Inducible kidney-specific Sgk1 knockout mice show a salt-losing phenotype

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Faresses N, Lagnaz D, Debonneville A, Ismailji A, Maillard M, Fejes-Toth G, Náray-Fejes-Tóth A, Staub O. Inducible kidney-specific Sgk1 knockout mice show a salt-losing phenotype. Am J Physiol Renal Physiol 302: F977–F985, 2012. First published February 1, 2012; doi:10.1152/ajprenal.00535.2011.—The expression of the serum- and glucocorticoid-regulated kinase 1 (Sgk1) is induced by mineralocorticoids and, in turn, upregulates the renal epithelial Na+ channel (ENaC). Total inactivation of Sgk1 has been associated with transient urinary Na+ wasting with a low-Na+ diet, while the aldosterone-mediated ENaC channel activation was unchanged in the collecting duct. Since Sgk1 is ubiquitously expressed, we aimed to study the role of renal Sgk1 and generated an inducible kidney-specific knockout (KO) mouse. We took advantage of the previously described TetOn/CreLoxP system, in which rtTA is under the control of the Pax8 promoter, allowing inducible inactivation of the floxed Sgk1 allele in the renal tubules (Sgk1fl/fl/Pax8/LC1 mice). We found that under a standard Na+ diet, renal water and Na+/K+ excretion had a tendency to be higher in doxycycline-treated Sgk1 KO mice compared with control mice. The impaired ability of Sgk1 KO mice to retain Na+ increased significantly with a low-salt diet despite higher plasma aldosterone levels. On a low-Na+ diet, the Sgk1 KO mice were also hyperkalemic and lost body weight. This phenotype was accompanied by a decrease in systolic and diastolic blood pressure. At the protein level, we observed a reduction in phosphorylation of the ubiquitin protein-ligase Nedd4-2 and a decrease in the expression of the Na+–Cl–cotransporter (NCC) and to a lesser extent of ENaC.

ENaC; hypertension; Nedd4-2; sodium homeostasis; sodium-chloride cotransporter

SALT HOMEOSTASIS, MAINTAINED by the kidney, is important for the maintenance of blood homeostasis. Several mono- genic diseases, including Gitelman’s syndrome, Liddle’s syndrome or pseudohypoaldosteronism type I, are caused by mutations in genes encoding renal transport proteins, channels, or protein regulators (for a review, see Ref. 28). The reabsorption of Na+ is primarily regulated by aldosterone in the aldosterone-sensitive distal nephron (ASDN) that comprises the second part of the distal convoluted tubule (DCT2), the collecting tubule (CNT), as well as the cortical and medullary collecting duct (CCD and MCD, respectively) (30). The ASDN is characterized by the presence of several important players in aldosterone-dependent regulation, including the mineralocorticoid receptor (MR), the 11β-hydroxysteroid-dehydrogenase type II, serum- and glucocorticoid-regulated kinase 1 (Sgk1), neuronal precursor cell-expressed developmentally downregulated 4-2 (Nedd4-2), and several transport proteins such as the epithelial Na+ channel (ENaC), the thiazide-sensitive Na+–Cl–cotransporter (NCC), the ROMK channel, and the Na+/K+–ATPase (13). Aldosterone diffuses into the cell and binds the intracellular MR, which then enters into the nucleus and promotes a complex transcriptional and translational response (44). The molecular and cellular events of this response are poorly understood; however, it is clear that it leads to increased Na+ reabsorption and, under certain circumstances, increased K+ secretion involving NCC, ENaC, and ROMK (5). One of the earliest aldosterone-stimulated transcripts and proteins involves Sgk1, which has been shown to be induced within 30 min (11, 33). Sgk1 is a member of the ABC family of Ser/Thr kinases that also includes PKB/Akt (29). There are several translational isoforms of Sgk1 (6) and two close paralogs referred to as Sgk2 and Sgk3 (29).

