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Abstract

Insulin and growth factors activate the phosphatidylinositol-3-kinase pathway, leading to stimulation of several kinases including serum- and glucocorticoid-inducible kinase isoform SGK3, a transport regulating kinase. Here, we explored the contribution of SGK3 to the regulation of renal tubular phosphate transport. Coexpression of SGK3 and sodium-phosphate cotransporter IIa significantly enhanced the phosphate-induced current in Xenopus oocytes. In sgk3 knockout and wild-type mice on a standard diet, fluid intake, glomerular filtration and urine flow rates, and urinary calcium ion excretion were similar. However, fractional urinary phosphate excretion was slightly but significantly larger in the knockout than in wild-type mice. Plasma calcium ion, phosphate concentration, and plasma parathyroid hormone levels were not significantly different between the two genotypes, but plasma calcitriol and fibroblast growth factor 23 concentrations were significantly lower in the knockout than in wild-type mice. Moreover, bone density was significantly lower in the knockouts than in wild-type mice. Histological analysis of the femur did not show any differences in cortical bone but there was slightly less prominent trabecular bone in sgk3 knockout mice. Thus, SGK3 has a subtle but significant role in the regulation of renal tubular phosphate transport and bone density. Kidney International advance online publication, 30 March 2011; doi:10.1038/ki.2011.67.
Phosphaturia in gene-targeted mice lacking functional SGK3s

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Running head: SGK3-sensitive phosphate excretion
Abstract

Insulin and growth factors activate the phosphatidylinositol (PI)-3-kinase-pathway, leading to stimulation of several kinases including the ubiquitously expressed serum- and glucocorticoid-inducible kinase isoform SGK3. This kinase has previously been shown to stimulate a number of transport systems. The present study aimed to define the contribution of SGK3 to the regulation of renal tubular phosphate transport. Coexpression of SGK3 indeed significantly enhanced the phosphate-induced current and protein expression in NaPiIIa-expressing *Xenopus* oocytes. To determine the *in vivo* significance of SGK3-dependent regulation of phosphate transport, SGK3 knockout mice (*sgk3KO*) and their wild type littermates (*sgk3WT*) were analyzed. Under standard diet, fluid intake, urinary flow rate, glomerular filtration rate, and urinary calcium excretion were similar in *sgk3KO* mice and *sgk3WT* mice. The fractional urinary phosphate excretion was, however, significantly larger in *sgk3KO* than in *sgk3WT* mice. Plasma calcium and phosphate concentration were not significantly different between *sgk3KO* mice and *sgk3WT* mice. Plasma concentration of PTH was similar in both genotypes, but plasma 1,25(OH)₂D₃ and FGF23 concentrations were significantly lower in *sgk3KO* than in *sgk3WT* mice. Moreover, bone density was slightly, but significantly lower in *sgk3KO* than in *sgk3WT* mice. Histological analysis of the femur bone did not show any differences in cortical bone but showed slightly less prominent trabecular bone in *sgk3KO* mice. The observations disclose a subtle but significant role of SGK3 in the regulation of renal tubular phosphate transport and bone density.

**Key words:** Phosphate, Calcium, mineralization, PTH, 1,25(OH)₂D₃, FGF23, insulin
**Introduction**

Phosphate is a critically important component of bone minerals [Berndt and Kumar 2007; Liu and Quarles 2007]. Accordingly, adequate mineralization of bone depends on the precise tuning of phosphate balance, which is a function of intestinal absorption and renal excretion [Berndt and Kumar 2007; Murer et al., 2000; Takeda et al., 2004]. The latter depends on cellular uptake of phosphate across the apical membrane of proximal tubular cells [Forster et al., 2006], which is mainly accomplished by the Na⁺-coupled phosphate transporter NaPiIIa [Forster et al., 2006; Murer et al., 2000]. The carrier is downregulated by parathyroid hormone PTH [Murer et al., 2000], a hormone at least in part effective through cAMP. In contrast, renal tubular phosphate reabsorption is stimulated by insulin [Allon 1992; DeFronzo et al., 1976] and by insulin-like growth factor IGF1 [Feld and Hirschberg 1996; Jehle et al., 1998]. Little is known, however, about the signaling involved in the insulin- and IGF1-mediated regulation of phosphate excretion.

