Activation of the renal Na^+:Cl^- cotransporter by angiotensin II is a WNK4-dependent process

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Pseudohypoaldosteronism type II is a salt-sensitive form of hypertension with hyperkalemia in humans caused by mutations in the with-no-lysine kinase 4 (WNK4). Several studies have shown that WNK4 modulates the activity of the renal Na^+Cl^- cotransporter, NCC. Because the renal consequences of WNK4 carrying pseudoaldosteronism type II mutations resemble the response to intravascular volume depletion (promotion of salt reabsorption without K^+ secretion), a condition that is associated with high angiotensin II (AngII) levels, it has been proposed that AngII signaling might affect WNK4 modulation of the NCC. In *Xenopus laevis* oocytes, WNK4 is required for modulation of NCC activity by AngII. To demonstrate that WNK4 is required in the AngII-mediated regulation of NCC in vivo, we used a total WNK4-knockout mouse strain ($WNK4^{-/-}$). WNK4 mRNA and protein expression were absent in $WNK4^{-/-}$ mice, which exhibited a mild Gitelman-like syndrome, with normal blood pressure, increased plasma renin activity, and reduced NCC expression and phosphorylation at T-58. Immunohistochemistry revealed normal morphology of the distal convoluted tubule with reduced NCC expression. Low-salt diet or infusion of AngII for 4 d induced phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and of NCC at S-383 and T-58, respectively, in $WNK4^{+/+}$ but not $WNK4^{-/-}$ mice. Thus, the absence of WNK4 in vivo precludes NCC and SPAK phosphorylation promoted by a low-salt diet or AngII infusion, suggesting that AngII action on the NCC occurs via a WNK4-SPAK–dependent signaling pathway. Additionally, stimulation of aldosterone secretion by AngII, but not by a high-K^+ diet, was impaired in $WNK4^{-/-}$ mice.

distal tubule | diuretics | thiazide | renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system (RAAS) is a key modulator of blood pressure. The blockade of this system has proven to be effective in the treatment of arterial hypertension. Recent studies suggest that hypertension induced by angiotensin II (AngII) results primarily from the renal effects of this hormone. An elegant set of renal transplant experiments between wild-type and AngII type 1 (AT1) receptor-knockout mice has revealed that arterial hypertension induced by AngII requires the presence of AT1 receptors in the kidney but not in any other tissue (1) and that hypertension mostly is the consequence of an increase in renal salt reabsorption (2). To date, all monogenic diseases featuring altered blood pressure in which the causative gene has been uncovered have been found to be caused by mutations in genes encoding proteins that are either the effectors or the modulators of salt-reabsorption pathways (3). The study of one of such disease has revealed interesting molecular mechanisms for the regulation of distal nephron salt reabsorption.

Pseudohypoaldosteronism type II (PHAII), also known as "familial hyperkalemic hypertension" or "Gordon's syndrome," is an autosomal dominant disease featuring arterial hypertension and hyperkalemia with metabolic acidosis and hypercalciuria (4). PHAII is the mirror image of Gitelman's syndrome, an autosomal recessive disease exhibiting arterial hypotension, hypokalemic metabolic alkalosis, and hypocalciuria, which is caused by inactivating mutations of the renal thiazide-sensitive Na^+Cl^- cotransporter, NCC (5). One type of PHAII is caused by missense mutations in the with-no-lysine kinase 4 (WNK4) (6). Numerous studies in *Xenopus laevis* oocytes, Cos-7 cells, HEK-293 cells, and BAC transgenic mice have shown that WNK4 is a negative modulator of NCC activity that becomes an activator when its primary structure is changed by the PHAII- type mutations (for extensive review, see ref. 7). It has been shown that WNK4 interacts with and activates SPAK/OSR1 (8). The active WNK4–SPAK complex thus is able to phosphorylate and activate NCC, at least in part, by increasing its traffic to the plasma membrane (9–11). The ability of WNK4 to phosphorylate SPAK/OSR1, and thus, NCC could be a subject to modulation: An inactive state would result in the high-jacking of nonphosphorylated NCCs, whereas an active state would result in phosphorylation and activation of NCC. This active state could be what PHAII mutations in WNK4 mimic (12). The observation that NCC activity is associated with its phosphorylation of N-terminal threonines 53 and 58 and serine 71 (13) opened the possibility to assess NCC "activity" in vivo indirectly using specific phospho antibodies (14).

In addition to increasing the activity of the NCC, the PHAII-mutant WNK4 increases activity of the apical epithelial sodium channel (ENaC) (15) and distal paracellular chloride transport (because of its action on claudins) (16, 17) while strongly inhibiting the renal outer medullary potassium channel (ROMK) (18). The combination of these effects induces salt reabsorption and prevents K^+ secretion, mimicking what occurs in the distal nephron during a low-salt diet or hypovolemia, conditions characterized by the activation of the RAAS. We thus proposed that PHAII-type mutations confer a gain of function to WNK4 that mimics the effect produced by AngII upon the WNK4–SPAK–NCC pathway (19). This hypothesis was supported by observations made in *Xenopus laevis* oocytes and murine distal convoluted tubule (mpkDCT) cells in which AngII induces a WNK4-SPAK–dependent increase in NCC phosphorylation and activity that can be prevented with the specific AT1 receptor blocker, losartan (19). The positive effect of AngII on NCC activity was suggested previously by Sandberg et al. (20), who demonstrated that AngII increases NCC trafficking to the apical plasma membrane in rat distal convoluted tubule (DCT) cells. Using adrenalectomized rats, Van der Lubbe et al. (21) reported that AngII increases NCC T53 and T58 phosphorylation, implying that AngII is able to activate the NCC by an aldosterone-independent mechanism. The goal of the present study was to

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use an in vivo model to determine whether WNK4 is required for the AngII-induced activation of SPAK and NCC.

