examined the total amount of the FGF adaptor protein FRS2α and its phosphorylated active form p-FRS2α. p-FRS2α mediates the

Table 3 PTH and FGF23 values in plasma from C57/*BL6* mice during 4-h feeding chronic and acute high and low dietary phosphate

*$p<0.05$ versus chronic diet

	High Pi diet ($n=8$)	High to Low Pi diet ($n=10$)	Low Pi diet ($n=9$)	Low to High Pi diet ($n=9$)
PTH (pg/ml)	623±156	61±23*	127±22	576±147*
FGF23 (pg/ml)	395±74	340±41	218±42	454±67

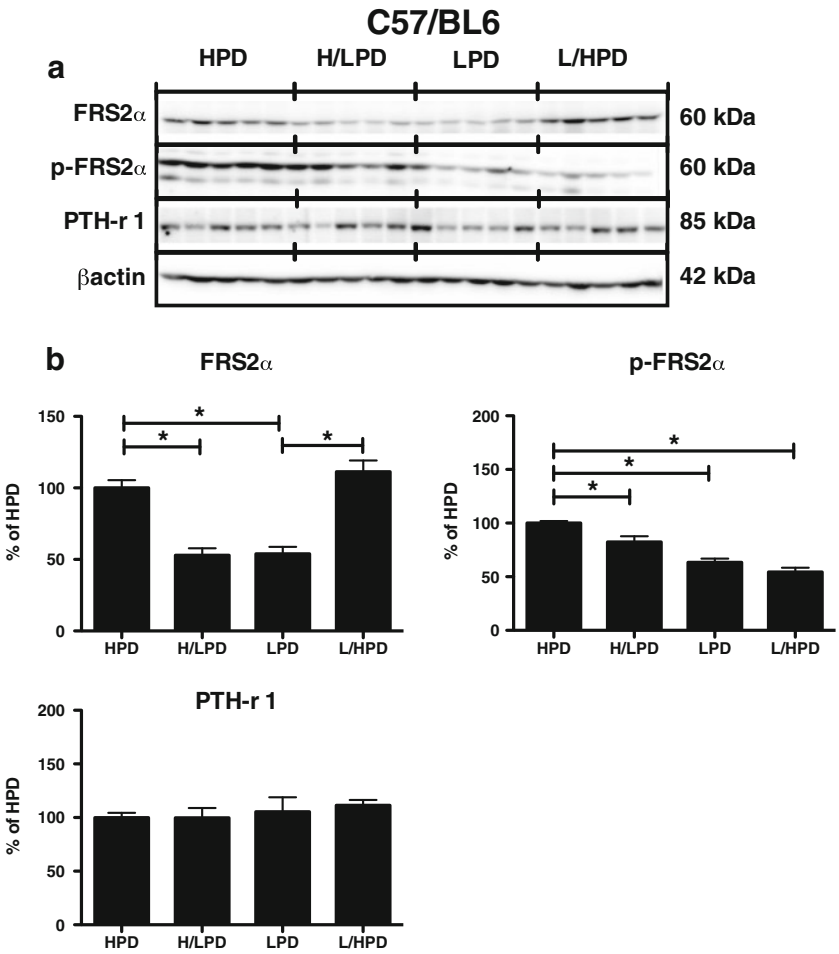

Fig. 10 FRS2α and p-FRS2α adapt to dietary phosphate intake whereas the PTH receptor 1 is not altered. Brush border membranes and whole-kidney homogenates were prepared from mice receiving only for 4 h/day high (HPD) or low (LPD) phosphate diets for 4 days. Some mice were switched on the last day from high to low phosphate (H/LPD) or from low to high phosphate diet (L/HPD). **a** Representative immunoblottings for FRS2α, p-FRS2α, and PTH receptor 1 during acute phosphate diets. All blots were normalized against total protein amount stained on Coomassie blue gels. **b** *Bar graph* summarizing data from immunoblots showing mean± SEM (n=5 mice/group). All data were normalized to the HPD group (set as 100 %). FRS2α expression in whole-kidney homogenates was acutely modulated by dietary phosphate. p-FRS2α expression in whole-kidney homogenates was modulated only during the acute HPD to LPD switch. BBM expression of the PTH receptor 1 was not modulated by phosphate diets. *p<0.05