The signaling of insulin involves the serum- and glucocorticoid-inducible kinase SGK3 [Kobayashi et al., 1999], which, similar to protein kinase B [Alessi et al., 1996] and SGK1 [Kobayashi and Cohen 1999], is activated by phosphatidylinositol (PI)-3-kinase and phosphoinositide-dependent kinase PDK1. SGK3 is known to regulate a wide variety of transport systems [Lang et al., 2006].

The present study thus explored the possibility that SGK3 may be involved in the regulation of renal tubular transport. To this end, the in vitro regulation of NaPiIIa by SGK3 was studied in the heterologous *Xenopus* oocyte expression system and in vivo significance of SGK3-sensitive phosphate transport elucidated by analyzing gene-targeted mice lacking functional SGK3 (sgk3<sup>KO</sup>) as well as their wild type littermates (sgk3<sup>WT</sup>) [McManus et al., 2005].
Results

A first series of experiments analysed the in vitro influence of the serum- and glucocorticoid-inducible kinase isoform SGK3 on NaPiIIa, the major renal tubular phosphate transporter. Exposure of noninjected Xenopus oocytes to phosphate (3 mM) in the bath solution did not induce a significant current, indicating that these oocytes do not express significant endogenous electrogenic phosphate transport (Fig. 1A). In oocytes injected with cRNA encoding NaPiIIa, however, the addition of phosphate (3 mM) induced an inward current (Ip), indicating that SGK3 was indeed effective by stimulating NaPiIIa. The membrane abundance of NaPiIIa in oocytes was determined by a chemiluminiscence-based assay to further confirm the effect of SGK3 on NaPiIIa expression. As shown in Fig 1B, coexpression of SGK3 significantly increased the surface expression of NaPiIIa by a similar factor as it stimulated phosphate induced currents.

Experiments in brush border membrane vesicle preparations from sgk3KO and sgk3WT were performed to determine the sodium-dependent transport of phosphate ex-vivo. However, no significant difference in total and phosphonoformic acid (PFA, 6 mM) resistant transport was observed between sgk3KO and sgk3WT (Fig. 2). Similarly, the protein expression of the phosphate transporters NaPiIIa, NaPiIIC, and Pit2 in brush border membrane fractions was not different between the genotypes (Fig. 2). Also mRNA levels of these transporters was unaltered in sgk3KO mice (data not shown).

A further series of experiments explored whether SGK3 participates in the regulation of renal phosphate excretion in vivo. To this end, metabolic cage experiments were performed in sgk3KO and sgk3WT mice. As shown in Table 1, the body weight was similar in sgk3KO mice and sgk3WT mice. Fluid intake tended to be slightly higher in sgk3KO than in sgk3WT mice, a difference,
however, not reaching statistical significance (Table 1). Food intake was slightly but significantly higher in \( sgk3^{KO} \) than in \( sgk3^{WT} \) mice (Table 1).

No significant differences between the genotypes were observed in the plasma \( Ca^{2+} \) and phosphate concentration (Table 1, Fig. 3). Similarly, the plasma glucose concentration was not significantly different between genotypes (data not shown).

The urinary flow rate and creatinine clearance each tended to be slightly higher in \( sgk3^{KO} \) mice than in \( sgk3^{WT} \) mice, a difference, however, not reaching statistical significance (Table 1). The absolute (Table 1) and fractional (Fig. 3) excretion of \( Ca^{2+} \) were not significantly different between the two genotypes. In contrast, the absolute (Table 1) and fractional (Fig. 3) excretion of phosphate were significantly higher in \( sgk3^{KO} \) mice than in \( sgk3^{WT} \) mice. In neither the \( sgk3^{WT} \) nor the \( sgk3^{KO} \) mice glucosuria was observed (data not shown).