Results

WNK4$^{-/-}$ Knockout Mice Exhibit a Mild Gitelman-Like Syndrome. From crosses of heterozygous WNK4$^{+/-}$ mice, wild-type, heterozygous, and homozygous mice were born at the expected Mendelian frequencies and showed normal growth and development (Table S1). WNK4$^{+/+}$ and WNK4$^{-/-}$ mice were identified by PCR assays on DNA from tail biopsies (Fig. S1*B*). The absence of WNK4 expression in the WNK4$^{-/-}$ mice was confirmed by real-time PCR and Western blot analysis (Fig. S1 *C* and *D*).

On a normal diet, WNK4$^{-/-}$ mice displayed mild hypokalemia, hypochloremia, metabolic alkalosis, and hypomagnesemia compared with wild-type littermates (Table 1). WNK4$^{-/-}$ mice showed greater urinary Na^+, K^+, and Cl^- excretion than wild-type mice. No difference was observed in urinary Ca^{2+} excretion. WNK4$^{-/-}$ mice exhibited a higher urinary volume, but no difference in body weight was observed. Thus, WNK4$^{-/-}$ mice did not appear to be dehydrated (Table 1). The ability to concentrate urine was similar in wild-type and WNK4$^{-/-}$ mice, as suggested by a dehydration test (Fig. S2). Hence, polyuria was probably the consequence of an increased thirst drive in the WNK4$^{-/-}$ mice, perhaps because of hypokalemia (22).

Similar systolic blood pressure was observed in the wild-type and WNK4$^{-/-}$ groups (117.2 ± 2.6 vs. 116.8 ± 3.2 mmHg; P = NS) (Fig. 1*A*); however, higher levels of plasma renin activity (PRA) were observed in the WNK4$^{-/-}$ mice (24.7 ± 1.8 in ng·mL^{-1}·h^{-1} WNK4$^{-/-}$ mice vs. 15.7 ± 1.3 ng·mL^{-1}·h^{-1} in WNK4$^{+/+}$; $P < 0.05$) (Fig. 1*B*). Interestingly, aldosterone levels in the two groups were similar despite elevated PRA (Fig. 1*C*). Thus, the phenotype of WNK4$^{-/-}$ mice is reminiscent of a mild Gitelman syndrome without arterial hypotension and hypocalciuria.

NCC Expression, Phosphorylation, and Activity Are Reduced in WNK4$^{-/-}$ Mice. Under basal conditions, the expression levels of NKCC2, Nedd4-2, OSR1, and SPAK were similar for WNK4$^{+/+}$ and WNK4$^{-/-}$ mice. Additionally, levels of SPAK phosphorylation at the T loop and S motif of the kinase (T-243 and S-383, respectively) were similar in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice (Fig. 2*A*). In contrast, NCC expression and phosphorylation at T-58 were markedly lower in WNK4$^{-/-}$ mice (~10% of wild-type levels for NCC and practically undetectable for phosphorylated NCC). Phosphorylation of T-58 has been shown to be critical for cotransporter activity (13, 14). NCC expression also was lower at the mRNA level (44% of wild-type expression level) (Fig. 2*B*). Immunohistochemistry of kidney sections confirmed the lower levels of phosphorylated NCC. No differences in number, apical surface, or cell size were detected in NCC-positive tubules (Fig. 2*C* and Table S2).

Decreased NCC activity was confirmed by showing that the natriuretic effect of hydrochlorothiazide was almost abolished in WNK4$^{-/-}$ mice (Fig. 2*D*). Na^+ excretion in the WNK4$^{+/+}$ mice was decreased between 6–24 h after thiazide injection; this decrease is explained as the response elicited after a period of loss (0–6 h) to restore the animal´s Na^+ store. As expected, this decrease was not observed in the WNK$^{-/-}$ mice. Interestingly, the inhibition of NCC was compensated, at least in part, by activation of ENaC in downstream nephron segments, as demonstrated in vivo by the increased natriuretic effect of amiloride in WNK4$^{-/-}$ mice (Fig. 2*E*) and in vitro by the increased rate of amiloride-sensitive sodium reabsorption measured in microperfused kidney collecting ducts (CCDs) (Fig. 2*F*). In addition, the increased rate of K^+ excretion (Table 1) was consistent with activation of ENaC.

Response of WNK4$^{-/-}$ Mice to Low-Salt Diet. During a low-salt diet, urinary Na^+ excretion was reduced similarly in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice (Fig. S3*A*). No difference in Ca^{2+} excretion was observed at any time point during the experiment (Fig. S3*B* but increased Cl^- and K^+ excretion were observed at several time points (Fig. S3 *C* and *D*). No differences were observed in systolic blood pressure. At days 0 and 4, blood pressure in WNK4$^{+/+}$ mice was 104 ± 5 and 106 ± 11 mmHg, respectively and in WNK4$^{-/-}$ mice was 112 ± 2 and 108 ± 2 mmHg, respectively (P = NS). Thus, the ability to reach Na^+ balance was not affected in the WNK4$^{-/-}$ mice, suggesting that the increased activity of ENaC in the connecting tubule and/or collecting duct (Fig. 2) probably is sufficient to allow the WNK4$^{-/-}$ mice to reach balance.

