Loss of WNK3 is compensated for by the WNK1/SPAK axis in the kidney of the mouse

Katharina Mederle,1 Kerim Mutig,2 Alexander Paliege,2 Isabel Carota,1 Sebastian Bachmann,2 Hayo Castrop,1 and Mona Oppermann3

1Institute of Physiology, University of Regensburg, Regensburg, Germany; 2Department of Anatomy, Charité, Berlin, Germany; and 3Children’s Hospital, University Medical Center, University of Regensburg, Regensburg, Germany

Submitted 21 May 2012; accepted in final form 18 February 2013


The WNK (with no lysine) kinase family includes several members of which WNK1, kidney-specific WNK1 (ks-WNK1), WNK3, and WNK4 are expressed in the kidney (27, 30, 32). The diversity of the WNK family is further expanded by the multiple splice isoforms of each WNK kinase. WNK-dependent phosphorylation of target proteins modulates the activity of several renal transporters and channels that are crucially involved in electrolyte reabsorption, including NKCC2, NCC, eNaC, and ROMK (9). In addition, there are kinase-independent effects of WNKs on renal ion transport (10, 13). The clinical relevance of WNK kinases with respect to renal salt reabsorption is demonstrated by rare hereditary diseases such as pseudohypoaldosteronism type II (PHAII) (4, 6, 10, 11, 30, 31). In individuals with PHAII, gain-of-function mutations in WNK1 or missense mutations in WNK4 result in inappropriately increased renal salt reabsorption and, consequently, in volume-dependent hypertension. For WNK3 no corresponding mutations have been described to date.

In vitro studies using the Xenopus oocyte expression system have indicated that WNK3 is a positive regulator of NKCC2, NCC, and eNaC (5, 21, 22, 24, 25, 33). Thus kinase-active WNK3 enhances the surface expression of NKCC2 and NCC in oocytes. For NKCC2, this stimulation of surface trafficking involves the phosphorylation of two threonine residues that are also involved in the vasopressin-dependent membrane trafficking and activation of the transporter (25). In addition, the activation of NKCC2 by intracellular Cl− depletion crucially depends on WNK3 activity and in WNK3’s downstream target SPAK (22). WNK3 therefore may function as a Cl− sensor in cells of the thick ascending limb (TAL) (20). In line with this hypothesis, transfection of Xenopus oocytes with a mutated WNK3 lacking the kinase function abolished the Cl−-dependent activation of NKCC2 (22). Like WNK2, also NCC, which is located downstream of NKCC2 in the distal convoluted tubule (DCT), is activated by kinase-active WNK3 (25, 33). In contrast to wild-type WNK3, a kinase-inactive WNK3 not only failed to trigger in vitro NCC surface trafficking but even markedly reduced the membrane localization of the cotransporter (25).

The evaluation of WNK3 function in vivo has been hampered by the lack of pharmacological tools to specifically inhibit this kinase. To investigate the renal function of WNK3 in vivo, we generated a WNK3-deficient mouse strain by gene targeting. Because NKCC2, NCC, and eNaC constitute the major tubular sodium transport pathways, we hypothesized that WNK3-dependent modulations of transporter activity would have a substantial impact on overall renal tubular function and, consequently, on salt and water homeostasis.

WNK3-deficient mice were viable and showed no gross abnormalities. Renal function and specifically tubular function in WNK3−/− mice were largely preserved. The limited impact of the loss of WNK3 on tubular function might be due to a striking compensatory upregulation of the WNK1/SPAK axis in WNK3-deficient mice.