The renal phosphate excretion was further studied under a low-phosphate diet. The urinary phosphate excretion was still significantly higher in \( sgk3^{KO} \) (0.17 ± 0.03 µmol/24 h/g BW; \( n = 5 \)) when compared to \( sgk3^{WT} \) (0.10 ± 0.01 µmol/24h/g BW; \( n = 6 \)) mice. To further analyze the renal function of those mice, acute inulin clearance studies were performed under anaesthesia. The GFR as determined from the inulin clearance was not significantly different between \( sgk3^{WT} \) (119 ± 28 µl/min; \( n = 5 \)) and \( sgk3^{KO} \) (92 ± 22 µl/min; \( n = 5 \)) mice. In this series, the fractional excretion of phosphate was again higher in \( sgk3^{KO} \) (2.4 ± 0.3%; \( n = 5 \)) than in \( sgk3^{WT} \) (1.7 ± 0.1%; \( n = 5 \)) mice.

The phosphaturia could have been secondary to altered levels of hormones involved in phosphate homeostasis. Therefore, the plasma levels of parathyroid hormone (PTH), calcitriol (1,25(OH)\(_2\)D\(_3\)) and FGF 23 were determined. As illustrated in Fig. 4, the PTH plasma concentration was not significantly different between the genotypes, while the plasma 1,25(OH)\(_2\)D\(_3\) and plasma FGF23 concentrations were significantly lower in \( sgk3^{KO} \) mice compared to \( sgk3^{WT} \) mice. Lower FGF23 levels are consistent with a compensatory change in response to renal phosphate losses.

The renal loss of phosphate is expected to foster demineralization of bone. Thus, the bone density was determined in \( sgk3^{KO} \) and \( sgk3^{WT} \) mice. As shown in Fig. 5A, the bone density was
indeed lower in $sgk3^{KO}$ than in $sgk3^{WT}$ mice. A histological analysis of the femur showed no obvious differences in the cortical bone between the two genotypes but revealed that the trabecular bone appeared to be less prominent in $sgk3^{KO}$ mice than in $sgk3^{WT}$ mice (Fig. 5B).
Discussion

The present observations disclose a novel function of SGK3, i.e. the stimulation of renal tubular phosphate transport. According to the experiments on *Xenopus* oocytes, coexpression of SGK3 leads to a marked increase in NaPiIIa activity. The *in vivo* relevance of SGK3-sensitive NaPiIIa regulation is underscored by the phosphaturia of *sgk3*KO mice.

The phosphaturia of *sgk3*KO mice was not due to an increased plasma phosphate concentration and occurs without significant alterations of the PTH plasma concentration. The hormone is well known to downregulate renal phosphate transport [Murer et al., 2000], and its release is stimulated by an enhanced plasma phosphate concentration [Martin et al., 2005]. The plasma levels of 1,25(OH)\(_2\)D\(_3\) were significantly decreased in *sgk3*KO mice. The rate-limiting enzyme in the generation of 1,25(OH)\(_2\)D\(_3\) is the renal 1α-hydroxylase [Kato 1999; Portale and Miller 2000], which is stimulated by PTH [Portale and Miller 2000] and cellular phosphate depletion [Perwad et al., 2005]. A decreased NaPiIIa activity is expected to decrease the entry of phosphate into proximal tubule cells and thus to rather decrease the phosphate concentration in proximal tubular cells. A decreased cellular phosphate concentration is in turn expected to increase the 1,25(OH)\(_2\)D\(_3\) formation. Possibly, SGK3 participates in the regulation of 1,25(OH)\(_2\)D\(_3\) formation, which has previously been shown to be stimulated by IGF1 [Menaa et al., 1995]. As 1,25(OH)\(_2\)D\(_3\) stimulates renal tubular phosphate transport [Kurnik et al., 1987], decreased 1,25(OH)\(_2\)D\(_3\) formation could contribute to the phosphaturia of *sgk3*KO mice. Moreover, 1,25(OH)\(_2\)D\(_3\) is a powerful stimulator of intestinal phosphate transport [Brown et al., 2002]. It is, however, not required for the adaptive increase in intestinal phosphate absorption in dietary phosphate depletion [Capuano et al., 2005]. FGF23 inhibits 1α-hydroxylase expression but FGF23 levels were decreased and therefore cannot account for lower 1,25(OH)\(_2\)D\(_3\) concentrations.
We did not observe a significant decrease of the plasma phosphate concentration despite the phosphaturia. Enhanced phosphate excretion must thus be paralleled by enhanced intestinal phosphate absorption or phosphate loss from bone. In an earlier study, faecal phosphate excretion was not significantly different between SGK3\(^{KO}\) and wild type mice [Sandu et al., 2005]. However, the food intake is significantly higher in \(sgk3^{KO}\) mice than in \(sgk3^{WT}\) mice and thus, intestinal phosphate absorption might indeed be increased.