As previously reported (23), the low-salt diet promoted an increase in NCC expression and phosphorylation in the WNK4$^{+/+}$ mice (Fig. 3*A*). Total levels of SPAK remained similar, but SPAK phosphorylation increased with the low-salt diet in the WNK4$^{+/+}$ mice. In contrast, increased expression and/or phosphorylation of the NCC or SPAK were not observed in WNK4$^{-/-}$ mice exposed to the low-salt diet. The absence of an increase in NCC phosphorylation in WNK4$^{-/-}$ mice was not due to the complete absence of NCC protein, as demonstrated in Figs. 2*C* and 3*B*.

NCC Response to AngII Infusion Is Impaired in WNK4$^{-/-}$ Mice. Mice were infused with AngII at a nonpressor dose (400 μg·kg^{-1}·d^{-1}) (24) for 4 d through miniosmotic pumps. As expected, no changes were observed in blood pressure in either WNK4$^{+/+}$ or WNK4$^{-/-}$ mice. Correct hormone infusion was confirmed by measuring AngII levels in the urine. As observed in previous reports (21), AngII induced an increase in NCC (T-58) and SPAK (S-383) phosphorylation in the WNK4$^{+/+}$ mice (379 ± 94% and 217 ± 13%, respectively; $P < 0.05$ relative to vehicle-infused mice) (Fig. 4*A*). Total NCC and SPAK protein increased slightly, but the difference did not reach significance (Fig. 4*A*). The effect of AngII infusion on NCC phosphorylation was observed even in the context of mineralocorticoid receptor blockade achieved through treatment with spironolactone (Fig. 4*B*). This result is consistent with previous observations (21) suggesting that AngII promotes NCC phosphorylation in an aldosterone-independent manner. Interestingly, no such increases in NCC or SPAK phosphorylation were observed in WNK4$^{-/-}$ mice (Fig. 4*A*). Notably, although SPAK expression was normal in the WNK4$^{-/-}$ mice, AngII did not induce its phosphorylation. In agreement with these data, AngII infusion decreased urinary salt excretion at day 1 in WNK4$^{+/+}$ mice but not in WNK4$^{-/-}$ mice (Fig. 4*C*). The difference in the response to AngII between

Table 1. Plasma and urine electrolytes of WNK4$^{+/+}$ and WNK4$^{-/-}$ mice on a normal diet

Electrolyte	WNK4$^{+/+}$	WNK4$^{-/-}$
Plasma		
Na^+ (mM)	152.61 ± 0.53 (15)	153.19 ± 1.06 (19)
K^+ (mM)	4.00 ± 0.08 (15)	3.46 ± 0.1 (19)***
Cl^- (mM)	118.24 ± 0.52 (15)	113.92 ± 1.28 (19)*
CO_2 (mM)	13.93 ± 0.65 (15)	16.02 ± 0.58 (19)*
Ca^{2+} (mg/dL)	8.43 ± 0.09 (15)	8.38 ± 0.15 (19)
Mg^{2+} (mg/dL)	2.31 ± 0.07 (15)	1.97 ± 0.07 (19)**
Creatinine (mg/dL)	0.15 ± 0.02 (15)	0.17 ± 0.02 (19)
Urine		
Na^+ (mmol/mmol Cr)	40.88 ± 1.78 (9)	49.07 ± 2.3 (7)*
K^+ (mmol/mmol Cr)	70.44 ± 5.87 (9)	109.27 ± 5.03 (7)**
Cl^- (mmol/mmol Cr)	58.82 ± 4.12 (9)	81.42 ± 3.36 (7)**
Ca^{2+} (mg/mg Cr)	0.11 ± 0.01 (9)	0.11 ± 0.014 (7)
Mg^{2+} (mg/mg Cr)	1.24 ± 0.09 (9)	1.66 ± 0.21 (7)
Creatinine (mg/dL)	37.7 ± 6.26 (9)	18.64 ± 2.25 (7)*
Urinary volume (mL)	0.84 ± 0.11 (6)	1.98 ± 0.10 (10)***
Creatinine clearance (mL/min)	0.25 ± 0.07 (8)	0.18 ± 0.06 (6)
Weight (g)	25.94 ± 2.57 (19)	25.32 ± 0.61 (13)
Food intake (g)	3.07 ± 0.15 (9)	3.32 ± 0.034 (7)
Water intake (mL)	3.83 ± 0.64 (6)	5.25 ± 0.43 (10)

Values are presented as the mean ± SE. The number of animals per group (n) is given in parentheses. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

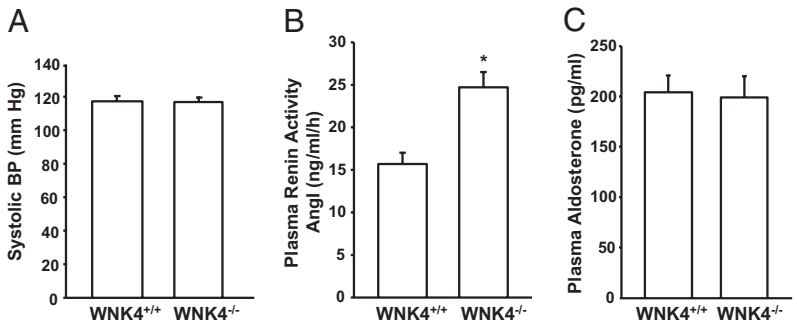

Fig. 1. Normal systolic blood pressure (BP) (*A*) with increased PRA (*B*) and normal aldosterone (*C*) in WNK4$^{-/-}$ mice. Values are presented as the mean ± SE; *$P < 0.001$ vs. wild type.

