Original Paper

Enhanced FGF23 Serum Concentrations and Phosphaturia in Gene Targeted Mice Expressing WNK-Resistant Spak

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Key Words

pH_i • Na⁺ • phosphate cotransporter • FGF23 • bone density

Abstract

Background: The WNK-dependent STE20/SPS1-related proline/alanine-rich kinase (SPAK) regulates the renal thiazide sensitive NaCl cotransporter (NCC) and the renal furosemide sensitive Na⁺,K⁺,2Cl⁻ cotransporter (NKCC2) and thus participates in the regulation of renal salt excretion, extracellular fluid volume and blood pressure. Inhibition of NCC leads to anticalciuria. Moreover, NCC is also expressed in osteoblasts where it is implicated in the regulation of bone mineralization. Osteoblasts further influence mineral metabolism by releasing the phosphaturic hormone FGF23. The present study explored, whether SPAK participates in the regulation of calcium-phosphate homeostasis. **Methods:** FGF23 serum levels and phosphate homeostasis were analyzed in gene targeted mice expressing SPAK resistant to WNK-dependent activation (spak^{tg/tg}) and in mice expressing wild type SPAK (spak^{wt/wt}). **Results:** Serum FGF23 level was significantly higher, urinary phosphate excretion significantly larger and serum phosphate concentration significantly lower in spak^{tg/tg} mice than in spak^{wt/wt} mice. Urinary calcium excretion was significantly decreased in spak^{tg/tg} mice. Serum levels of calcitriol and PTH were not significantly different between the genotypes. Bone density was significantly increased in spak^{tg/tg} mice compared to spak^{wt/wt} mice. Treatment of spak^{wt/wt} mice with HCT increased FGF23 serum levels, and led to phosphaturia and hypophosphatemia. **Conclusions:** SPAK is a strong regulator of FGF23 formation, bone mineralization and renal Ca²⁺ and phosphate excretion.

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Introduction

SPAK (SPS1-related proline/alanine-rich kinase) is a key regulator of renal tubular salt transport and blood pressure [1-3]. SPAK is under control of the with-no-K(Lys) (WNK) kinases [1, 4-8], which are similarly decisive for renal salt excretion and blood pressure regulation [7, 9-12]. Mutations within WNK kinases cause Gordon's syndrome, a monogenic disease leading to hypertension and hyperkalaemia [6, 8, 13, 14]. SPAK and the related oxidative stress-responsive kinase 1 (OSR1) kinase modify renal salt excretion and blood pressure at least partially through activation of the NaCl cotransporter (NCC) and the Na⁺,K⁺,2Cl⁻ cotransporter (NKCC2) [4, 5, 7, 15-25].

Inhibition of NCC with thiazides is followed by anticalciuria [26]. Moreover, thiazide sensitive NCC activity is a powerful regulator of bone differentiation and bone mineralisation [27, 28]. Thus, at least in theory, WNK sensitive regulation of SPAK could participate in the regulation of Ca²⁺ and phosphate metabolism. As a matter of fact, a WNK4 gene variant has been shown to be associated with osteoporosis [29]. However, nothing is known about a role of SPAK in the regulation of renal tubular transport of Ca²⁺ and phosphate. Renal phosphate excretion is under regulation by dietary phosphate intake, acid-base status, parathyroid hormone, 1,25-(OH)₂ vitamin D₃, FGF23, insulin and insulin-like growth factor IGF1 [30-36]. Signaling known to regulate renal tubular phosphate transport include protein kinases A and C, ERK1/2, Klotho and the PI3K/PKB/GSK3 kinase cascade [37-44].

The present study addressed the putative role of SPAK in the regulation of renal Ca²⁺ and phosphate metabolism. To this end, urinary Ca²⁺ and phosphate output as well as serum Ca²⁺, phosphate, PTH, 1,25(OH)₂D₃ and FGF23 concentrations were determined in gene targeted mice expressing WNK-resistant SPAK (*spak^{tg/tg}*) [1] and in the respective wild type mice (*spak^{wt/wt}*). The observations point to an effect of SPAK on the regulation of bone mineralization and FGF23 release as well as renal tubular Ca²⁺ and phosphate transport.