In summary, the overall relevance of WNK3 for the renal reabsorption of NaCl appears to be limited and can be largely compensated for by the activation of WNK3-independent pathways, including WNK1-dependent pathways. Consequently, our data suggest that WNK3 may serve as a member of a kinetic network that facilitates the fine-tuning of renal transepithelial NaCl transport.
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METHODS

Generation of WNK3-deficient mice. WNK3-deficient mice were generated according to standard gene trap protocols. A mouse embryonic stem cell clone (strain 129SvEv) that had inserted in the WNK3 gene was expanded and injected into recipient blastocysts harvested from C57BL/6 mice. Offspring chimeric mice were bred with C57BL/6 wild types to establish germline transmission. All steps involved in the generation of the WNK3−/− line were performed at Lexicon Pharmaceuticals. Unless otherwise stated, all experiments were done in 6- to 8-wk-old male WNK3−/− and wild-type littermates. Due to the location of the WNK3 gene on the X-chromosome, heterozygous females and wild-type males were used for breeding, resulting on average in 50% wild-type and 50% WNK3−/− male littermates. Animal care and experimentation were approved by the local government (Regierung der Oberpfalz, 54 –2532.1–11/12) and carried out in accordance with the National Institutes of Health principles as outlined in their Guide for the Care and Use of Laboratory Animals.

Genotyping was performed using primers for the wild-type allele (flanking the gene trap vector insertion site), 5′-AGCCCAAACCATCAATTCA-3′ (sense) and 5′-GGTAACATGATTGTGC-3′ (antisense). Due to the insertion of 5.1 kb-gene trap cassette no amplicon was obtained under standard conditions in the mutated allele. For the mutated allele the same sense primer was combined with an antisense primer located in the gene trap cassette (5′-ATAAACCTTTGCAGTGAC-3′). Thirty-eight cycles of PCR amplification were done according to the following protocol: annealing for 45 s at 60°C, extension for 45 s at 72°C, and denaturation for 30 s at 94°C.

RT-PCR for evaluation of WNK3 transcripts. In the case of a successful targeting of WNK3 in the WNK3−/− strain, the expression of transcript sequences located downstream of the gene trap vector insertion site should be absent. To measure possible run-through transcript or splicing events of the targeting vector, RNA was isolated from brain and kidney tissue and was used for qPCR after reverse transcription. The following primers were used to amplify transcript regions located downstream of the vector integration site: exons 5, 5′-GTGAAAGAAATAATTGAGG-3′ (sense); exons 6 5′-TAGCAAGGAAGATGATGTC-3′ (antisense); exon 17, 5′-ATCATTTCAGCGGGGTCCTG-3′ (sense); exon 18a, 5′-CATCAGACCAAAACACAGTC-3′ (antisense); exon 18b, 5′-CCACTCCGTGAAGGACAAGC-3′ (antisense); and exon 22, 5′-GATGGTCTGAAGGAGGGGAGA-3′ (antisense). After reverse transcription, real-time PCR was performed using the light cycler system (Roche, Mannheim, Germany) and Sybr Green for detection of dsDNA. Dilution series of cDNA standards indicated a detection limit at 0.01% of the wild-type cDNA.

Assessment of urine volume, osmolarity, and concentrating ability. To determine 24-h urine volume, urine was collected in metabolic cages after animals were accustomed to the metabolic cages for 3 days. Urine osmolarities under ambient conditions and after 48 h of water restriction were determined in spot urine samples. Urine osmolarities under ambient conditions and after 48 h of water restriction were determined in spot urine samples. Urine osmolarity was determined by the freezing-point depression method.

Fig. 1. Gene trap targeting strategy of WNK3. A: schematic representation of the insertion site of the gene trap targeting vector into intron 5 of the WNK3 gene on chromosome X. B: RT-PCR analysis of WNK3 transcripts in the kidney and thymus of wild-type and WNK3−/− mice.