WNK4$^{+/+}$ and WNK4$^{-/-}$ mice was not caused by altered AT1 expression in the WNK4$^{-/-}$ mice kidney (Fig. 4*D*). Intriguingly, AngII infusion did not promote the expected increase in plasma aldosterone concentration in the WNK4$^{-/-}$ mice, which was indeed observed in the WNK4$^{+/+}$ mice (Fig. 4*E*).

Response of WNK4$^{-/-}$ Mice to Changes in Potassium Intake. Mice were fed with normal (1.2%), low- (0%), or high- (5%) K^+ diets for a period of 4 d. At the end of this period, plasma K^+ levels remained within physiological limits in WNK4$^{+/+}$ mice on low- and high-K^+ diets. In contrast, plasma K^+ levels were lower in WNK4$^{-/-}$ mice kept on a normal diet (3.26 ± 0.17 mM vs. 3.99 ± 0.12 mM in wild-type mice). Hypokalemia was aggravated further in WNK4$^{-/-}$ mice subjected to a low-K^+ diet (2.03 ± 0.11 mM vs. 3.44 ± 0.2 mM in wild-type mice). On a high-K^+ diet, the difference between genotypes in plasma K^+ no longer was observed (Fig. 5*A*). In contrast to observations with AngII, the high-K^+ diet increased the plasma aldosterone concentration in both WNK4$^{+/+}$ (866 ± 135 pg/mL vs. 232 ± 33 pg/mL on a normal diet) and WNK4$^{-/-}$ mice (1,467 ± 343 pg/mL vs. 234 ± 30 pg/mL on a normal diet) (Fig. 5*B*).

Discussion

In this study, we characterized a WNK4-total knockout mouse strain to define the role of this kinase in renal ion handling. Surprisingly, the total absence of WNK4 resulted in an incomplete form of Gitelman's disease with mild hypokalemia, metabolic alkalosis, hypochloremia, and hypomagnesemia but without hypocalciuria or arterial hypotension. Biochemical analysis revealed a significant decrease in the expression, phosphorylation, and activity of NCC without changes in NKCC2, Nedd4-2, SPAK, or OSR1 expression. Sodium balance was maintained, at least in part, through increased activity of ENaC in the distal nephron.

Surprisingly, different phenotypes similar to Gitelman result from two opposite WNK4 models: the complete absence (this study) or an overexpression of WNK4 (11). Absence of WNK4 results in hypokalemia, metabolic alkalosis, hypochloremia, and hypomagnesemia without changes in blood pressure or urinary Ca^{2+} excretion (Table 1). In contrast, overexpression of WNK4 leads to hypotension with hypocalciuria without changes in the other electrolytes (11). Intriguingly, NCC is reduced in both models. The difference could be caused by the consequences of the absence versus overexpression of WNK4 in other renal transport pathways. In the absence of WNK4, ENaC is activated