Sgk1 has been shown to stimulate ENaC activity or transcellular Na+ currents when coexpressed in heterologous systems such as Xenopus laevis oocytes (3, 32) and in other systems (15), but it was not clear whether this involved only changes in cell surface expression (3) or also in open probability (45). It was proposed (12, 40) that Sgk1 phosphorylates the ubiquitin-protein ligase Nedd4-2 involved in negative regulation of ENaC (1, 20, 23, 41), thereby inhibiting the interaction between Nedd4-2 and ENaC. Such inhibition would then lead to reduced ubiquitylation, internalization, and degrada- tion of ENaC. It was also shown that phosphorylation of Nedd4-2 creates binding sites for 14-3-3 proteins, which sterically interfere with Nedd4-2–ENaC interaction (8, 10, 27). Sgk1 has been studied in vivo in two different constitutive knockout (KO) models. In the first model, Sgk1 was inactivated by deleting exons 4–11 encoding the kinase domain (48). These mice are viable and maintain, under a standard salt diet, indistinguishable water and Na+ excretion, despite higher circulating aldosterone, suggesting that the mechanisms controlling Na+ homeostasis and volume control are affected. Under a low-Na+ diet, the KO mice lose weight, have a higher urinary flow rate, increased Na+ excretion, hyperkalemia, lower blood pressure, and highly increased plasma aldosterone levels, demonstrating that Sgk1 plays a role in Na+ and water homeostasis. Intriguingly, these mice have increased proximal-tubular Na+ and fluid reabsorption and a rather weak reduction of amiloride-sensitive transepithelial potential difference in isolated, perfused collecting ducts and hardly any effect on the expression or apical localization of α-ENaC (21, 48). Conversely, Sgk1 deletion has a more pronounced effect on the inhibition of NCC expression and phosphorylation (43). A pharmacological analysis using different diuretic inhibitors showed that inhibition of ENaC by triamterene in Sgk1−/− mice leads to severe body weight loss, as well as increases in circulating aldosterone, urea, and K+ concentration, suggesting that Sgk1 may be involved in functions other than ENaC regulation. Other inhibitors (canrenone, furosemide, or thia-

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zide) did not show any differences between KO and control animals (7).

The second model was generated by crossing floxed Sgk1 mice (the flox sites encompassing exons 2–6) with constitutive and ubiquitous Cre-deleter mice (Cre under the control of a CMV promotor), allowing the removal of K127 (essential for Sgk1 kinase activity) and resulting in a missense mutation starting at codon 139 and a premature stop codon at 159 (16). Similarly to the other mouse model, these mice are viable and fertile although the litter sizes are relatively small. Moreover, there are no obvious differences between wild-type and KO mice under a standard salt diet. However, under a low-salt diet, KO mice increased both urinary Na$^+$ and K$^+$ excretion, and surprisingly displayed increased ENaC activity, when whole cell currents in CCD were measured. On the other hand, they exhibited decreased γ-ENaC cleavage as well as diminished NCC protein expression. These data suggest that Sgk1 is not primarily involved in ENaC regulation, but likely in another transport system, such as the NCC.

The two KO models represent constitutional inactivation of Sgk1, hence it is possible that Sgk1 in other tissues may influence and affect renal regulation. Moreover, mice inactivated for Sgk1 may develop compensatory mechanisms to deal with the loss of the kinase. Therefore, we developed a novel model, in which the floxed Sgk1 mice generated by Fejes-Toth et al. (16) were bred with mice expressing the Cre-recombinase under the control of a tetracycline-inducible promotor, allowing the expression of the Cre-recombinase in the entire renal tubule (42). We confirm that NCC is a major target of Sgk1 and that ENaC protein, but not mRNA expression, is reduced. Moreover, we demonstrate for the first time in vivo that Nedd4-2 phosphorylation is strongly reduced in Sgk1 KO mice, indicating that these sites are indeed primary targets of this kinase in the nephron.

MATERIALS AND METHODS

Generation and induction of renal tubule-specific Sgk1 KO mice. Inducible renal tubule-specific Sgk1 knockout mice were generated by combined use of Tet-On and Cre-loxP systems. Pax8-rTA transgenic mice, which express the reverse tetracycline-dependent transactivator (rTA) in all proximal and distal tubules, and the entire collecting duct system of both embryonic and adult kidneys, were bred with TRE-LC1 transgenic mice, which express Cre recombinase under the control of a rTA-response element (42). Double transgenic Pax8-rTA/TRE-LC1 mice (Pax8/LC1), which allow doxycycline-inducible renal tubule-specific Cre-mediated recombination, were bred with mice homozygous for the Sgk1 floxed allele (16) to obtain double transgenic Sgk1fl/fl/Pax8/LC1 mutants. Double transgenic homozy-
gous Sgk1Pax8/LC1 mice (KO) and simple transgenic homozygous Sgk1Pax8 or Sgk1LC1 littersmates (controls) were treated with doxycycline (2 mg/ml in 2% sucrose drinking water) for 15 days to induce the mutation.