The phosphaturia of \(sgk3^{KO}\) mice was not paralleled by a decrease of phosphate transport across brush border membrane vesicles or of mRNA and protein abundance of the phosphate transporters. The lack of significant differences between tissue of \(sgk3^{WT}\) mice and \(sgk3^{KO}\) mice is in seeming contrast to the powerful effect of SGK3 on NaPiIIa expression in \(Xenopus\) oocytes. It must be kept in mind, though, that in vivo the effect of SGK3 is paralleled by a similar effect of PKB/Akt [Kempe et al., 2010] and thus, knockout of one of the kinases in mice does not abrogate SGK3/PKB/AKT-dependent regulation of NaPiIIa and is thus not expected to yield similarly strong effects as overexpression of SGK3 in \(Xenopus\) oocytes. The phosphaturia of \(sgk3^{KO}\) mice confirms that SGK3 indeed participates in renal tubular phosphate reabsorption. It remains elusive, though, why the phosphaturia is not reflected by respective alterations of carrier protein abundance and phosphate uptake in brush border vesicles. Possibly, SGK3 does not only affect NaPiIIa abundance in a heterologous expression system such as oocytes, but may also stimulate the activity of phosphate transporter in vivo without altering their cell surface expression.

The \(sgk3^{KO}\) mice suffer from a subtle but significant decrease of bone mass, which may again be partially due to decreased formation of 1,25(OH)\(_2\)D\(_3\). 1,25(OH)\(_2\)D\(_3\) is known to counteract apoptosis of osteoblasts [Morales et al., 2004] and thus enhances bone mineralization [van Driel et al., 2004]. The effect of 1,25(OH)\(_2\)D\(_3\) in osteoblasts is mediated by the phosphoinositide-3-kinase pathway [Zhang and Zanello 2008], and may thus involve at least partially SGK3. The demineralization of bone may further be due to phosphate depletion, as
phosphate inhibits the formation of new osteoclasts and stimulates apoptosis of mature osteoclasts [Kanatani et al., 2003]. The present observations do not rule out the participation of further mechanisms. For instance, SGK3 shares several functions with Akt2/PKBβ [Lang and Cohen 2001], which has been shown to confer survival of osteoblasts [Chaudhary and Hruska 2001] and osteoclasts [Kwak et al., 2008; Lee et al., 2001].

In conclusion, the present observations reveal a novel function of SGK3, i.e. its involvement in the regulation of 1,25(OH)2D₃ plasma concentration, renal phosphate excretion, and mineralization of bone.
Methods

In vitro experiments

For generation of cRNA, constructs were used encoding wild type human NaPiIIa [Busch et al., 1995] and human SGK3 [Kobayashi et al., 1999]. The cRNA was generated as described previously [Henrion et al., 2009]. SGK3 cDNA was kindly provided by Sir Philip Cohen, the cDNA encoding NaPiIIa by Heini Murer, University of Zurich. For electrophysiology, _Xenopus_ oocytes were prepared as previously described [Boehmer et al., 2008a; Boehmer et al., 2008b]. Wild type SGK3 (7.5 ng) and 15 ng of NaPiIIa cRNA were injected (one day) after preparation of _Xenopus_ oocytes. All experiments were performed at room temperature 4-5 days after injection. Two electrode voltage-clamp recordings were performed at a holding potential of -50 mV [Laufer et al., 2009]. The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A converter and Chart V.4.2 software for data acquisition and analysis (Axon Instruments) [Gehring et al., 2009]. The control solution (superfusate / ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES, pH 7.4. Phosphate (3 mM) was added to induce NaPiIIa-dependent currents. The flow rate of the superfusion was 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s. All experiments were repeated with at least 3 batches of oocytes; in all repetitions qualitatively similar data were obtained.