Materials and Methods

Animals

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Blood was drawn, urine collected or tissue isolated from sex- and age-matched 3-8-month-old homozygous SPAK knockin mice (*spak^{tg/tg}*) and respective wild type mice (*spak^{wt/wt}*), kindly provided by Dario Alessi. As described earlier [1] in the knockin mice the T-loop Thr residue in SPAK (Thr243) was mutated to Ala to prevent activation by WNK isoforms. Mice had free access to control diet (sniff, Soest, Germany) containing 7000 mg/kg phosphorus or to phosphate-depleted diet (Altromin, Lage, Germany) containing 131 mg/kg phosphate and to tap drinking water ad libitum. Where indicated, the mice were treated with hydrochlorothiazide (Sigma Aldrich, Germany), which was first dissolved in DMSO and then in drinking water at the dose of 600 mg/l for 12 days. To obtain serum, mice were anaesthetized with diethylether (Roth, Karlsruhe, Germany) and blood was drawn into capillaries by puncturing the retroorbital plexus.

To determine urinary parameters, the mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany) as described previously. They were allowed a 2 day habituation period during which food and water intake, urinary flow rate and phosphate excretion were recorded every day to ascertain that the mice were adapted to the new environment. Subsequently, 24 h collection of urine was performed for three consecutive days in order to obtain the urinary parameters. This procedure was repeated under low-phosphate diet. To assure quantitative urine collection, metabolic cages were siliconized, and urine was collected under water-saturated oil.

The phosphate concentration was determined colorimetrically utilizing a commercial diagnostic kit (Roche Diagnostics, Mannheim, Germany). ELISA kits were employed for determination of the serum intact parathormone concentration (Immutopics, San Clemante, USA), of 1,25(OH)₂D₃ concentration (IDS Diagnostics, Frankfurt/Main, Germany) and of FGF23 concentration (Immutopics).

Bone density

For the analysis of bone density, animals were sacrificed and legs were amputated and fixated in PFA (paraformaldehyde). The samples were scanned with a high resolution X-ray computed tomography (Inveon SPECT/CT) scanner (Siemens Preclinical Solutions, Knoxville, TN, USA) using a field of view of $3.6 \times 3.6 \times 3.6 \text{ cm}^3$. The X-ray tube parameters were set at 80 kVp and 500 μA . The images were acquired with 400 angular projections (exposure time 1000 ms per projection) over 200 degree and binned with a factor of two, yielding a reconstructed pixel size of $\sim 35 \mu\text{m}$. The total scan time was 12 minutes. Reconstructed CT images were analyzed with the Inveon Research Workplace software (Siemens Preclinical Solutions, USA), by drawing a standard-sized container around the femur and applying a region growth routine to segment the trabecular bone structure. For all samples, the same upper and lower density threshold was applied and the relative numbers of trabecular bone density compared.

SPAK transcripts in UMR106 cells

Rat osteosarcoma UMR106 cells (10^6) were cultured in Dulbecco's MEM medium, supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin, at 5% CO_2 and 37°C for 2 days. Cells were lysed, total RNA isolated (Mini kit, Qiagen, Hilden, Germany) and 1 μg transcribed into cDNA using reverse transcriptase (Roche Diagnostics GmbH, Roche Applied Science, Mannheim Germany). Aliquots of cDNA, corresponding to equal amounts of RNA, were used for quantification of mRNA. Specific primers for rat used were:

Sense primer: AAGTCATGGAACAGGTGAGAGGC

Antisense primer: TTCAGTCTTATGAAGGTGACCGC

The mRNA was subjected to RT-PCR analysis using the light cycler system (Roche Diagnostics GmbH, Roche Applied Science, Mannheim Germany). The reaction mixture of 20 μl contained cDNA, 10 mM MgCl_2 , 0.5 μM of each primer, and 1 μl Master Syber Green I mix (LightCycler™FastStart Master Syber Green, Roche). The transcript level of the housekeeping gene GAPDH of the sample was taken as reference using a commercial primer kit (Search LC, Heidelberg, Germany). Amplification of the target DNA was performed during 35 cycles, each of 10 s at 95°C, 10 s at 68°C and 16 s at 72°C. The product size was analysed on 1.5% agarose gel.

Statistics

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. All data were tested for significance using unpaired Student's t-test or ANOVA, as appropriate. Only results with $p < 0.05$ were considered statistically significant.