**Metabolic cages.** Induced mice were fed with a standard (0.18% Na⁺; Sniff, Germany) or low-Na⁺ diet (≤0.01%) for 4 days and placed in metabolic cages to measure body weight (BW), water and food consumption, and urine collection for 24 h. Animal studies were carried out in accordance with Swiss animal welfare regulations and after evaluation and written consent of the veterinarian office of the Canton of Vaud, Switzerland.

Urinary and plasma Na⁺ and K⁺ were measured using a flame photometer (Cole-Palmer Instruments, Vernon Hills, IL). Mice were anesthetized by isoflurane inhalation for blood collection and euthanized by cervical dislocation for tissue collection. Plasma aldosterone was measured by RIA (Coat-a-Count; Diagnostic Products).

**Blood pressure analysis.** Blood pressure measurements were done by the Cardiovascular Assessment Facility of the University of Lausanne. Noninvasive blood pressures were obtained from mice using the Blood Pressure Analysis System from Visitech Systems (Apex, NC). The pulse is detected on the tail, distal to the tail cuff, with a photoelectric sensor. The detected pulse is displayed on the computer monitor, and the cuff is inflated by the system. The mice are placed on a warm platform to increase blood flow to the tail to improve pulse detection. The mice are trained on the machine for 3 consecutive days, and the final values are obtained on days 4 and 5.

**Immunoblotting.** For tissues protein extraction, the kidneys were homogenized by ultrason in extraction solution (250 mM sucrose, 150 mM NaCl, 30 mM Tris-HCl, pH 7.5, and Complete protease inhibitor cocktail 1 tablet/20 ml; Roche). Before use, 1 mM DTT, 100 μM sodium fluoride, and 10 μM disodium pyrophosphate were added to the extraction solution. After centrifugation for 15 min at 1,500 g and 4°C, supernatants were collected and assayed for total protein.

**Antibodies.** The antibodies used were anti-Sgk1 (1/1,000, Sigma), anti-Nedd4-2 (1:1,000, Abcam), anti-P222-Nedd4-2 (1:500, provided by D. Alessi), anti-P238-Nedd4-2 (1:500) (18), anti-α-, β-, and γ-ENaC and anti-NCC (1:500) (14). Secondary antibodies coupled with horseradish peroxidase (GE Healthcare) were used diluted at 1:25,000.

**Real-time quantitative PCR.** Total RNA of half of a mouse kidney was extracted using a TissueLyser (Qiagen) and an RNAquous Kit (Ambion). RNA (1 μg) was reverse transcribed using SuperscriptII RT (Invitrogen) and 1 μg of random hexamer primers (Invitrogen) in a total volume of 20 μl. Quantitative real-time PCR was performed in replicate for each sample using the Applied Biosystems 7500 Fast Real-Time PCR System. TaqMan Gene Expression Assays (Applied Biosystems) for Sgk1 (Mm00441380_m1), NCC (Mm00490213_m1), and GAPDH (Mm99999915_g1) as a housekeeping gene, and TaqMan Universal PCR Master Mix (Applied Biosystems). Diluted reverse-transcribed samples (total RNA of 5 ng) were amplified in a final volume of 20 μl. The amount of Sgk1, NCC, or α-ENaC mRNA was normalized to GAPDH mRNA expression.

**Statistical analysis.** All measurements are presented as means ± SE. The data were analyzed using unpaired two-tailed Student’s t-test KO vs. controls, if not stated otherwise. The animal number measured for the experiment is indicated on each graph.