Detection of NaPiIIa cell surface expression by chemiluminescence

Defolliculated oocytes were incubated with primary rabbit anti-NaPiIIa antibody (diluted 1:500, Lifespan Biosciences, Seattle, WA, USA) and secondary, peroxidase-conjugated goat anti-rabbit antibody (diluted 1:1000, Cell Signaling, Danvers, MA, USA). Individual oocytes were placed in 96 well plates with 10 µl of Super Signal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA). Chemiluminescence of single oocytes was quantified in a luminometer (WalterWallac2 plate reader, Perkin Elmer, Jügesheim, Germany) by integrating the signal over a
period of 1s. Results display normalized arbitrary light units which are proportional to the detector voltage.

**In vivo function**

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities.

Generation and basic properties of the SGK3 knockout mice (sgk3KO) were described previously [McCormick et al., 2004]. The mice were compared to their wild type littermates (sgk3WT) and genotyped by PCR on tail DNA using SGK3- and neo-R-specific primers as previously described [McCormick et al., 2004].

The mice (age 3.5-6 months) were fed a control diet (C1314, Altromin, Lage, Germany) or 4 days low phosphate diet (C 1048 Altromin, Germany) with free access to tap drinking water.

For evaluation of renal excretion, both sgk3KO and sgk3WT mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany) for 24 hour urine collection as described previously [Vallon 2003]. They were allowed a 3 day habituation period during which food and water intake, urinary flow rate, urinary excretion of salt, fecal excretion and body weight were recorded every day to ascertain that the mice were adapted to the new environment. Subsequently, 24 h collection of urine was performed for several consecutive days in order to obtain the urinary parameters. To assure quantitative urine collection, metabolic cages were siliconized, and urine was collected under water-saturated oil.

To obtain blood specimens, animals were lightly anesthetized with diethylether (Roth, Karlsruhe, Germany), and about 150 μl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus.

Urinary phosphate concentration was determined colorimetrically utilizing a commercial diagnostic kit (Roche Diagnostics, Mannheim, Germany). Plasma and urinary calcium and plasma phosphate concentration were determined by a photometric method according to the
manufacturer’s instructions (dri-chem clinical chemistry analyzer FUJI FDC 3500i, Sysmex, Norsted, Germany). Creatinine concentration in urine was determined using the Jaffe reaction (Sigma, St. Louis, USA), creatinine concentration in serum was measured using an enzymatic kit (creatinine PAP, Lehmann, Berlin, Germany) according to the manufacturer’s instructions. Plasma parathyroid hormone concentration was measured using an ELISA kit (Immunotopics, San Clemente, USA). A radioimmunoassay kit was employed to determine the concentration of 1,25(OH)2-vitamin D3 (IDS, Boldon, UK) in plasma. Plasma FGF23 was determined using a ELISA kit (Immunotopics, San Clemente, USA).

**Clearance experiments**

Clearance experiments were performed in anaesthetized SGK3 wild type and knockout mice (n = 5 in each group) as described previously [Huang et al., 2004; Huang et al., 2006]. Briefly, mice were anesthetized intraperitoneally with 80 mg/kg sodium pentobarbital. Body temperature was maintained at 37.5°C by placing the animals on an operating table with a servo-controlled heating plate. The trachea was cannulated for free air breathing throughout the experiment. The left femoral artery was cannulated for arterial blood pressure recording and blood sample withdrawal. The right jugular vein was cannulated for continuous maintenance infusion of 0.85% NaCl at a rate of 8µl/min. For assessment of GFR, [3H]-inulin was added into 0.85% NaCl solution in order to deliver 20µCi/h radioactive inulin. Urine samples were collected via a catheter inserted into the urinary bladder. After surgical preparation, mice were allowed to stabilize for 20 min. Subsequently, to determine renal function, a 60-min urine collection period was performed. Blood samples (80 µl) were collected immediately before and after urine collections. Blood pressure was recorded before every blood sample collection. Urinary flow rate was determined gravimetrically. Concentrations of [3H]-inulin in plasma and urine were measured by liquid-phase scintillation counting. The glomerular filtration rate was calculated according to standard formulas.
Brush border membrane preparation and phosphate transport assays