down-stream signals from the FGF1 receptor upon stimulation by the FGF23/klotho complex [27]. Total FRS2α followed dietary phosphate intake increasing with a high phosphate diet and decreasing during a low phosphate diet. p-FRS2α exhibited also changes during chronic switches but had a slower response than FRS2α during the acute HPD to LPD switch and did not acutely adapt during the LPD to HPD switch (Fig. 10) suggesting reduced FGF23 signaling during acute low Pi intake despite elevated circulating FGF23.

Time course of regulatory mechanisms involved in the acute HPD to LPD switch

To better characterize the adaptive mechanisms involved in the acute HPD to LPD switch, we performed an additional time course experiment with four groups of WT mice adapted to high phosphate and acutely switched to low phosphate diet after 2, 4, and 8 h, respectively. Plasma PTH level was drastically reduced 2 h after switching to low phosphate while plasma FGF23 level remained unchanged for at least 8 h (Fig. 11 and Table 4). PTH receptor 1 exhibited no change while total p-FRS2α and FRS2α decreased after 2 and 4 h, respectively of HPD to LPD switch (Fig. 12). Finally, NaPi-IIa expression in the brush border of these animals gradually increased 4 h after changing to low phosphate (Fig. 13)

Discussion

The acute and chronic effects of dietary phosphate on phosphate (Pi) transport in the proximal tubule have been studied in great details [12, 33, 38, 39]. Taken together, these studies demonstrate that phosphate intake has a direct effect on kidney Pi transport and occurs acutely mainly by trafficking of transporters into or out of the brush border membrane and chronically also by transcriptional and translational mechanisms [12, 33, 37]. In the kidney, three transporters have been identified at the brush border membrane of proximal tubule cells, NaPi-IIa, NaPi-IIc, and Pit-2. In rodents, the modulation of their expression and function by dietary Pi had only been studied for NaPi-IIa in some details but little is known about the relative contribution and fast adaption of NaPi-IIc and Pit-2 [29, 43, 59]. Moreover, the correlation with the regulation of the phosphaturic hormones PTH and

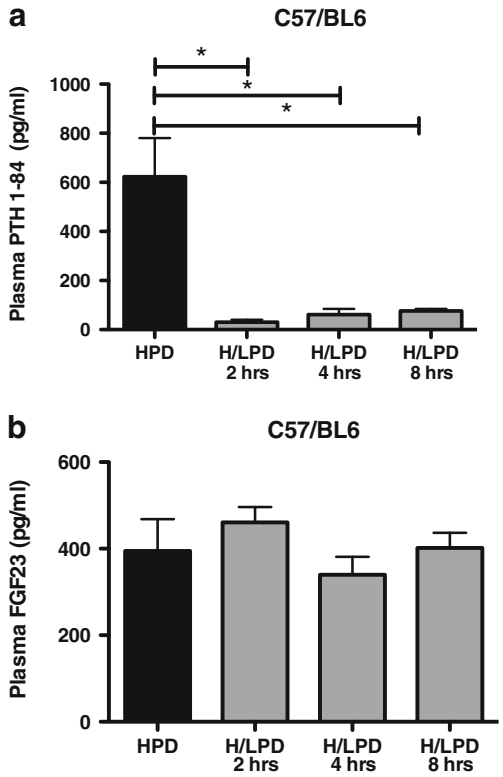

Fig. 11 Plasma PTH and FGF23 changes over 8 h after switching from high to low dietary phosphate intake. Mice were trained to eat for 4 h a day during 4 days high phosphate diet. On the fifth day, mice were subdivided into four groups: group 1 receiving a HPD, groups 2, 3, 4 receiving LPD for 2, 4, and 8 h, respectively. **a** Plasma PTH was reduced in all groups receiving the LPD whereas **b** FGF23 remained high until 8 h LPD. *$p<0.05$ versus HPD. ($n=5$ to 10 mice/group)

FGF23 during acute changes in phosphate intake has not been reported to date [8].