Results

In order to elucidate, whether phosphate metabolism is sensitive to WNK dependent regulation of SPAK, experiments were performed in SPAK knockin mice ($\text{spak}^{\text{tg/tg}}$), which were carrying a WNK-insensitive $\text{T}243\text{A}$ SPAK mutant. The animals were compared to respective wild type mice ($\text{spak}^{\text{wt/wt}}$). Urinary phosphate excretion was significantly higher in $\text{spak}^{\text{tg/tg}}$ mice than in $\text{spak}^{\text{wt/wt}}$ mice (Fig. 1). As shown in Fig. 1, a low-phosphate diet decreased the urinary phosphate excretion to similarly low levels in $\text{spak}^{\text{tg/tg}}$ and $\text{spak}^{\text{wt/wt}}$ mice. In contrast to urinary phosphate excretion, urinary Ca^{2+} excretion was significantly lower in $\text{spak}^{\text{tg/tg}}$ than in $\text{spak}^{\text{wt/wt}}$ mice. Low phosphate diet increased urinary Ca^{2+} excretion in both genotypes. During low phosphate diet urinary Ca^{2+} excretion was still significantly lower in $\text{spak}^{\text{tg/tg}}$ than in $\text{spak}^{\text{wt/wt}}$ mice.

In theory, the phosphaturia of $\text{spak}^{\text{tg/tg}}$ mice could have been due to an increased serum phosphate concentration. Conversely, phosphaturia due to decreased renal tubular phosphate transport should result in a decrease of serum phosphate concentration. Thus, serum phosphate concentration was determined. As shown in Fig. 2, the serum phosphate concentration was significantly lower in $\text{spak}^{\text{tg/tg}}$ mice than in $\text{spak}^{\text{wt/wt}}$ mice. Thus, the phosphaturia of $\text{spak}^{\text{tg/tg}}$ mice was not due to hyperphosphatemia but obviously resulted in hypophosphatemia. Dietary phosphate depletion decreased the serum phosphate

Fig. 1. Urinary phosphate and Ca^{2+} excretion in $\text{spak}^{\text{wt/wt}}$ and $\text{spak}^{\text{tg/tg}}$ mice. Arithmetic means $\pm$ SEM ($n = 10-12$) of urinary phosphate (A) and Ca^{2+} (B) excretion in homozygous SPAK knockin mice ($\text{spak}^{\text{tg/tg}}$, black bars) and wild type mice ($\text{spak}^{\text{wt/wt}}$, white bars) under normal diet (left bars) and following dietary phosphate depletion (right bars). * ($p < 0.05$) indicates significant difference from $\text{spak}^{\text{wt/wt}}$ mice, # ($p < 0.05$) indicates significant difference from control diet.

Fig. 2. Serum phosphate and Ca^{2+} concentrations in $\text{spak}^{\text{wt/wt}}$ and $\text{spak}^{\text{tg/tg}}$ mice. Arithmetic means $\pm$ SEM ($n = 10-12$) of serum phosphate (A) and Ca^{2+} (B) concentration in homozygous SPAK knockin mice ($\text{spak}^{\text{tg/tg}}$, black bars) and wild type mice ($\text{spak}^{\text{wt/wt}}$, white bars) under normal diet (left bars) and following dietary phosphate depletion (right bars). * ($p < 0.05$) indicates significant difference from $\text{spak}^{\text{wt/wt}}$ mice, # ($p < 0.05$) indicate significant difference from control diet.

concentration in both, $\text{spak}^{\text{tg/tg}}$ mice and in $\text{spak}^{\text{wt/wt}}$ mice. Under phosphate depleted diet, the serum phosphate concentration remained still lower in $\text{spak}^{\text{tg/tg}}$ mice than in $\text{spak}^{\text{wt/wt}}$ mice, a difference, however, not reaching statistical significance. Serum Ca^{2+} concentration was not significantly different between $\text{spak}^{\text{tg/tg}}$ and $\text{spak}^{\text{wt/wt}}$ mice and was not significantly modified by low phosphate diet.

As phosphaturia could have resulted from altered hormone levels, the serum concentration of parathyroid hormone (PTH), calcitriol ($1,25(\text{OH})_2\text{D}_3$), and fibroblast growth factor 23 (FGF23) were determined. As shown in Fig. 3, the serum concentrations of PTH (Fig. 3A) and of $1,25(\text{OH})_2\text{D}_3$ (Fig. 3B) were similar in $\text{spak}^{\text{tg/tg}}$ mice and in $\text{spak}^{\text{wt/wt}}$ mice. The serum FGF23 concentration was, however, significantly higher in $\text{spak}^{\text{tg/tg}}$ mice than in $\text{spak}^{\text{wt/wt}}$ mice (Fig. 3C).