Fig. 2. Renal blood flow (RBF) and renal vascular resistance. A: RBF was measured by Doppler flowmetry in anesthetized wild-type (WT) and WNK3−/− (WNK3-KO) mice (n = 5 each). B: renal vascular resistance calculated from RBF and mean arterial pressure in wild-type and WNK3−/− mice (n = 5 each).
Renal blood flow measurement. Mice were anesthetized with 100 mg/kg thiobutabarbital (inactin) intraperitoneally and 100 mg/kg ketamine subcutaneously. Body temperature was maintained at 38.0°C by placing the animals on an operating table with a servo-controlled heating plate. The trachea was cannulated, and a stream of 100% oxygen was blown toward the tracheal tube throughout the experiment. The left femoral artery was catheterized with hand-drawn polyethylene tubing for continuous measurement of arterial blood pressure. A catheter was also inserted into the right jugular vein for an intravenous maintenance infusion of saline at a rate of 12 ml/h/kg body wt. The bladder was catheterized for urine collections. For measurements of total renal blood flow (RBF) the right renal artery was approached from a flank incision and carefully dissected free to permit placement of an ultrasonic flow probe (0.5PSB nanoprobe) connected to a TS402 perivascular flowmeter module (Transonic Systems, Ithaca, NY). RBF signals were digitized and analyzed using PowerLab software (ADInstruments, Colorado Springs, CO). Mean arterial blood pressure was divided by RBF to obtain renal vascular resistance (RVR).

Measurement of blood and plasma volume. Blood and plasma volume were determined by the Evans blue dilution method, as described previously (18).

Determination of glomerular filtration rate. Glomerular filtration rate (GFR) of conscious mice was measured by FITC-labeled inulin clearance after a single retroorbital injection and consecutive blood sampling from the tail vein (19).

Blood pressure measurement. Systolic blood pressures and heart rates of conscious mice were determined by tail-cuff manometry (Visitech Systems). Animals were conditioned by placing them into the holding device on 3 consecutive days before the first measurement. Blood pressure was determined for 3 days in a row, and values were calculated as averages of these three measurements for each individual mouse.

Determination of plasma aldosterone and renin concentration. For plasma aldosterone and renin concentrations measurements, blood was collected from conscious mice by puncture of the submandibular vessels with a 19-gauge needle. Approximately 60 μl of blood was collected into an EDTA-containing microhematocrit tube. Red blood cells and plasma were separated by centrifugation; the plasma was frozen until used for aldosterone or renin measurements. Using a 20-fold dilution of 12 μl of plasma, renin was measured by radioimmunoassay (DiaSorin, Stillwater, MN) as generation of ANG I following addition of excess rat substrate (plasma renin concentration, PRC), with final plasma dilutions varying between 1:500 and 1:1,000. ANG I generation was determined for a 3-h incubation period at 37°C and expressed as hourly average. In each assay, substrate without plasma was incubated for the same time, and any background ANG I formation was subtracted from the plasma-containing samples. In addition, background ANG I levels were determined in a plasma aliquot kept frozen without the addition of substrate until assaying.

Determination of WNK1 and WNK4 mRNA expression. Total RNA was isolated from kidneys of WNK3−/− and wild-type mice using Trizol reagent (Invitrogen). After reverse transcription, real-time PCR was performed for WNK1 and WNK4 using the light cycler system.

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Urine Osmolality (Baseline) $p = .86$

Urine Osmolality (48h Water Restriction) $p = .41$

(No text from the image)
The absolute GFRs were 315 by FITC-inulin clearance measurements in conscious mice, and 121.9 min/ml in wild-type mice (Fig. 2). Systolic blood pressure (mmHg) in WNK3 measured in conscious mice averaged 121.9 in wild-type mice and 119.3 in WNK3-deficient mice, a level that was not significantly different from the absence of wild-type transcripts in WNK3-deficient mice, a level that was not significantly different from wild-type mice. RBF was

\[ \text{RBF (ml/min)} = \frac{\text{Urea production (mmol/min) \times 1000}}{\text{weight in kg}} \]

Similarly, no difference was observed when the GFR was related to the animals’ body weight, resulting in a GFR of 1.389 ± 0.067 ml·min⁻¹·100 g⁻¹ in WNK3−/− and 1.362 ± 0.062 ml·min⁻¹·100 g⁻¹ in wild-type mice (P = 0.81), as shown in Fig. 4. These results demonstrate that renal vascular function under baseline conditions was not markedly affected by the loss of WNK3.