Here, we demonstrated, using C57/BL6 and NaPi-IIa$^{-/-}$ mice that NaPi-IIa is the main adaptive NaPi transporter required for acute and chronic dietary phosphate changes in mice. Moreover, we show that plasma levels of PTH but not FGF23 correlate with NaPi-IIa brush border membrane abundance, and that the FGF receptor adaptor protein FRS2α but not the PTH receptor 1 is regulated during the adaption to low phosphate suggesting that PTH may play an important role in the very acute regulation of NaPi-IIa during dietary adaption and that the kidney is able to adapt its sensitivity to FGF23 by regulating FGF receptor signaling.

NaPi-IIa is the main phosphate transporter regulated by dietary phosphate intake in the mouse proximal tubule

Our data demonstrate that both C57/BL6 mice and NaPi-IIa$^{-/-}$ mice were able to acutely regulate their plasma and urine phosphate levels. However, NaPi-IIa$^{-/-}$ mice reduced their blood Pi level more drastically when acutely challenged with low phosphate diet than C57/BL6. Moreover, they exhibited higher phosphaturia than C57/BL6 mice whatever the diet. Our data confirm previous observations on the role of NaPi-IIa in chronic high and low phosphate diets in mice [7] and highlight the importance of NaPi-IIa in renal Pi handling during acute changes in phosphate intake in rodents. NaPi-IIa$^{-/-}$ mice maintain their phosphate homeostasis, at least to some degree, by increasing the intestinal absorption of phosphate. This had been suggested as compensatory mechanism but never been tested before [7]. 1,25$(OH)_2D_3$ is elevated in NaPi-IIa$^{-/-}$ mice and may contribute to the adaptive response of the intestine observed in our study [7]. However, the small intestine is also able to adapt to low phosphate intake independent from vitamin D_3 or the vitamin D_3 receptor and thus vitamin D_3 independent mechanisms may be also involved [17]. Whether NaPi-IIa is equally important in human kidney for phosphate reabsorption and the fast adaption to dietary phosphate intake remains to be established. In contrast to rodents, NaPi-IIc plays a more important role in human kidney, at least for total phosphate reabsorption [9]

In C57/BL6 mice, NaPi-IIa protein is very rapidly regulated by dietary phosphate intake. In the case of adapting to acute low phosphate diets, this involves most likely only translational or posttranslational mechanisms and trafficking of pre-synthesized NaPi-IIa transporter proteins. At the mRNA level, NaPi-IIa and NaPi-IIc were modulated chronically and also acutely after 4 h of switching from low to high phosphate diet. Both immunoblotting and immunohistochemistry demonstrated a relocation of NaPi-IIa into the brush border membrane after ingesting low phosphate and this was detectable after 4-h feeding. Similarly, intake of high phosphate caused downregulation and removal of NaPi-IIa transporters from the BBM within 4 h demonstrating the importance of trafficking for the regulation of NaPi-IIa function. Removed NaPi-IIa transporters are then routed to lysosomes for degradation [26] whereas NaPi-IIc may be recycled [43] or directly degraded [32, 44]. In our experiments, we were unable to detect intracellular NaPi-IIc proteins by

Table 4 PTH and FGF23 values in plasma from C57*BL6* during a high to low phosphate diet switch

	High Pi diet ($n=8$)	2-h high to low Pi diet ($n=5$)	4-h high to low Pi diet ($n=10$)	8-h high to low Pi diet ($n=5$)
PTH (pg/ml)	623±156	30±10*	61±23*	75±8*
FGF23 (pg/ml)	395±74	461±36	340±41	402±35