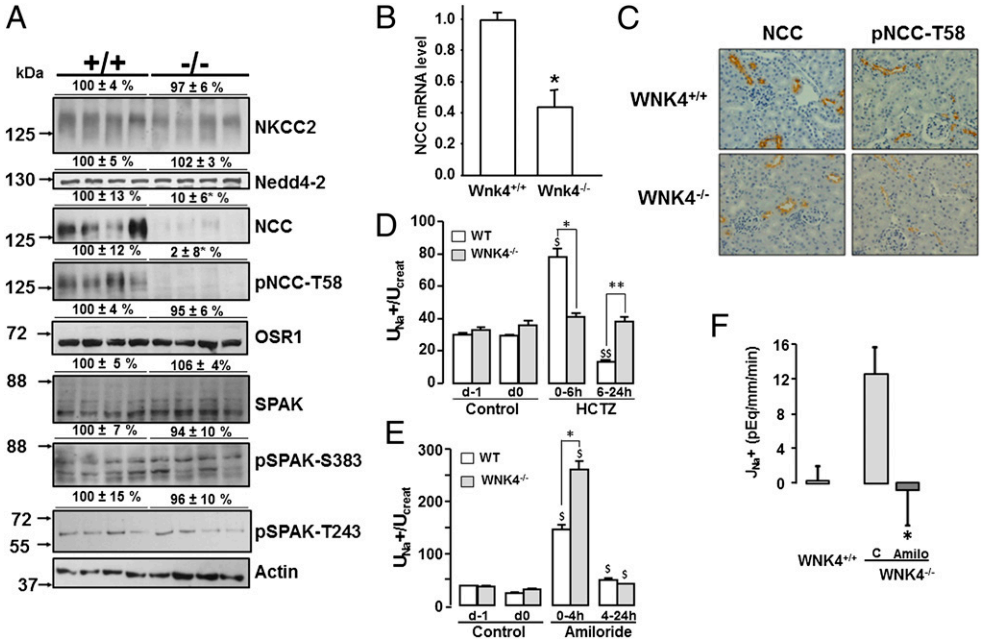

Fig. 2. Effects of WNK4 absence on distal nephron Na^+ transport pathways. (*A*) Western blot analysis of kidney protein samples of WNK4$^{+/+}$ and WNK4$^{-/-}$ mice on a normal diet. Representative blots are shown for each protein analyzed. The numbers displayed are the results of the densitometric analysis of at least two blots per assay, expressed as a percentage ± SE of wild type (100%). *$P < 0.0001$ vs. wild type. (*B*) NCC expression at the mRNA level measured through real-time PCR. RNA samples extracted from WNK$^{+/+}$ (n = 12) or WNK4$^{-/-}$ (n = 10) kidneys were used. Values shown are the relative abundance of NCC mRNA to 18S mRNA. (*C*) Immunohistochemical analysis of NCC and phosphorylated NCC (pNCC). Kidney slices from WNK4$^{+/+}$ and WNK4$^{-/-}$ mice were treated simultaneously with the same antibody and detection solutions. (Magnification: 400×.) (*D* and *E*) Effect of (*D*) hydrochlorothiazide (HCTZ) and (*E*) amiloride on Na^+ excretion. Urinary excretion of Na^+ before (days −1 and 0) and after a single administration at time 0 of HCTZ (50 mg/kg body weight, i.p.) (*D*) or amiloride (5 mg/kg body weight, i.p.) (*E*) to WNK4$^{+/+}$ mice (open bars) and WNK4$^{-/-}$ mice (gray bars). n = 6 mice per group except in the WNK4$^{+/+}$ amiloride group (n = 9), *$P < 0.05$ and **$P < 0.005$ vs. WNK4$^{+/+}$; $^{\$}P < 0.005$ and $^{\$\$}P < 0.001$ vs. control. (*F*) CCDs from WNK4$^{-/-}$ mice display amiloride-sensitive Na^+ reabsorption. Sodium flux (JNa^+) in microperfused CCDs from WNK$^{+/+}$ mice (n = 6) and WNK4$^{-/-}$ mice (n = 4) treated or not treated with 10 μM amiloride (amilo). *$P < 0.05$ vs. CCDs not treated with amiloride. All data are presented as the mean ± SE.

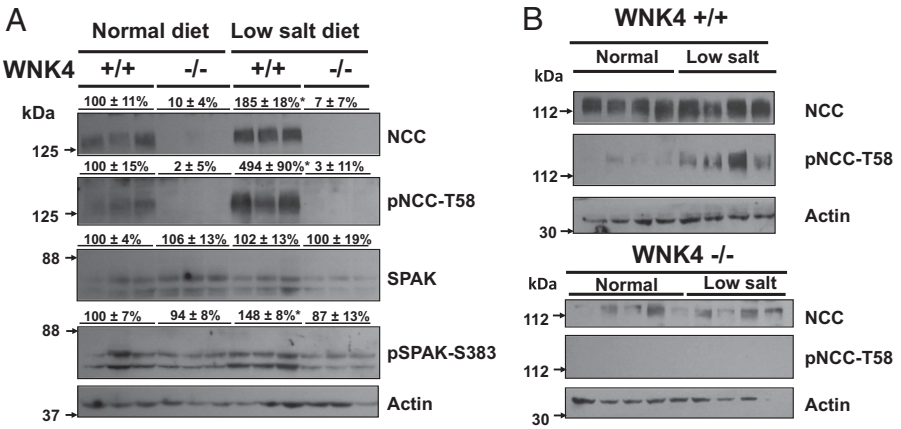

Fig. 3. NCC expression and phosphorylation are not stimulated in WNK4$^{-/-}$ mice on a low-salt diet. (*A*) Representative Western blots of kidney proteins from WNK4$^{+/+}$ and WNK4$^{-/-}$ mice on a normal or low-salt diet (4 d). The results from the densitometric analysis in which at least three blots were included per assay and at least six samples from different mice were analyzed per group are shown above each blot as the mean ± SE. $P < 0.0005$ vs. normal diet. Two bands are seen using the SPAK antibodies. These bands were reported previously in kidney (36) and represent isoforms of SPAK (34). Thus, both bands were used for the densitometric analysis. (*B*) WNK4$^{+/+}$ and WNK4$^{-/-}$ mice were studied separately to allow longer exposure times during the film scan for the WNK4$^{-/-}$ blots so that NCC expression could be detected. No increase with a low-salt diet was observed. NCC phosphorylation (at T58) could not be detected even on a low-salt diet.

(Fig. 2 *E* and *F*), and, presumably, ROMK is activated also [WNK4 is an inhibitor of this channel (18)], further contributing to the K^+ wasting already expected from the decreased NCC activity. Increased ENaC activity probably is sufficient to compensate for NCC deficiency in terms of Na^+ reabsorption; thus, these animals are normotensive. In contrast, WNK4 overexpression will exert some degree of inhibition not only on NCC but also upon ENaC and ROMK (15, 18). Thus, K^+ (and H^+) wasting in response to NCC loss probably is counteracted by a decreased activity of ENaC/ROMK, preventing important urinary K^+ losses. However, decreased Na^+ reabsorption by NCC cannot be compensated by ENaC, leading to hypotension. Regarding hypocalciuria, WNK4 has been shown to increase TRPV5 activity (25). Thus, an overexpression of WNK4 would increase Ca^{2+} reabsorption in the DCT, leading to hypocalciuria.