**Drinking behavior and urine excretion (metabolic cage studies).** To address the net impact of WNK3 on renal sodium reabsorption and urinary concentrating ability, in a first set of experiments ambient urine osmolarity was determined in spot urine samples from 40 WNK3−/− and 45 wild-type mice. The

<table>
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<th>Electrolytes</th>
<th>Wild type</th>
<th>WNK3−/−</th>
<th>P value</th>
<th>Wild type</th>
<th>WNK3−/−</th>
<th>P value</th>
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<td>Cl⁻, mmol/l</td>
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<td>2.30 ± 0.05 (n = 8)</td>
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<td>K⁺, mmol/24 h</td>
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Values are means ± SE.

**Statistics.** Data are expressed as means and SE. Statistical comparisons were made by Student’s t-test or by ANOVA with Bonferroni post hoc test when necessary.

**RESULTS**

**Generation of WNK3-deficient mice.** WNK3−/− mice were generated by transfection of mouse embryonic stem cells with a gene trap targeting vector. A shown in fig. 1A, the gene trap vector integrated into intron 4 of the WNK3 gene. Analysis of the WNK3 transcript by RT-PCR using primers located downstream of the insertion site in exons 5/6 and revealed the absence of wild-type transcripts in WNK3−/− mice (Fig. 1B); consequently, there were no abnormal splice events in the mutated WNK3-transcript that would remove the targeting vector. The DNA region downstream of the targeting vector insertion site encodes 1,346 of 1,710 (79%) of the WNK3 gene, including two subdomains of the kinase domain (8). WNK3 possesses alternate exons 18a, 18b, and 22 that are specific for brain WNK3 (isoforms 1 and 2) and kidney WNK3 (isoform 3) (8). All of these exons are located downstream of the vector insertion site and they were absent in the gene trap WNK3−/− strain as determined by qPCR using exon-specific primers located in exon 17 (sense primer) and exon 18a, 18b, and 22 (antisense primers), respectively. WNK3−/− mice were viable and showed no gross anatomic, behavioral, or fertility abnormalities. The mice were born at the Mendelian ratios for X-linked inheritance.

**Renal vascular function, RBF, and GFR.** To assess renal vascular and glomerular function, RBF, and the GFR were determined in WNK3−/− and wild-type mice. RBF was determined by Doppler flowmetry in the renal artery of anesthetized mice. RBF averaged 1.42 ± 0.04 ml/min in WNK3-deficient mice, a level that was not significantly different from that of wild-type controls (1.35 ± 0.09 ml/min, n = 5, P = 0.52). The mean arterial blood pressure during the experiment was similar in both genotypes (97 ± 5 and 91 ± 5 mmHg in WNK3−/− and wild-type mice, respectively; P = 0.43). Consequently, the renal vascular resistance averaged 68.3 ± 4 mmHg × min/ml in WNK3−/− mice, and 67.4 ± 5 mmHg × min/ml in wild-type mice (Fig. 2). Systolic blood pressure measured in conscious mice averaged 121.9 ± 1.3 mmHg (n = 8) in WNK3−/− and 119.3 ± 1.5 mmHg (n = 12) in WNK3+/+ mice (P = 0.24) (Fig. 3). The GFR, as determined by FITC-inulin clearance measurements in conscious mice, was virtually identical in WNK3-deficient mice and wild types. The absolute GFRs were 315 ± 13 and 343 ± 22 µl/min in WNK3−/− and +/+ mice, respectively (n = 7, P = 0.30).
ambient urine osmolarities averaged 1,819 ± 61 and 1,804 ± 62 mosmol/kg in WNK3−/− and wild-type mice, respectively (P = 0.86). To further address the urinary concentrating ability, mice were not allowed access to drinking water for 48 h. After 48 h of water restriction, the urine osmolarity increased to 3,200 ± 180 mosmol/kg in WNK3-deficient mice, similar to the increase observed in wild-type animals (3,440 ± 220 mosmol/kg, n = 11 each; P = 0.41) (Fig. 5A).

Similarly, when urine was collected from mice in metabolic cages over 24 h, the urine volume did not differ between genotypes (1.55 ± 0.08 and 1.63 ± 0.06 ml/24 h in WNK3−/− and +/+ mice, respectively, n = 16; P = 0.42). Consequently, the drinking volume was also similar between WNK3−/− and wild-type mice (4.41 ± 0.19 and 4.53 ± 0.16 ml/24 h in WNK3−/− and wild-type mice, respectively; P = 0.61) (Fig. 5B). As summarized in Table 1, the plasma electrolyte concentrations and renal 24-h electrolyte excretion were similar in both genotypes.