**RESULTS**

**Generation of inducible nephron-specific Sgk1 KO mice.** To inactivate Sgk1 in an inducible fashion in the kidney, we used double transgenic mice expressing Cre recombinase and firefly luciferase under the control of a Tet on promoter (TRE-LC1) and rtTA directed by the Pax8 promotor. This system allows expression of rtTA in the renal tubular system except in the glomerulus (42). These mice were bred with mice expressing the floxed gene of Sgk1 (16), yielding mice (Sgk1floXflo/XPax8-rTA/LC1 or Sgk1Pax8/LC1, referred to as KO mice) allowing the tetracycline-dependent inactivation of Sgk1 in the entire nephron and the collecting duct (Fig. 1A). To induce recombination and inactivation of the Sgk1 gene, mice were treated for 15 days with doxycycline-containing drinking water. As a control, we used littersmates that were missing either the Pax8-rTA, i.e., Sgk1LC1 mice, or the TRE-LC1, i.e., Sgk1Pax8 mice (referred to as control mice, Fig. 1A). PCR analysis of genomic DNA showed that recombination occurred in the kidney, and in the liver, consistent with the observation done by Traykova-Brauch et al. (42) that there is some Pax8 promoter activity in a small subset of hepatocytes. In control mice, no recombination of Sgk1 was observed (Fig. 1B). Western blots of total kidney lysates as well as quantitative real-time PCR demonstrated that Sgk1 mRNA and protein expression was largely reduced (Fig. 1, C and D). The 40% remaining Sgk1 mRNA may be derived from the nontubular cells of the kidney. However, we cannot exclude the possibility that some residual Sgk1 expression remains along different segments of the nephron.

To assess the physiological effects of Sgk1 disruption, we followed body weight variation of the KO mice compared with controls. We found that KO mice, which were given a normal-salt diet, were able to maintain normal growth body weight (Fig. 2A). However, when mice were kept on a low-Na⁺ diet, KO mice started to lose weight despite no difference in food consumption between the two groups (neither under a normal-salt diet, normal-salt diet. Mice were placed in metabolic cages and changes in body weight (A), food consumption (B), and urinary volume (C) were assessed at day 4 of the indicated diet. *P < 0.05.
nor low-Na\textsuperscript+ diet) (Fig. 2B). On the other hand, KO mice were increasing their urinary volume relative to water intake, when being kept on a low-Na\textsuperscript+ diet (Fig. 2C). These results suggest that Sgk1 disruption disturbs kidney function.

\textit{Sgk1Pax8/LC1} mice display a salt-losing phenotype. To examine the capability of the kidney to handle salt homeostasis, we placed control and KO mice into metabolic cages under a normal- and low-Na\textsuperscript+ diet and followed plasma and urinary excretion of Na\textsuperscript+ and K\textsuperscript+. Under the normal diet, no difference was observed in urinary Na\textsuperscript+ and K\textsuperscript+ excretion in both genotypes (Fig. 3, A and B). However, under a low-Na\textsuperscript+ diet, the urinary Na\textsuperscript+ and K\textsuperscript+ excretion rates were significantly greater in KO mice (Fig. 3, C and D). The plasma analysis revealed no difference in Na\textsuperscript+ or K\textsuperscript+ concentrations under a low-salt diet (Fig. 3, C and D), but aldosterone levels were elevated in KO compared with control mice (Fig. 3E). We measured systolic and diastolic blood pressure by the tail cuff method. As shown in Fig. 3F, KO mice exhibited lower systolic and diastolic blood pressure under both a normal- and low-Na\textsuperscript+ diet. This result diverges from previous observations where the total KO mice displayed no difference in blood pressure under a normal-salt diet (48).

\textit{Nedd4-2 phosphorylation is reduced in Sgk1Pax8/LC1} mice. We wondered whether the phosphorylation level of Nedd4-2 was affected, as it was shown previously in vitro experiments that Sgk1 is able to phosphorylate Nedd4-2 (8, 12, 17, 22, 39). To the best of our knowledge, this has never been demonstrated in vivo. We used two different anti-phospho Nedd4-2 antibodies, one directed against \textit{X. laevis} Nedd4-2 S444 (S328 in mice) described previously (18), and a second one directed against \textit{X. laevis} Nedd4-2 S328 (S222 in mice), which we received from Dr. Alessi’s laboratory. To characterize these antibodies, we first carried out experiments in Hek293 cells, in which we cotransfected murine wild-type Nedd4-2, S328A, or S222A mutants, together with Sgk1 (Fig. 4A). The cell lysates were blotted with the corresponding phospho-antibodies or with an antibody recognizing Nedd4-2 (24). The phospho-antibody against phosphorylated serine 222 detected