*$p<0.05$ vs high Pi diet

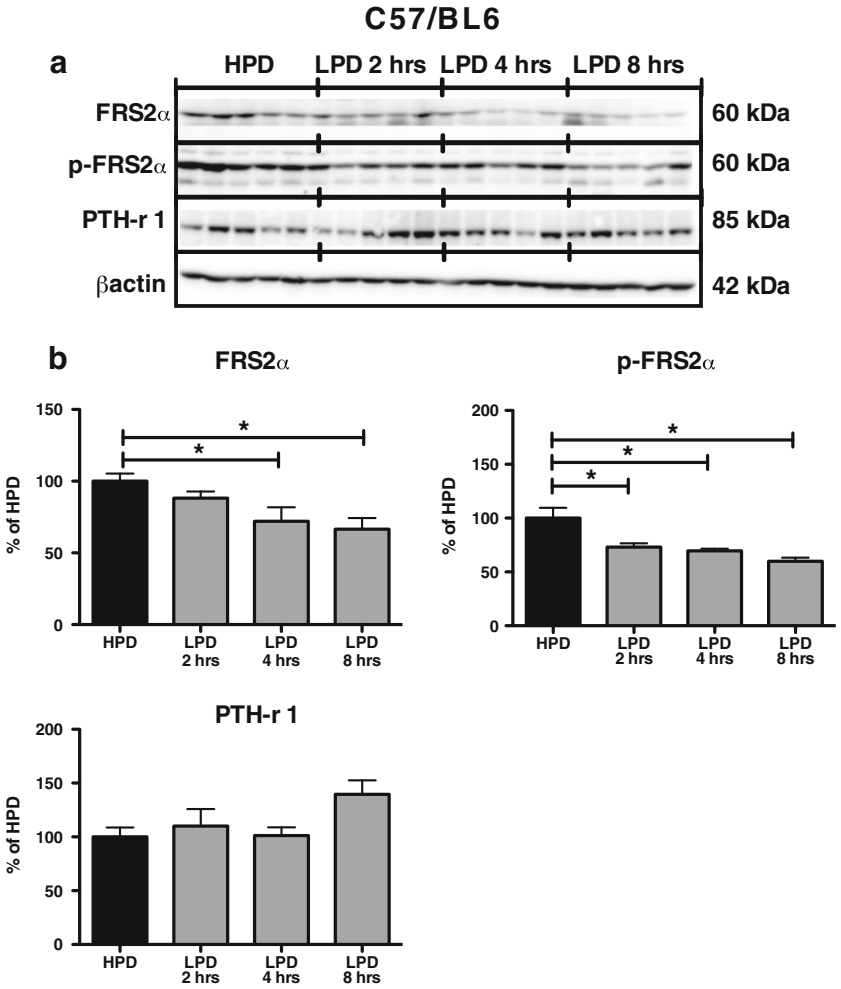

Fig. 12 The adaptor protein FRS2α and its phorphorylated form p-FRS2α are acutely modulated after switching from high to low dietary phosphate intake. Mice were trained to eat 4 h/day during 4 days HPD. On the fifth day, mice were subdivided into four groups: group 1 receiving HPD, and groups 2, 3, 4 feeding LPD for 2, 4, or 8 h, respectively. Whole-kidney homogenates and BBM were prepared and FRS2α p-FRS2α, and PTH receptor 1 abundance were determined by western-blotting. **a** Representative immunoblottings for FRS2α, p-FRS2α, and PTH receptor 1 during a 2, 4, and 8 h HPD to LPD switch. All blots were normalized against total protein amount stained on Coomassie blue gels. **b** *Bar graph* summarizing data from immunoblotting showing mean±SEM (n=5 mice/group). All data were normalized to the HPD group (set as 100 %). FRS2α and p-FRS2α whole-kidney expression decreased after respectively a 4- and 2-h feeding LPD. n=5 mice/group. BBM expression of the PTH receptor 1 was unchanged during the treatments. *p<0.05 versus HPD

immunohistochemistry consistent with degradation of NaPi-IIc proteins.