BBMV were prepared from rat kidney cortex and outer medulla using the Mg\textsuperscript{2+} precipitation technique as described previously [Biber et al., 2007]. The phosphate transport rate into BBMVs 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 Pi was made with 0.125 mM K\textsubscript{2}HPO\textsubscript{4} and \textsuperscript{32}P (1 μCi/ml) to yield a final concentration 0.1 mM close to the expected apparent KmPi for Na\textsuperscript{+}-dependent transport in renal BBMVs. The stop solution contained 100 mM mannitol, 5 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM Pi. Na\textsuperscript{+} dependence was established by incubating BBMVs 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. In order to distinguish between Na\textsuperscript{+}-dependent Pi uptake mediated by SLC34 family members (e.g. NaPiIIa and NaPiIIc) and other Na\textsuperscript{+}-dependent phosphate transporters such as SLC20 family members (e.g. Pit1 and Pit2) trisodium phosphonoformic acid (PFA, final concentration 6 mM) added to the same solution with 107 mM NaCl was used. PFA has previously been shown to have a higher selectivity for SLC34 than SLC20 phosphate transporters at this concentration [Villa-Bellosta et al., 2009] The total protein concentration was measured using the Bio–Rad Protein Assay kit, Bio–Rad, Hercules, CA, USA. BBMVs were stored at −80°C until further use.

Western blotting

After measurement of the protein concentration (Bio-Rad, Hercules, CA), 10 μg of renal brush border membrane proteins were solubilized in loading buffer containing DTT and separated on 8% polyacrylamide gels. For immunoblotting, the proteins were transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore,
Bedford, MA, USA). After blocking with 5% milk powder in Tris-buffered saline/0.1% Tween-20 for 60 min., the blots were incubated with the primary antibodies: rabbit polyclonal anti-NaPiIIa (1:6000) [Custer et al., 1994] rabbit polyclonal anti-NaPiIIc (1:10000) [Nowik et al., 2008], rabbit polyclonal anti-Pit-2 (1:3000), (kindly provided by Dr. V. Sorribas, University of Zaragosa, Spain) [Villa-Bellosa et al., 2009] and mouse monoclonal anti-β-actin antibody (42 kD; Sigma, St. Louis, MO; 1:5000) either for 2 h at room temperature or overnight at 4°C. Membranes were then incubated for 1 h at room temperature with secondary goat anti-rabbit or donkey anti-mouse antibodies 1:5000 linked to alkaline phosphatase (Promega, USA) or HRP (Amersham). The protein signal was detected with the appropriate substrates (Millipore) using the DIANA III-chemiluminescence detection system (Raytest, Straubenhardt, Germany). All images were analysed using the software Advanced Image Data Analyser AIDA, Raytest to calculate the protein of interest/β-actin ratio.

**Determination of bone density**

For the analysis of bone density, animals were sacrificed and hind legs were removed and fixed in formalin. The samples were scanned with a high resolution microCAT-II (Siemens, Germany) small animal computed tomography (CT) scanner using a field of view of 3.1 x 3.1 x 4.8 cm³. The X-ray tube parameters were set at 80 kVp and 400 µA. The images were acquired with 720 angular projections (exposure time 1200 ms per projection) over 360° and binned with a factor of two, yielding a spatial resolution of ~38 µm. The total scan time was 24 minutes. Reconstructed CT images were analyzed with the Inveon Research Workplace software (Siemens, Germany) by drawing a standard-sized area around the femur and applying a region growth routine to segment the trabecular bone structure. For all samples, the same upper and lower density thresholds were applied comparing the relative numbers of trabecular bone density.
**Bone histology**

For morphological bone analysis, femurs of $sgk3^{KO}$ and $sgk3^{WT}$ mice ($n = 5 - 6$ in each group) were fixed in 4.5% buffered formalin (Roti®-Histofix 4.5%, Roth, Karlsruhe, Germany) for at least 24 hours. Decalcification was performed in EDTA solution at room temperature for 2-3 days. The decalcified bones were embedded in paraffin and cut in 2-3 µm thick sections and stained with H&E.