![Fig. 3. Sgk1Pax8/LC1 mice have a deficiency to regulate Na\textsuperscript+ and K\textsuperscript+ excretion, leading to hypotension. A and B: mice were placed in metabolic cages, and urine Na\textsuperscript+ and K\textsuperscript+ in controls and KO mice on a standard and low-Na\textsuperscript+ diet (4 days) were measured. C–E: plasma concentration of Na\textsuperscript+, K\textsuperscript+, and aldosterone were measured at day 4 of a low-Na\textsuperscript+ diet. F: systolic and diastolic blood pressure were measured by tail-cuff at day 4 of the indicated diet. *P < 0.05, **P < 0.01, ***P < 0.001.](http://ajprenal.physiology.org/)

\textsuperscript{F}980 IN VIVO REGULATION OF Na-Cl\textsuperscript+ COTRANSPORTER BY Sgk1

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wild-type phospho-Nedd4-2 in the presence of Sgk1, but was unable to reveal the S222A Nedd4-2 mutant. Similarly, the phospho-antibody directed against the phosphorylated serine 328 recognized wild-type Nedd4-2 but not Nedd4-2 S328A. These results suggest that these antibodies are specific for the corresponding phosphorylated sites on Nedd4-2.

We then carried out immunoblot analysis on whole kidney lysates of either control or KO mice that were kept on a low-Na\(^+\)/H\(^+\) diet (Fig. 4B). No difference was seen with the Nedd4-2 antibody, showing that Sgk1 does not affect the expression of Nedd4-2. However, hardly any signal was detected by the two phospho-antibodies in KO mice, suggesting that in the kidney, under a low-Na\(^+\)/H\(^+\) diet, phosphorylation of these sites is primarily dependent on the presence of Sgk1.

**Sgk1\(^{Pax8/LC1}\) mice have lower ENaC protein expression.**

Given that the Sgk1-mediated phosphorylation of Nedd4-2 was impaired in KO mice, we carried out another set of immunoblot analyses to check the expression of the three ENaC subunits (\(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC). We observed that full-length \(\alpha\)-ENaC (90 kDa) as well as the cleaved form of 30-kDa were decreased in KO mice, but the change was only significant for the cleaved form (Fig. 5A). Similarly, \(\beta\)- and \(\gamma\)- both the full-length and cleaved form of \(\gamma\)-ENaC were reduced (Fig. 5A). When looking at ENaC mRNA levels, we observed no difference between control and KO mice (Fig. 5B). Taken together, these results suggest that Sgk1 is important for the regulation of ENaC protein expression and is involved in the regulation of the proteolytic cleavage of \(\alpha\)- and \(\gamma\)-ENaC.

**NCC is strongly downregulated in Sgk1\(^{Pax8/LC1}\) mice.** As it was previously suggested that NCC is regulated via Sgk1 (4, 16, 37, 43), we wondered whether this protein may be affected in the Sgk1 KO mice. We therefore blotted kidney lysates with anti-NCC antibodies (31) (Fig. 6A). We found that NCC expression was largely diminished (by 80%) in KO mice compared with control mice, suggesting that Sgk1 plays a major role in the regulation of NCC protein expression. By quantitative RT-PCR, we found that NCC mRNA was not affected, indicating that Sgk1-mediated regulation of NCC occurred at the protein level (Fig. 6B).

A large fraction of Na\(^+\) reabsorption in the proximal part of the nephron involves the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC2) in the thick ascending limb of Henle’s loop and the Na\(^+\)/H\(^+\) exchanger (NHE3) in proximal tubules. As it was shown in vitro that Sgk1 is able to regulate these Na\(^+\) transporters (26, 50), we investigated these transporters but found no difference in NKCC2 and NHE3 expression in kidney lysates of control and KO mice (Fig. 7).

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**Fig. 4. Sgk1 is crucial for Nedd4-2 phosphorylation.** A: HEK-293 cells were cotransfected with Sgk1 and Nedd4-2 wt, Nedd4-2 S222A, or Nedd4-2 S328A. Cell lysates were subjected to immunoblotting using antibodies against Nedd4-2, P-serine 222 Nedd4-2, P-serine 328 Nedd4-2, and Sgk1. B: kidney lysates from control and KO mice were subjected to immunoblotting using antibodies against Nedd4-2, P-serine 222 Nedd4-2, and P-serine 328 Nedd4-2.