Fig. 3. Serum concentration of PTH, 1,25(OH)₂D₃ and FGF23 in *spak*^{wt/wt} and *spak*^{tg/tg} mice. Arithmetic means ± SEM (n = 10-12) of serum PTH (A), 1,25(OH)₂D₃ (B), and FGF23 (C) in *spak*^{wt/wt} mice (white bars) and *spak*^{tg/tg} mice (black bars) under normal diet (upper bars) and following dietary phosphate depletion (lower bars). ** (p<0.01) indicates significant difference from *spak*^{wt/wt} mice (Student's t-test), # (p<0.05) indicates significant difference from control diet.

Dietary phosphate depletion significantly increased 1,25(OH)₂D₃ concentration and significantly decreased serum concentration of PTH in both, *spak*^{tg/tg} and *spak*^{wt/wt} mice. Phosphate depletion decreased serum FGF23 concentrations, an effect reaching statistical significance only in *spak*^{wt/wt} mice (Fig. 3A-C).

As SPAK stimulates the thiazide sensitive NaCl cotransporter, additional experiments were performed to elucidate whether thiazide treatment similarly influences FGF23 serum levels. As illustrated in Fig. 4, hydrochlorothiazide treatment was followed by a significant increase of FGF23 serum levels, which was paralleled by phosphaturia and hypophosphatemia, thus mimicking the phenotype of *spak*^{tg/tg} mice.

Further experiments were performed to elucidate whether the enhanced FGF23 release was paralleled by altered bone density. As shown in Fig. 5, bone density was significantly higher in *spak*^{tg/tg} mice than in *spak*^{wt/wt} mice.

In order to test, whether Spak is expressed in osteoblasts, mRNA encoding Spak was determined by RT-PCR. As illustrated in Fig. 6, the SPAK transcript was indeed detected in rat osteosarcoma UMR106 cells.

Discussion

The present observations reveal a completely novel function of WNK/SPAK signaling, i.e. its participation in the regulation of calcium phosphate metabolism and bone density.

Fig. 4. Effect of hydrochlorothiazide on serum FGF23 levels, urinary phosphate output and serum phosphate concentration in *spak*^{wt/wt} mice. Arithmetic means $\pm$ SEM (n = 5) of serum FGF23 (A), urinary phosphate excretion (B) and serum phosphate concentration (C) in *spak*^{wt/wt} mice without (white bars) and with (black bars) treatment with hydrochlorothiazide. * (p < 0.05) indicates significant difference from untreated *spak*^{wt/wt} mice (Student's t-test).

Fig. 5. Bone density in *spak*^{wt/wt} and *spak*^{tg/tg} mice. Arithmetic means $\pm$ SEM (n = 7 each group) of bone density in *spak*^{wt/wt} mice (white bars) and *spak*^{tg/tg} mice (black bars). * p < 0.05 vs. respective value of *spak*^{wt/wt} mice.

Fig. 6. Spak transcripts in UMR106 cells. Original gel displaying amplified fragments of Spak mRNA (left lane) and Gapdh mRNA (middle lane) from UMR106 cells.

According to the present observations, WNK insensitivity of SPAK decreases urinary Ca^{2+} excretion, increases bone density, upregulates FGF23 release, and increases urinary phosphate excretion.

The phosphaturia in *spak*^{tg/tg} mice was paralleled by hypophosphatemia and was thus not due to increased serum phosphate concentration. Conversely, the urinary phosphate loss presumably accounts for the hypophosphatemia of the *spak*^{tg/tg} mice. In theory, the phosphaturia of *spak*^{tg/tg} could have resulted from increased serum levels of PTH, a powerful hormone fostering internalization and degradation of NaPiIIa [34, 38], the most important renal tubular phosphate transporter [45-47]. Thus, PTH leads to phosphaturia and hypophosphatemia [34, 38]. The PTH serum concentration tended, however, to be rather decreased in *spak*^{tg/tg} mice. Thus, increased PTH plasma concentrations do presumably not