In parallel to the changes in phosphate transporter expression in the brush border membrane, we found adaption of Na^+-dependent phosphate transport activity in brush border membrane vesicles prepared from C57/BL6 mice. In contrast, Na^+-dependent Pi-uptake into BBMV was very low and remained unchanged in NaPi-IIa$^{-/-}$ mice whatever the diet. The remaining activity in knock-out BBM is approximately reduced to 30 % under chronic high phosphate diet which is similar to what was described earlier [50, 56]. This result indicates that NaPi-IIc and Pit-2 transport activities are low in mice and may already be maximally stimulated in NaPi-IIa-deficient mice as compensatory mechanism. Of note, NaPi-IIc and Pit-2 were not able to compensate for the lack of NaPi-IIa as indicated by higher urinary phosphate losses under all conditions. However, NaPi-IIa$^{-/-}$ mice were able to modulate total urinary phosphate excretion reducing phosphate excretion with low phosphate intake. Several mechanisms may be responsible for this adaption including lower filtered phosphate load due to lower circulating plasma phosphate levels, changes in glomerular filtration rate, or phosphate absorption in segments other than the proximal tubule. Micropuncture studies suggested also some phosphate absorption in distal portions of the nephron [36] and more recently the presence of NaPi-IIb has been suggested [54].

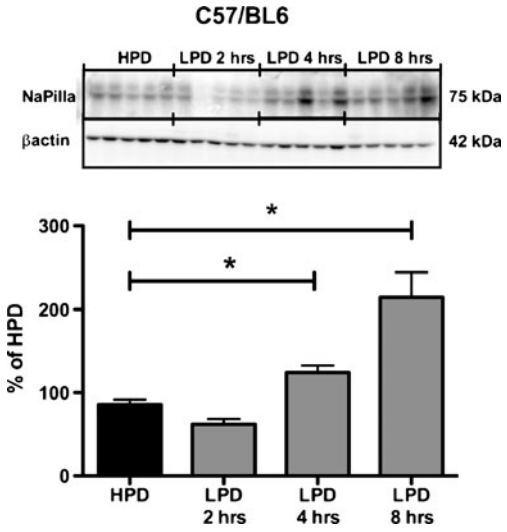

Fig. 13 Dietary phosphate intake induces rapid regulation of NaPi-IIa protein abundance. Mice were trained to eat 4 h/day during 4 days HPD. On the fifth day, mice were subdivided into four groups group 1 receiving HPD, and groups 2, 3, or 4 feeding LPD for 2, 4, or 8 h, respectively. BBM were prepared and NaPi-IIa abundance was determined by western-blotting. BBM NaPi-IIa levels were significantly increased after 4 h after feeding LPD. n=5 to 8 mice/group, *p<0.05 versus HPD

NaPi-IIa is upregulated despite persistently high FGF23 level

Changes in dietary phosphate intake were paralleled by plasma PTH levels; high phosphate intake associated with higher PTH levels and low phosphate diet associated with reduced PTH levels. The changes in PTH levels were achieved within at least 4 h after switching to high Pi intake and within less than 2 h after ingesting low phosphate diets. Previous experiments in thyroparathyroidectomized rats showed that adaption from high to low Pi diet did not increase NaPi-IIa abundance within 4 h whereas switching from low to high Pi reduced NaPi-IIa protein after 4 h [55]. Since animals were not substituted with thyroid hormone, no conclusions on the adaption from high to low Pi diets can be made. Moreover, shorter time points than 4 h were not investigated. The adaption to chronic Pi restriction intake in thyroparathyroidectomized rats demonstrated that neither PTH nor thyroid hormone are the major regulator in this setting [53, 62]. Despite changes in PTH levels, the abundance of the PTH receptor 1 in the brush border membrane as well as in total kidney remained stable. Whether the receptor is regulated on the subcellular level, i.e., by endocytosis and recycling, remains to be examined in more detail.