Consistent with these data on renal reabsorptive function, the plasma volume as determined by the Evans blue dilution method did not differ between WNK3−/− and wild-type mice (3.59 ± 0.13% of body wt; n = 13) and wild-type controls (3.56 ± 0.13% of body wt; n = 12; P = 0.88). The hematocrit of WNK3−/− and +/+ mice averaged 49.9 ± 0.6 and 49.5 ± 0.5%, resulting in a calculated blood volume of 7.3 ± 0.3 and 7.0 ± 0.2% of body wt, respectively (P = 0.55) (Fig. 6).

**Plasma renin and aldosterone concentrations.** The plasma renin concentration and the plasma aldosterone concentration were determined in WNK3−/− and WT mice. PRC averaged 80 ± 13 ng ANG I·ml⁻¹·h⁻¹ in WNK3−/− mice (n = 14), a value not significantly different from that for wild-type controls (94 ± 18 ng ANG I·ml⁻¹·h⁻¹, n = 13; P = 0.54). Furthermore, as summarized in Table 2, the plasma aldosterone concentration was similar in WNK3−/− and wild-type mice (312.9 ± 39.38 pg/ml, n = 8, and 223.9 ± 22.64 pg/ml, n = 9, respectively; P = 0.06).

**Assessment of the compensatory regulation of other WNK kinases.** Given the complex interplay among various WNK kinases in the kidney, we addressed changes in the expression levels of WNK kinases other than WNK3, because these other kinases may functionally compensate for the loss of WNK3 in the knockout mouse strain. The WNK4 mRNA expression levels in the kidney as determined by quantitative real-time RT-PCR were similar in WNK3−/− and wild-type mice, averaging 1.55 ± 0.10 and 1.25 ± 0.24 rU, respectively (P = 0.29, Fig. 7A). In contrast to WNK4 levels, the abundance of WNK1 mRNA was markedly upregulated in WNK3−/− mice compared with the levels in wild-type mice. The renal WNK1 mRNA levels were 1.25 ± 0.10 rU in WNK3-deficient mice and 0.69 ± 0.08 rU in wild-type controls (P = 0.003) (Fig. 7A). To distinguish between WNK1 isoforms, isoform-specific qRT-PCR was used for quantification of ks-WNK1 and L-WNK1. Expression levels of ks-WNK1 were similar in WNK3−/− compared with wild-type mice (1.07 ± 0.04 vs. 1.25 ± 0.08 rU, P = 0.13; n = 5) whereas L-WNK1 was significantly increased in WNK3−/− mice averaging 1.52 ± 0.31 compared with 0.65 ± 0.25 in wild-types (P = 0.01; n = 5) (Fig. 7B).

**Determination of pNKCC2, pNCC, eNaC, and pSPAK/OSR levels in WNK3-deficient mice.** In view of the upregulation of WNK1 in WNK3−/− mice we next determined the localization and abundance of the WNK1 downstream targets pSPAK/OSR1 and the effectors pNKCC2, pNCC, and eNaC. As shown in the immunostaining in Figs. 8 and 10, the expression levels of pSPAK/OSR1, pNKCC2, and pNCC under baseline conditions did not considerably differ between WNK3−/− and wild-type mice. To challenge renal salt conservation, mice

Table 2. Plasma renin concentration and plasma aldosterone concentration in wild-type and WNK3−/− mice

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<th>Wild Type</th>
<th>WNK3−/−</th>
<th>P Value</th>
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<tr>
<td>PRC, ng ANG I·ml⁻¹·h⁻¹</td>
<td>94.24 ± 18.55 (n = 13)</td>
<td>80.24 ± 13.36 (n = 14)</td>
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<tr>
<td>PAC, pg/ml</td>
<td>312.9 ± 39.38 (n = 8)</td>
<td>223.9 ± 22.64 (n = 9)</td>
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Values are means ± SE. PRC, plasma renin concentration; PAC, plasma aldosterone concentration.