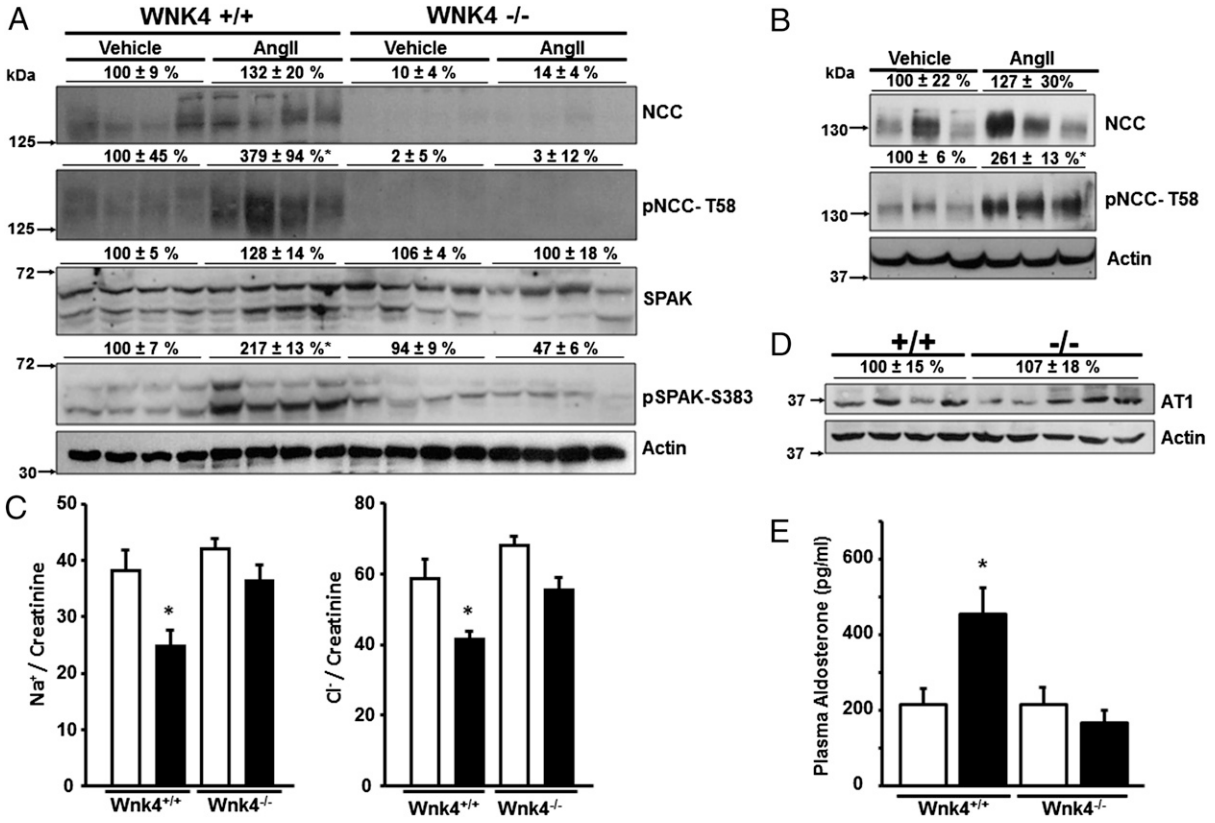

Fig. 4. Response to AngII infusion is altered in WNK4$^{-/-}$ mice. (*A*) Representative Western blots of kidney proteins from WNK4$^{+/+}$ and WNK4$^{-/-}$ mice infused with vehicle or AngII (400 $\mu g \cdot kg^{-1} \cdot d^{-1}$). The results from densitometric analysis are expressed as percentages of wild-type levels above each blot (100%). At least two blots were included per assay, and at least six samples from different mice were analyzed per group. *$P < 0.05$ vs. vehicle. (*B*) NCC expression and phosphorylation (T58) in wild-type mice treated with spironolactone and infused with vehicle or AngII. For densitometric data shown above the blot, two blots were used for a total of six samples from per group. *$P < 0.05$ vs. vehicle. (*C*) Urinary Na^+ and Cl^- excretion of WNK4$^{+/+}$ ($n = 6$) and WNK4$^{-/-}$ ($n = 6$) mice at day 1 of infusion with vehicle (open bars) or AngII (black bars). Values are presented as the mean ± SE; *$P < 0.05$ vs. vehicle. (*D*) Kidney AT1 expression in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice. (*E*) Plasma aldosterone concentration (mean ± SE) of WNK4$^{+/+}$ and WNK4$^{-/-}$ mice infused with vehicle (open bars) or AngII (black bars). *$P < 0.05$ vs. vehicle. Measurements were performed in duplicate. Samples from six different animals per group were studied.