**Fig. 5. Sgk1 deletion decreased epithelial sodium channel (ENaC) expression.** A: kidney lysates from control and KO mice fed with low-salt diet were subjected to immunoblotting using antibodies against full-length \(\alpha\)-ENaC, cleaved \(\alpha\)-ENaC, \(\beta\)-ENaC, and \(\gamma\)-ENaC, and actin as a loading control. The expression level of the different ENaC subunits was quantified, and values were normalized to actin and are displayed as means \(\pm\) SE. *\(P<0.05\), **\(P<0.01\). B: the abundance of \(\alpha\)-ENaC mRNA in total kidneys was measured by quantitative real-time RT-PCR (RT-PCR). Values are means \(\pm\) SE relative to GAPDH.
DISCUSSION

The role of Sgk1 in vivo was studied previously in two different mouse models in which Sgk1 activity was constitutively inactivated (16, 48). Both models were able to maintain normal Na\(^+\)/H\(^+\) balance when kept under a standard Na\(^+\)/H\(^+\) diet but displayed Na\(^+\)/H\(^+\) and K\(^+\)/H\(^+\) wasting when kept on a low-Na\(^+\) diet. Here, we wished to know the role of renal Sgk1 in adult mice. We generated an inducible KO mouse in which the Sgk1 gene could be inactivated within the nephron by treatment with doxycycline in the drinking water. As shown in Fig. 1B, the Sgk1 gene was spliced in the kidney and in the liver, compatible with previously reported data that show that the Pax8promotor is leaky in a small subset of hepatocytes (42). In the kidney of mice kept on a low-Na\(^+\) diet, expression of Sgk1 mRNA and protein was largely decreased in the KO mice (Fig. 1C). Remaining traces of Sgk1 may be due to nonepithelial renal cells that are not affected by the Pax8 promotor. Hence, these mice represent a convenient system to study the role of Sgk1 in adult mice, which is expressed in the epithelial cells of the nephron and the collecting duct, as the deletion is induced at the age of 3 wk. However, we cannot entirely rule out the fact that Sgk1 deletion could be partial in the kidney and that certain effects in these mice are due to inactivation of Sgk1 in the liver.

Similarly to the total KO models, the Sgk1\(^{\text{Pax8/LC1}}\) mice showed no obvious defect in the handling of Na\(^+\) and K\(^+\) under a normal-Na\(^+\) diet but displayed decreased blood pressure, which is different from the total KO mice (48). When animals are provided a low-Na\(^+\) diet, they lose weight due to increased urine production. Although the plasma Na\(^+\) and K\(^+\) levels are not changing, they have increased urinary Na\(^+\) and K\(^+\), higher plasma aldosterone, and decreased systolic and diastolic blood pressure. These observations are comparable to the phenotype found in the total Sgk1 KO mice. However, given the strong reduction of the Na\(^+\) transport system in the distal part of the nephron (NCC and ENaC), we expected a greater defect in Na\(^+\) conservation.

This observation leads us to suggest that Sgk1 is not the major regulator of Na\(^+\) reabsorption by the nephron, but rather that other kinases and signaling pathways were involved. For ENaC, in vitro (2) as well as in vivo (7) studies showed that Sgk1 regulation is an addition, but not the only mechanism of aldosterone-dependent regulation. Concerning NCC, other signaling pathways were shown to regulate its activity and cell surface expression (i.e., WNK or SPAK/OSR1). It is possible that an increase in phosphorylation of NCC induces an increase in transporter activity without any increase in transporter expression (35, 36).