contribute to the phosphaturia of *spak^{tg/tg}* mice. In theory, phosphaturia may result from altered activity of calcitriol ($1,25(\text{OH})_2\text{D}_3$), which increases plasma phosphate and plasma Ca^{2+} concentration mainly by stimulating Ca^{2+} and phosphate absorption in intestine [48-51]. Serum $1,25(\text{OH})_2\text{D}_3$ concentration was, however, not significantly different between *spak^{tg/tg}* mice and *spak^{wt/wt}* mice. The $1,25(\text{OH})_2\text{D}_3$ serum levels increased following phosphate depletion, which has previously been shown to stimulate renal 1α -hydroxylase and thus $1,25(\text{OH})_2\text{D}_3$ formation [52]. The 1α -hydroxylase is further stimulated by PTH [53]. The upregulation of $1,25(\text{OH})_2\text{D}_3$ during dietary phosphate depletion presumably contributed to the decrease of urinary phosphate output.

The decrease of renal tubular phosphate reabsorption may well have resulted from increased release of FGF23, which is known to decrease renal tubular phosphate transport and plasma phosphate concentration [54]. FGF23 formation is stimulated by $1,25(\text{OH})_2\text{D}_3$ and FGF23 in turn decreases the formation of $1,25(\text{OH})_2\text{D}_3$ [55, 56]. FGF23 formation is further stimulated by PTH, increased phosphate intake and hyperphosphatemia [57-59]. Accordingly, phosphate deficient diet decreased serum FGF23 levels. However, the enhanced FGF23 serum level of *spak^{tg/tg}* mice cannot be explained by PTH or hypophosphatemia. In theory, SPAK may influence FGF23 release from osteoblasts indirectly, by stimulating the NaCl cotransporter NCC. Along those lines, hydrochlorothiazide indeed increased FGF23 release, an effect paralleled by phosphaturia and hypophosphatemia. Thus, inhibition of NCC mimics the phenotype of *spak^{tg/tg}* mice. Along those lines, NCC activity plays a pivotal role for bone differentiation and bone mineralisation [27]. The present observations do, however, not allow discriminating, whether the effect of hydrochlorothiazide on FGF23 plasma concentration was secondary to inhibition of NCC in kidney or in bone.

The phosphaturia of *spak^{tg/tg}* mice is paralleled by anticalciuria, which may again be an indirect effect of impaired NaCl cotransporter activity. In view of the present observations it is tempting to speculate that the phosphaturia is the result of the following sequence of events: Lack of renal tubular SPAK activity decreases the activity of the NaCl cotransporter in early distal tubule. The volume depletion triggers a compensatory increase of NaCl reabsorption in proximal renal tubules and Henle's Loop. The enhanced proximal tubular and loop Na^+ reabsorption is paralleled by a similar increase of Ca^{2+} reabsorption resulting in anticalciuria. The renal Ca^{2+} retention enhances bone mineralization, which triggers FGF23 release. The enhanced FGF23 levels cause phosphaturia. Along those lines, inhibition of the NaCl cotransporter with hydrochlorothiazide similarly leads to anticalciuria, an effect again at least partially explained by compensatory increase of proximal tubular Na^+ reabsorption with parallel increase of renal tubular Ca^{2+} reabsorption [26]. The rather constant plasma concentration of Ca^{2+} does not support the assumption that Ca^{2+} retention drives enhanced bone mineralisation in *spak^{tg/tg}* mice. It should be kept in mind, though, that it is the free ionized Ca^{2+} rather than total Ca^{2+} , which is relevant for bone mineralisation. The hypophosphatemia decreases complexation of Ca^{2+} by phosphate and the free Ca^{2+} concentration may, at least in theory, be indeed (slightly) higher in *spak^{tg/tg}* mice and *spak^{wt/wt}* mice.

Conclusion

WNK/SPAK signaling participates in the regulation of FGF23 release as well as renal tubular Ca^{2+} and phosphate transport. WNK resistance of SPAK activity leads to increased FGF23 release, anticalciuria, phosphaturia, hypophosphatemia and enhanced bone density. The present observations thus disclose a novel, powerful mechanism contributing to the regulation of mineral metabolism and a novel functional role of WNK/SPAK signaling.

Conflict of Interests

The authors declare that they have no competing financial interests to disclose.

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