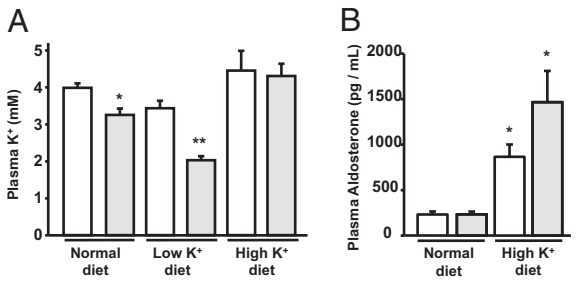

Fig. 5. Response of WNK4$^{-/-}$ mice to changes in dietary K^+ content. (*A*) Plasma K^+ concentration in WNK4$^{+/+}$ (open bars) and WNK4$^{-/-}$ (gray bars) mice kept on a normal, low-, or high-K^+ diet. Samples from nine different animals were studied per group, except for the WNK4$^{+/+}$ normal-diet group (n = 8) and WNK4$^{-/-}$ low-K^+ group (n = 10). Values are presented as the mean ± SE; $*P < 0.005$ and $**P < 0.00005$ vs. WNK4$^{+/+}$. (*B*) Plasma aldosterone concentration of WNK4$^{+/+}$ (open bars) and WNK4$^{-/-}$ (gray bars) mice subjected to a normal or high-K^+ diet. From left to right, n = 7; n = 10; n = 8; n = 9; $*P < 0.005$ vs. normal diet.

The effect of WNK4 harboring PHAII-type mutations on the distal nephron ion transport systems (NCC, ENaC, ROMK, Claudins) resembles what occurs during a low-salt diet or hypovolemia (26) in which the RAAS is activated. Thus, we proposed in a previous study that AngII could be a hormonal signal involved in switching WNK4 to the functional state promoting NCC-ENaC activation, with increased ROMK inhibition stimulating volume retention without K^+ wasting. This hypothesis was supported by observations in *Xenopus* oocytes in which WNK4 was required for AngII to increase the activity of NCC in a SPAK-dependent fashion and in mpkDCT cells in which AngII induced SPAK and NCC phosphorylation (19). Additionally, AngII increased the surface expression and phosphorylation of NCC by an aldosterone-independent mechanism in the distal tubule of rats (20, 21, 27). Supporting this proposal, we observed that renal sodium excretion and blood pressure were normal in WNK4$^{-/-}$ mice, but the activity of AngII was increased, constituting the expected physiological response when a component of a negative feedback system is lost. Additionally, our data confirmed that absence of WNK4 precluded the increase in NCC and SPAK phosphorylation induced by a low-salt diet or AngII infusion, suggesting that a WNK4–SPAK complex is part of the pathway through which AngII induces activation of NCC in the DCT.

These results may be controversial because of the low expression of NCC, even though we showed that NCC expression is not lost completely (Fig. 3*C*). Nevertheless, observations on SPAK help us resolve this issue. Under basal conditions, SPAK expression and phosphorylation in the kidney were similar in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice. However, a low-salt diet or AngII infusion increased SPAK phosphorylation in the WNK4$^{+/+}$ mice but not in the WNK4$^{-/-}$ mice (Figs. 3 and 4), strongly supporting the idea that WNK4 is implicated in SPAK and therefore NCC activation through AngII. The conservation of basal SPAK phosphorylation in the WNK4$^{-/-}$ mice suggests that kinases other than WNK4 mediate this phosphorylation. SPAK phosphorylation, however, cannot be traduced into NCC activation. Thus, a WNK4–SPAK–NCC complex apparently is required for SPAK to phosphorylate NCC.

As discussed above, PHAII mutations in WNK4 may be mimicking the NCC-activating state of WNK4 that is induced by AngII. In this scenario, WNK4 harboring PHAII mutations behaves as if AngII were constitutively acting upon the WNK4–SPAK–NCC pathway, activating NCC in the DCT and inhibiting ROMK in the collecting duct (28), resulting in hypertension and hyperkalemia. If so, the NCC-inhibiting and NCC-activating forms of the WNK4–SPAK–NCC complex could coexist under physiological conditions, with the ratio of the two depending on the activity of the RAAS. A recent study supports this possibility by showing that the NCC phosphorylation by the WNK4–SPAK/OSR1 complex is modulated by calcium concentration in the wild-type WNK4 but not in the PHAII-mutant WNK4 (12). An increase in calcium concentration shifts the kinase activity of WNK4 toward SPAK/OSR1–NCC into high gear. Accordingly, at low calcium, WNK4 may have an inhibitory effect on NCC, because the complex is less active, high-jacking nonphosphorylated NCCs, whereas at higher calcium levels NCC becomes active as the ability of the complex to phosphorylate SPAK/OSR1 and NCC increases. The PHAII mutations in the acidic motif of WNK4 disrupt the Ca^{2+}-sensing mechanism, probably locking the kinase at the state induced by elevated calcium.