**Statistical analysis**

Data are provided as arithmetic means ± SEM, $n$ represents the number of independent experiments. All data were tested for significance using ANOVA (Fig. 1) or paired or unpaired Student’s t-test. Only results with $p < 0.05$ were considered statistically significant.

**Disclosure**

All authors of this manuscript state that they have neither financial nor any other conflicts of interest to disclose.

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Acknowledgments
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Table 1: Analysis of blood and urine of \textit{sgk3}^{KO} and \textit{sgk3}^{WT} mice

Body weight, food and fluid intake, plasma concentration and renal excretion of Ca\textsuperscript{2+} and phosphate, urinary flow rate, creatinine clearance, and fractional renal excretion of Ca\textsuperscript{2+} and phosphate in SGK3 knockout mice (\textit{sgk3}^{KO}) and wild type mice (\textit{sgk3}^{WT}). Arithmetic means ± SEM (n = 10 – 19 mice); * indicates significant difference between genotypes (\(p<0.05\)).

<table>
<thead>
<tr>
<th></th>
<th>\textit{sgk3}^{WT}</th>
<th>\textit{sgk3}^{KO}</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>23.8 ± 0.6</td>
<td>23.6 ± 0.6</td>
</tr>
<tr>
<td>Food intake (mg/g BW)</td>
<td>187 ± 7</td>
<td>207 ± 6*</td>
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<tr>
<td>Fluid intake (mg/g BW)</td>
<td>275 ± 15</td>
<td>304 ± 16</td>
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<tr>
<td>([\text{Ca}^{2+}]_{\text{plasma}}) (mg/dl)</td>
<td>9.31 ± 0.08</td>
<td>9.33 ± 0.11</td>
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<tr>
<td>([\text{P}<em>i]</em>{\text{plasma}}) (mg/dl)</td>
<td>7.04 ± 0.21</td>
<td>7.18 ± 0.22</td>
</tr>
<tr>
<td>Urine Ca\textsuperscript{2+} (µmol/24h/g BW)</td>
<td>0.30 ± 0.02</td>
<td>0.31 ± 0.03</td>
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<tr>
<td>Urine P\textsubscript{i} (µmol/24h/g BW)</td>
<td>0.50 ± 0.12</td>
<td>0.97 ± 0.18*</td>
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<tr>
<td>Urinary flow rate (µl/24h/g BW)</td>
<td>48.5 ± 6.3</td>
<td>61.1 ± 5.7</td>
</tr>
<tr>
<td>Creatinine clearance (µl/min/g BW)</td>
<td>4.8 ± 0.6</td>
<td>6.1 ± 0.9</td>
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<tr>
<td>Fractional renal Ca\textsuperscript{2+} excretion (%)</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Fractional renal P\textsubscript{i} excretion (%)</td>
<td>4.6 ± 0.9</td>
<td>7.6 ± 1.4*</td>
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**Figure Legends**

**Fig. 1: Coexpression of SGK3 stimulates electrogenic phosphate transport in NaPiIIa-expressing Xenopus oocytes**

A. Arithmetic means ± SEM (n = 11-18) of phosphate (3 mM)-induced currents (I_{Pi}) in *Xenopus* oocytes injected with water (H2O) or cRNA encoding SGK3 or NaPiIIa, or cRNA encoding both, SGK3 and NaPiIIa. * indicates significant difference from absence of cRNA encoding NaPiIIa. ### indicates significant difference from absence of cRNA encoding SGK3 (p<0.001).

B. Arithmetic means ± SEM (n = 42-60) of the normalized chemiluminescence intensity of NaPiIIa expression in *Xenopus* oocytes injected with water (left bar), with cRNA encoding NaPiIIa (middle bar), or with cRNA encoding both, NaPiIIa and SGK3 (right bar). * indicates statistically significant difference from absence of NaPiIIa cRNA (p<0.05). ### indicates difference from absence of SGK3 cRNA (p<0.001).