The signals stimulating PTH secretion in response to elevated phosphate intake or reducing PTH levels after ingestion of low phosphate diets are not clear to date. While we cannot rule out small changes in ionized calcium levels altering PTH secretion, direct effects of phosphate on PTH synthesis and secretion may also contribute to regulation of parathyroid function. Direct effects of extracellular phosphate on PTH secretion by isolated parathyroid cells or slices under conditions of fixed concentrations of extracellular calcium have been described suggesting that phosphate independent from ionized calcium can stimulate PTH secretion [1, 2, 41, 51]. The mechanism underlying this regulation is currently unknown.

In contrast to PTH, FGF23 levels remained high for at least 8 h after switching from high to low phosphate intake. Similarly, FGF23 did not change over a period of 6 h in healthy volunteers when plasma phosphate levels were acutely lowered [25]. Also in a more chronic setting, FGF23 levels decreased not before 16 h after switching healthy probands to a low phosphate diet [58]. Thus, FGF23 seems to adapt only slowly when the organism has to adapt from high to low phosphate intake. Nevertheless, in our model renal adaption of phosphate excretion, increased BBM phosphate transport activity, and enhanced NaPi-IIa protein expression in the brush border membrane occurred much faster than 8 h despite the high FGF23 levels. These data suggest that PTH and possibly other factors may be more relevant for the rapid adaption from low to high phosphate intake and that the proximal tubule is resistant to the action of FGF23 during the acute adaption to low phosphate diet. The pathway by which FGF23 regulates NaPi-IIa function and expression in the proximal tubule is controversial. FGF23 may act either indirectly on the proximal tubule via direct stimulation of a MAPK-dependent pathway in the distal tubule [22] or may directly elicit an intracellular signaling cascade in the proximal tubule [3, 6, 24]. However, in all models, FGF23 signals via FGF1c receptors and its downstream adaptor protein FRS2α. We show now that the total abundance of FRS2α and its phosphorylated form are differently modulated during changes in dietary phosphate intake providing a possible explanation for the apparent resistance of the kidney to high circulating FGF23 levels during the switch from high to low Pi intake.

Regulatory network in the renal adaption to dietary phosphate intake

Several factors contribute to the adaption of the kidney to changes in phosphate intake. FGF23 and PTH contribute importantly to the downregulation of renal phosphate transporters, albeit with different time courses. In addition, sFRP4, MEPE, and dopamine have been identified as potential regulators. sFRP4 acutely downregulates renal phosphate reabsorption and NaPi-IIa abundance in rat kidney [11]; however, mice lacking sFRP4 adapt normally to changes in phosphate intake [18]. MEPE reduces renal phosphate reabsorption [21, 31] and MEPE-deficient mice display hyperphosphatemia with elevated expression of

NaPi-IIa consistent with a phosphaturic role of MEPE [20]. However, the role of MEPE in the acute renal adaption to changes in dietary phosphate intake has not been determined. In addition, locally produced dopamine may contribute to the downregulation of NaPi-IIa during intake of high phosphate, an effect mediated by D1-like dopamine receptors [4, 61]. A role of the small intestine in sensing the phosphate load and inducing the renal adaption has been proposed but a subsequently published erratum indicates that very high phosphate loads were given [10]. Thus, even though several hormones besides PTH and FGF23 may be involved in adapting the kidney to increased dietary phosphate intake, the role of the small intestine remains to be clarified. The relative contribution of these factors to regulating renal phosphate handling may vary over time as indicated by the different time courses for PTH and FGF23 elevation and fall upon changes in dietary Pi intake. The response time and time course of other factors such as dopamine and MEPE have not been determined in enough detail to integrate them into a more detailed model to date.

In summary, we demonstrate that NaPi-IIa is the main phosphate transporter in mouse kidney and critical for the adaptive capacity of the proximal tubule to adapt to dietary phosphate intake. However, NaPi-IIa-deficient mice showed some ability to adapt their renal excretion of phosphate to dietary intake. During the acute adaption to low phosphate intake, NaPi-IIa is rapidly recruited to the brush border membrane within 4 h despite persistently high FGF23 level suggesting that PTH may mediate at least in part the acute adaption to dietary phosphate.

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