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Fig. 7. WNK1 and WNK4 expression in the kidneys of wild-type and WNK3−/− mice. A: WNK1 and WNK4 mRNA expression levels in kidneys from wild-type and WNK3−/− mice (n = 5 each). Data are given as relative units normalized to β-actin expression. B: mRNA expression of ks-WNK1 and L-WNK1 in kidneys from wild-type and WNK3−/− mice (n = 5 each). Data are given as relative units normalized to β-actin expression.
were fed in the next experiment a salt-restricted diet for 7 days [0.02% NaCl (wt/wt)]. The renal abundances of pSPAK/OSR, pNKCC2, and pNCC were slightly enhanced during salt restriction in wild types (Figs. 8 and 10). In contrast to wild-type mice, the abundances of renal pSPAK, pNCC, and pNKCC2 seemed markedly enhanced in WNK3−/− mice during salt restriction (Figs. 8 and 10). Confocal evaluation of phospho-SPAK/OSR1 signal intensities confirmed the induction of pSPAK/OSR1 during a low-salt diet in both genotypes with levels in WNK3−/− mice exceeding those of wild types in both the cortical TAL (cTAL) (14.971 ± 1.606 vs. 11.141 ± 1.289 rU; \( P = 0.047 \)) and the DCT (32.200 ± 3.064 vs. 14.487 ± 1.619; \( P < 0.0001 \)). Relative values are shown in fig. 9. Furthermore, immunohistochemical analysis of pNKCC2 revealed traffick-
pNKCC2 protein abundance more reliably. pNKCC2 protein abundance was also increased in WNK3−/− compared with wild types (97 ± 2 vs. 72 ± 5 rU; P = 0.003, n = 4), as summarized in Fig. 11.

In view of the upregulation of the WNK1/pSPAK/pNKCC2/pNCC axis in WNK3-deficient mice during salt restriction, we next determined if WNK1-dependent transepithelial salt transport may functionally compensate for the loss of WNK3. Because the levels of surface expression of pNCC and pNKCC2 were enhanced in WNK3−/− relative to wild-type mice when fed a low-salt diet, we determined the relative contribution of NCC- and NKCC2-dependent Na+ reabsorption in WNK3−/− and wild-type mice. To inhibit NCC and NKCC2, mice on a low-salt diet received single intraperitoneal injections of hydrochlorothiazide (30 mg/kg) and furosemide (40 mg/kg), respectively, and diuresis was measured over the following 4 h. During the 4−h period after hydrochlorothiazide injection, urine volume of WNK3−/− mice (n = 7) exceeded that of wild-type animals (n = 8) (0.92 ± 0.04 vs. 0.64 ± 0.06 ml; P = 0.003) (Fig. 12). During the 4−h period after furosemide injection, urine volume in WNK3−/− tended to be increased compared with wild type mice, without reaching levels of significance (1.09 ± 0.08 vs. 0.89 ± 0.09 ml; P = 0.14). In contrast to the situation of a pretreatment with a salt-restricted diet, the diuretic effects of hydrochlorothiazide and furosemide were similar in WNK3−/− and wild types when mice were fed standard rodent chow. Following a single intraperitoneal injection of hydrochlorothiazide (30 mg/kg), the 4−h urine volume of mice fed standard chow did not differ between genotypes (0.45 ± 0.07 ml, n = 7, and 0.41 ± 0.04 ml, n = 8, in WNK3−/− and wild-type mice, respectively; P = 0.63). Similar results were obtained in experiments with furosemide (40 mg/kg). Diuresis over a 4−h interval after furosemide injection averaged 1.16 ± 0.12 ml in WNK3−/− mice (n = 7), a value not significantly different from that for wild-type controls (1.04 ± 0.09 ml, n = 8; P = 0.43) (Fig. 12).