**Fig.2: Protein abundance of renal sodium-dependent phosphate cotransporters and sodium-dependent phosphate transport activity in brush border membrane vesicles (BBMV) of sgk3^KO and sgk3^WT mice.**

A. Original western blots for NaPiIIa, NaPiIIc, and Pit2. All membranes were stripped and reprobed for β-actin to control for loading.

B. Arithmetic means ± SEM (n = 6 each group) of relative band density of NaPiIIa (left panel), NaPiIIc (middle panel) and Pit2 (right panel).

C. Arithmetic means ± SEM (n = 5-6 each group) of the sodium-dependent transport rates into isolated BBMVs after 1 min in the absence (left bars) and presence (right bars) of 6 mM phosphonoformic acid (PFA) to block phosphate transport mediated by SLC34 family members.

**Fig.3: Fractional excretion of calcium and phosphate in sgk3^KO and sgk3^WT mice**
A. Arithmetic means ± SEM (n = 10-19 each group) of fractional urinary calcium (left panel) and phosphate (right panel) excretion in SGK3 knockout mice (sgk3KO, closed bars) and wild type mice (sgk3WT, open bars).* p<0.05 vs. respective value of sgk3WT mice.

B. Arithmetic means ± SEM (n = 10-19 each group) of plasma calcium (left panel) and phosphate (right panel) concentration in SGK3 knockout mice (sgk3KO, closed bars) and wild type mice (sgk3WT, open bars).

Fig. 4: Plasma PTH, FGF23 and 1,25(OH)2D3 concentrations in sgk3KO and sgk3WT mice

Arithmetic means ± SEM of plasma PTH (A) (n = 10 each group), 1,25(OH)2D3 (B) (n = 10-12 each group) and FGF23 (C) (n = 8 in each group) concentration in SGK3 knockout mice (sgk3KO, closed bars) and wild type mice (sgk3WT, open bars).* p<0.05 vs. respective value of sgk3WT mice.

Fig. 5: Bone density and histological analysis of femur bones from sgk3KO and sgk3WT mice

A. Arithmetic means ± SEM (n = 6 each group) of bone density in SGK3 knockout mice (sgk3KO, closed bars) and wild type mice (sgk3WT, open bars).* p<0.05 vs. respective value of sgk3WT mice.

B. Histology of bone in the SGK3 knockout mice (sgk3KO) and wild type mice (sgk3WT) representative for n = 5-6 each group. The cortical bone is similar but the trabecular bone is less prominent in SGK3 knockout (sgk3KO) than in wild type (sgk3WT) mice.
Figure 1

A. Normalised chemiluminescence signal (RLU) for H₂O, SGK3, NaPiIIa, and NaPiIIa.

B. Normalised chemiluminescence signal (RLU) for H₂O, NaPiIIa, and SGK3 NaPiIIa.
**Figure 2**

A. 

- **sgk3**
  - sgk3^{+++}
  - sgk3^{-/-}

- NaPi-IIa
- NaPi-IIc
- Pit-2
- β-actin

B.

- NaPi-IIa/β-actin
- NaPi-IIc/β-actin
- Pit-2/β-actin

C.

- Total Na^{+}-dependent Pi-uptake
- PFA-resistant Na^{+}-dependent Pi-uptake

- sgk3^{WT}
- sgk3^{KO}
Figure 3

A

plasma $\text{Ca}^{2+}$ [mg/dl]

B

FE $\text{Ca}^{2+}$ [%]

$s\text{gk3}^{\text{WT}}$

$s\text{gk3}^{\text{KO}}$

plasma $\text{P}_i$ [mg/dl]

FE $\text{P}_i$ [%]

*
Figure 4

A. PTH [pg/ml]

B. 1,25(OH)₂D₃ [pmol/ml]

C. FGF23 [pg/ml]

- sgk³WT
- sgk³KO

* indicates statistical significance.
Figure 5A

Mean of bone density

sgk3<sup>WT</sup>  sgk3<sup>KO</sup>
Figure 5B

WT

KO

epiphyisis
Growth plate
metaphysis
diaphysis

25x

100x

25x

100x