Although Sgk1 has been associated with ENaC regulation via Nedd4-2, the two total KO models showed only moder-
ate effects on ENaC function. In the first case, it was shown by microperfusion that amiloride had a rather weak effect on transepithelial Na⁺ transport in the collecting ducts (48). Moreover, pharmacological inhibitors indicated in this model, that ENaC, but not MR, NCC, or NKCC2, is essential for maintaining the Na⁺ balance, suggesting that ENaC activity is important and does not depend on Sgk1 activity (7). The second model demonstrated a defect in γ-ENaC processing but no evidence for an effect on ENaC function, as measured by whole cell patch-clamp studies (16). In our study, we noticed decreased expression of the βγ-subunits and a mild effect on the α subunit, without any change in α-ENaC mRNA expression. Moreover, we observed a slight reduction of α-, but not γ-ENaC cleavage. This is consistent with our previous data showing that ubiquitination of ENaC inhibits cleavage of α- and γ-subunits (38). Given that Ned4-2 phosphorylation is reduced in our model, Ned4-2 activity may be enhanced and consequently ENaC ubiquitination increased. The finding that α-ENaC mRNA was unchanged was surprising, as this mRNA is under the control of aldosterone; hence one would expect increased expression. We do not know the precise reasons for that, but one explanation may arise from the fact that Sgk1 positively controls α-ENaC gene transcription (9), likely by relieving repression via the Dot1a-Af9 complex (51).

Interestingly, we observed a dramatic effect on NCC expression, without an effect on NCC mRNA. The transporter was hardly detectable anymore. This effect was much stronger than the reduction in the corresponding total KO mouse using the same floxed allele (16), suggesting that in the total KO model compensatory mechanisms are active, which can partly increase NCC expression. Such mechanisms may turn on during the development into adulthood and can no more be compensated with the acute deletion of Sgk1 as shown here. It is likely that the increased Na⁺ load to theCNT and collecting ducts increases the driving force for K⁺ secretion (5). Furthermore, in our mouse model, the increase in K⁺ secretion additionally to a decrease in ENaC expression corroborate the work of Frindt and Palmer (19), which reveals the existence of ENaC independent K⁺ excretion; however, this will need to be further investigated in the future.

Two possible mechanisms have been proposed on how Sgk1 may regulate NCC expression and activity. First, Rozansky and collaborators (37) suggested that Sgk1 may phosphorylate WNK4, a kinase proposed to interfere with NCC expression, and interfere with the inhibitory function of WNK4. However, this model is based entirely on in vitro data and needs confirmation in animal models. Moreover, the proposed inhibitory role of WNK4 is still a matter of debate, as this has been observed so far primarily in overexpression systems, including oocytes, mammalian cells, and transgenic mice (25, 46, 49), whereas data from a hypomorphic WNK4 mouse, with exon 7 deleted and reduced WNK4 expression, rather suggest a stimulatory role for WNK4 (34). Alternatively, Sgk1 may regulate NCC via Ned4-2 inhibition. It has been demonstrated both in vitro and in vivo that Ned4d-2 is able to inhibit NCC, and Sgk1 can interfere with such inhibition, by phosphorylating Nedd4d-2 on Ser222 and 328 (4). Indeed, conditional kidney-specific Ned4d-2 KO mice show a dramatic increase in NCC proteins (4). Interestingly, these data also suggested that Sgk1 has to phosphorylate Ned4d-2 on both serines, 222 and 328, to interfere with Nedd4-2 action, in contrast to the situation with ENaC (4), where only one site (S328) appears to be important (12). The data presented here, showing that Sgk1 KO mice on a low-Na⁺ diet lose phosphorylation of these sites, further support such a model of Sgk1/Nedd4d-2-dependent regulation of NCC. To our knowledge, this is the first demonstration that Sgk1 phosphorylates Nedd4d-2 in vivo in mice under low-Na⁺ conditions.

To conclude, our data in an acute adult kidney-specific Sgk1 KO mouse suggest that Sgk1 plays a prime role in the regulation of NCC, which is strongly downregulated, likely due to reduced Ned4d-2 phosphorylation. The observed renal phenotype is consistent with such a mechanism, as these mice on a low-Na⁺ diet have high aldosterone, high natriuresis and kaliuresis, and lower blood pressure. Further investigation will be required to further characterize this phenotype and to understand the relationship of the Sgk1/Nedd4d-2 pathway to the WNK kinase pathway.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: N.F. and O.S. provided conception and design of research; N.F., D.L., A.D., A.I., and M.M. performed experiments; N.F. and D.L. prepared figures; N.F. and O.S. drafted manuscript; N.F., D.L., A.D., and M.M. analyzed data; N.F., A.D., and O.S. interpreted results of experiments; N.F. and D.L. prepared figures; N.F. and O.S. drafted manuscript; N.F., D.L., G.F.-T., A.N.-FT., and O.S. edited and revised manuscript; N.F., D.L., A.D., and O.S. approved final version of manuscript.

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