Fig. 9. Confocal evaluation of phospho-SPAK/OSR1 abundance under baseline conditions (black bars) and following a low-salt diet for 7 days (open bars) in kidneys from wild-type and WNK3−/− mice. pSPAK/OSR1 intensities were determined in the cTAL (A) and the DCT (B) of the kidney. Intensities for wild-type mice under a normal salt diet were set as 100%.

**DISCUSSION**

In the present study we addressed the role of WNK3 in kidney function using a WNK3-deficient mouse line. Because WNK3 was shown to be a potent stimulator of NKCC2 and NCC function in vitro (22, 25, 33), we hypothesized that the loss of WNK3 would lead to a salt-losing phenotype. In view of the results of the in vitro experiments in oocytes addressing the role of WNK3 for NKCC2 and NCC function, our study particularly focused on the TAL and DCT. Contrary to our hypothesis, the renal function of WNK3-deficient mice was largely unaltered relative to that of wild-type controls.

Modulation of NKCC2 or NCC transport activity, e.g., by the use of diuretics, leads to marked natriuresis and diuresis and compromises renal concentrating ability. A stimulatory effect of WNK3 on renal NKCC2- and NCC-dependent NaCl transport, as suggested from heterologous expression studies in *Xenopus* oocytes, was therefore assessed in WNK3−/− mice. For this purpose, drinking volume, urinary volume, urinary electrolyte excretion, and urine osmolarity under ambient conditions and after water restriction were assessed. All parameters for WNK3−/− mice were indistinguishable from those for wild-type animals, suggesting that the loss of WNK3 does not result in major net changes in the tubular reabsorptive capacity. Consistent with the unaltered activity of the major renal salt reabsorbing transport systems, the plasma volume and plasma electrolyte composition were similar in WNK3−/− mice and wild-type controls. Furthermore, the plasma renin concentration, a sensitive readout of alterations in renal reabsorptive and vascular function, was normal in WNK3−/− mice (2). The inconspicuous tubular phenotype of WNK3-deficient mice was
accompanied by normal systemic blood pressure, renal vascular resistance, and GFR, suggesting that changes in hemodynamics in WNK3−/− mice do not account for the overall inconspicuous renal phenotype.

At first glance, there appear to be two possible explanations for this apparent lack of a renal phenotype in WNK3-deficient mice: 1) in vivo, WNK3 might play a limited overall role in modulating the activity of the salt reabsorbing transport systems, and/or 2) the loss of WNK3 was compensated for by the induction of alternative signaling pathways. In the first case, WNK3 kinase expression levels and/or its specific activity in the kidney would be too low to facilitate considerable effects on transepithelial salt transporter systems. This possibility would imply that the WNK3 expression levels obtained in heterologous expression systems, such as *Xenopus laevis* oocytes, may exceed those of the native renal tissue substantially. In the second case, the loss of WNK3 would change the set point of the regulatory kinase network that modulates tubular transporters and channels. The resulting deviation would then initiate compensatory changes in the abundance or activity of alternate regulatory pathways. In accordance with our results, an independently generated strain of WNK3-deficient mice showed no major renal phenotype (17).

In view of the complex regulatory network consisting of several WNK kinases, downstream kinases such as SPAK and OSR1, and kinase-independent effects of WNKs, we determined if WNK3 ablation was accompanied by compensatory changes in other members of the WNK family. In accordance with this hypothesis, the mRNA abundance of WNK1 was markedly upregulated in WNK3−/− mice compared with wild