The observation that both the absence (in this study) and the overexpression of WNK4 (11) lead to decreased mRNA levels of NCC is intriguing and reveals that modulation of the *SLC12A3* gene transcription rate/mRNA stability is another poorly studied pathway for WNK4 regulation of NCC activity. A recent work shows that variations in salt diet in rats induce opposite effects in NCC and WNK4 mRNA levels (29). Interestingly, contradictory observations also exist in adult mice in which WNK4 expression is changed by pharmacologic interventions. Norepinephrine induces salt-sensitive hypertension with increased NCC expression and phosphorylation associated with WNK4 down-regulation (30). In contrast, tacrolimus induces salt-sensitive hypertension with increased NCC phosphorylation but is associated with WNK4 up-regulation (31). These results imply that in wild-type mice similar effects on NCC can also result from opposite changes in WNK4. These two models are expected to differ in RAAS activity because norepinephrine is known to stimulate renin secretion directly (32). Additionally, WNK4 may have other pathways for NCC and ion transport system regulation in the kidney. For instance, it is known that WNK4 inhibits the transient receptor potential canonical 3 channel, modulating the vascular tone, which in turn could modulate pressure natriuresis mechanisms (33).

In this study, we observed in the basal state that, although PRA was higher in WNK4$^{-/-}$ mice, aldosterone levels were similar in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice, suggesting a deficient response of the adrenal glands to AngII. This notion was supported by the observation that AngII infusion resulted in more than a twofold increase in plasma aldosterone in WNK4$^{+/+}$ mice but did not induce an increase in aldosterone in the WNK4$^{-/-}$ mice. However, a high-K^+ diet increased aldosterone secretion in both genotypes. These observations suggest that WNK4 may be implicated in the intracellular pathway through which AngII regulates aldosterone secretion. In this regard, SPAK-knockout mice also display decreased aldosterone secretion in the context of increased AngII levels, suggesting that SPAK is implicated in aldosterone secretion (34). Further investigation is required to clarify this issue.

In conclusion, the present study shows that total absence of WNK4 in mice is associated with an impaired ability of low-salt diet or AngII to promote phosphorylation of SPAK and NCC, suggesting that in the DCT the AngII-positive effect on the NCC is a WNK4-SPAK–dependent process.

Methods

Animal Studies. All experiments involving animals were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (35) and were approved by the Animal Care and Use Committee at our institutions. For every study performed, only male mice aged 12–16 wk were used.

Initial Characterization (Basal State). Mice were housed in metabolic cages with free access to food and water and were given a powder diet containing 0.49% NaCl (0.2% Na^+) for a period of 12 d during which three 24-h urine collections were performed. The first two collections were intended for adaptation to the cages. The results reported correspond to samples from the third collection. At the end of the experiment, mice were killed, and blood and kidneys were collected.

Salt-Balance Studies. Teklad custom normal diet (containing 0.49% NaCl) and NaCl-deficient diet (TD.96208 and TD.90228) were used. During a 4-d period, mice were given a powder diet (0.49% NaCl) and placed in metabolic cages daily for 2- to 3-h periods to adapt to the cages and diet. Then a 12-h urine

collection was performed while mice were on a normal diet. Mice then were switched to a NaCl-deficient diet, and four 12-h collections were performed over the next 4 d. The first collection began immediately after the diet was changed. All collections were used for urine analysis. Tail-cuff blood pressure measurements were done every day. At the end of the experiment, mice were killed, and blood and kidneys were collected.

Low- and High-Potassium Diets. Control (1.2% K^+), low- (0% K^+), and high- (5% K^+) potassium diets were obtained from TestDiet. Diets were prepared by modification of an AIN-93M semipurified diet. Tribasic potassium citrate was added to the 0% K^+ diet to make the 1.2% K^+ and 5% K^+ diets. After the 4-d period of adapting to the 1.2% K^+ powder diet, the diet was changed to 0% and 5% K^+ for some animals and kept at 1.2% K^+ for others. Four days later, mice were killed for urine and blood collection.

Response to Diuretics. Mice were acclimatized to individual metabolic cages (Techniplast) for at least 4 d before the study. Mice received a single dose of hydrochlorothiazide (50 mg/kg body weight, i.p.) or amiloride (5 mg/kg body weight, i.p.) after the first day. Then urine was collected for several periods (6- and 18-h urine collections for hydrochlorothiazide and 4- and 20-h urine collections for amiloride), and Na^+ and creatinine concentrations were determined.

Blood Pressure Measurement. Systolic blood pressure was measured when mice were awake using the noninvasive volume-pressure recording CODA system (Kent Scientific). For each mouse, sessions of 10 acclimation cycles and 20 measurement cycles were performed daily for a period of 5 d. Before this 5-d period, three measurements were performed over consecutive days for the purpose of adaptation. The blood pressure reported corresponds to the average of all the accepted measurement cycles according to software parameters.

AngII Infusion. Micro-osmotic pumps (Model 1007; Alzet, DURECT) were implanted s.c. to infuse AngII at a rate of 280 $ng \cdot kg^{-1} \cdot min^{-1}$ (400 $\mu g \cdot kg^{-1} \cdot d^{-1}$), a dose previously reported to lack pressor effects (24). The infusion lasted 4 d, during which two 24-h urine collections were performed, one starting the day after implantation and the second starting 24 h before the animal was killed. After the animals were killed, blood and kidneys were collected. Some animals were treated with spironolactone during the infusion period. Spironolactone was dissolved in olive oil and administered s.c. at 50 $mg \cdot kg^{-1} \cdot d^{-1}$.

Details on the generation of WNK4-knockout mice, immunoblot assays, immunohistochemistry, real-time PCR, in vitro microperfusion, and statistical analysis are provided in *SI Methods*.

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