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**Fig. 10. Immunostaining for pNCC (A–D) and pNKCC2 (E–H).** pNCC expression levels in wild-type (A) and WNK3−/− mice (C) receiving a standard diet. Following a low-salt diet for 7 days, pNCC expression levels were enhanced in WNK3−/− mice (D) compared with wild-type animals (B). Similarly, the renal expression of pNKCC2 appeared to be increased in WNK3−/− mice (H) compared with wild-type mice (F) under low-salt conditions. Baseline pNKCC2 expression levels were similar between wild-type (E) and WNK3−/− mice (G).
was accompanied by a higher abundance and greater membrane localization of pNCC and pNKCC2. These changes in transporter localization and abundance were apparently functionally relevant, as WNK3-deficient mice kept on a salt-restricted diet showed an increased sensitivity to the diuretics hydrochlorothiazide and furosemide compared with wild-type mice. In contrast, the diuretic effects of hydrochlorothiazide and furosemide were similar in WNK3+/- and wild-type mice fed standard rodent chow, suggesting that the upregulation of compensatory pathways in WNK3-deficient mice becomes obvious only during situations of increased renal reabsorptive demand. These data are in part in contrast to a recent report by Oi et al. (17). The authors did not detect differences in pNKCC2 and pNCC between WNK3+/+ and +/- mice during a salt-restricted diet. The reason for this discrepancy remains unclear but might be related to different experimental protocols in the two studies. Thus, conversely to the protocol used in the study by Oi et al., we induced the low-salt diet by a single injection of furosemide. The purpose of this procedure was to induce a salt and water deficit in the mouse that cannot be compensated for when the animal is fed a salt-restricted diet, resulting in an overall more severe salt deprivation. These more severe conditions might be the reason for more apparent changes in the expression levels of the phosphorylated transporters.

The ubiquitous WNK1 isoform L-WNK1 and the kidney-specific WNK1 isoform ks-WNK1 exert dual effects on major salt transporters and channels of the nephron including NKCC2, NCC, and NaCl (9, 12). L-WNK1 was shown to stimulate the activity of NKCC2 and NCC, and these effects are partially counteracted by ks-WNK1. ks-WNK1 was demonstrated to inhibit WNK3, which in turn phosphorylates NCC, resulting in an inhibitory component on NCC activity (7). Our data suggest, that the inhibitory function of ks-WNK1 is taken over by L-WNK1 due to specific upregulation of L-WNK1 during dietary salt restriction and, consequently, that the net effect of the compensatory upregulation of the WNK1 signaling pathway in WNK3-deficient mice during a low-salt diet is stimulatory. This hypothesis would be in line with findings in a strain of global heterozygous WNK1-deficient mice (34); the WNK1+/+ animals of this strain were shown to be hypotensive, suggesting again that the net effects of L-WNK1 and ks-WNK1 on tubular salt transport are stimulatory (34). An independently generated strain of WNK1+/- mice, however, was normotensive, indicating the large compensatory capacity depending on the genetic background (26).

A human counterpart of the loss of WNK3 was recently described in patients with a microdeletion in the X chromosome (Xp11.22) (22). Microdeletion Xp11.22, which involves several genes including WNK3, is associated with autistic disorder, intellectual disability, and cleft palate. Although the clinical picture of these patients most likely was related to the loss of function of the PHF8 gene (plant homeodomain finger protein 8), the possible involvement of WNK3 in the observed neurodevelopmental disorders remains to be evaluated (1, 23). As in WNK3-deficient mice, no apparent renal dysfunction has been reported for patients with microdeletion Xp11.22, suggesting that WNK3 is also dispensable in humans for kidney function or can be compensated for by other regulatory mechanisms (23).
In summary, our data obtained from WNK3-deficient mice suggest that the overall relevance of WNK3 for the regulation of bulk renal salt reabsorption is limited. WNK3 in vivo rather seems to be part of a regulatory kinase network that allows the fine tuning of tubular reabsorptive function. As a consequence, the loss of WNK3 can be largely compensated for by other regulatory mechanisms, in particular the WNK1-SPAK/OSR1 axis.

GRANTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB699/A4).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Authors contributed: K. Mederle, S.B., H.C., and M.O. conception and design of research; K. Mederle, K. Mutig, A.P., I.C., S.B., H.C., and M.O. analyzed data; K. Mederle, K. Mutig, A.P., I.C., H.C., and M.O. interpreted results of experiments; K. Mederle, K. Mutig, A.P., I.C., and M.O. prepared figures; K. Mederle and H.C. drafted manuscript; K. Mederle, K. Mutig, A.P., I.C., S.B., H.C., and M.O. approved final version of manuscript; S.B., H.C., and M.O. edited and revised manuscript.

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WNK3 AND KIDNEY FUNCTION


