Double Knockout of the Na\textsuperscript{+}-Driven Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} Exchanger and Na\textsuperscript{+}/Cl\textsuperscript{−} Cotransporter Induces Hypokalemia and Volume Depletion


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ABSTRACT

We recently described a novel thiazide–sensitive electroneutral NaCl transport mechanism resulting from the parallel operation of the Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger pendrin and the Na\textsuperscript{+}–driven Cl\textsuperscript{−}/2HCO\textsubscript{3}\textsuperscript{−} exchanger (NDCBE) in β-intercalated cells of the collecting duct. Although a role for pendrin in maintaining Na\textsuperscript{+} balance, intravascular volume, and BP is well supported, there is no \textit{in vivo} evidence for the role of NDCBE in maintaining Na\textsuperscript{+} balance. Here, we show that deletion of NDCBE in mice caused only subtle perturbations of Na\textsuperscript{+} homeostasis and provide evidence that the Na\textsuperscript{+}/Cl\textsuperscript{−} cotransporter (NCC) compensated for the inactivation of NDCBE. To unmask the role of NDCBE, we generated Ndcbe/Ncc double–knockout (dKO) mice. On a normal salt diet, dKO and single-knockout mice exhibited similar activation of the renin-angiotensin-aldosterone system, whereas only dKO mice displayed a lower blood K\textsuperscript{+} concentration. Furthermore, dKO mice displayed upregulation of the epithelial sodium channel (ENaC) and the Ca\textsuperscript{2+}–activated K\textsuperscript{+} channel BKCa. During NaCl depletion, only dKO mice developed marked intravascular volume contraction, despite dramatically increased renin activity. Notably, the increase in aldosterone levels expected on NaCl depletion was attenuated in dKO mice, and single-knockout and dKO mice had similar blood K\textsuperscript{+} concentrations under this condition. In conclusion, NDCBE is necessary for maintaining sodium balance and intravascular volume during salt depletion or NCC inactivation in mice. Furthermore, NDCBE has an important role in the prevention of hypokalemia. Because NCC and NDCBE are both thiazide targets, the combined inhibition of NCC and the NDCBE/pendrin system may explain thiazide-induced hypokalemia in some patients.


The kidney continuously alters the renal excretion of NaCl to match dietary intake to keep vascular volume constant. Any situation that leads to a net positive NaCl balance (\textit{i.e.}, NaCl retention) increases intravascular volume and favors a rise in BP. Conversely, a net decrease in NaCl balance reduces BP.\textsuperscript{1} Thiazide diuretics block renal NaCl uptake, thereby substantially decreasing the risk of death from hypertension\textsuperscript{2} and are, hence, the first choice in treatment of essential hypertension.\textsuperscript{3} Although the apical Na\textsuperscript{+}/Cl\textsuperscript{−} cotransporter (NCC) expressed in the distal convoluted tubule is acknowledged as the canonical target of thiazides,
part of their antihypertensive action may be attributed to the cortical collecting duct (CCD). In this part of the nephron, electronegic Na⁺ reabsorption is achieved in principal cells through the apical epithelial sodium channel (ENaC) in tandem with the basolateral Na⁺/K⁺-ATPase. Whereas this electronegic component of NaCl reabsorption in the CCD is not inhibited by thiazides, we recently described a novel thiazide-sensitive electroneutral NaCl uptake mechanism through β-intercalated cells of the CCD that results from the parallel operation of the Cl⁻/HCO₃⁻ exchanger pendrin and the Na⁺-driven Cl⁻/2HCO₃⁻ exchanger (NDCBE; Slc4a8). In sharp contrast to NaCl uptake via NCC and ENaC, NaCl uptake by intercalated cells is mainly energized by the V-type H⁺-ATPase instead of the Na⁺/K⁺-ATPase. In addition to its role in bicarbonate secretion, pendrin plays an essential role in maintaining sodium balance and thus, also normal intravascular volume and BP. Targeted inactivation of pendrin results in a lower BP and protects against mineralocorticoid-induced hypertension. Conversely, mice overexpressing pendrin in ICs exhibited a delayed increase in urinary NaCl excretion when exposed to a high-salt diet and developed hypertension. However, a role for NDCBE, the functional partner of pendrin, in volume and electrolyte homeostasis has not been shown here.

Here, we show that NDCBE expression is upregulated by either primary or secondary hyperaldosteronism. Its genetic disruption causes only subtle perturbations in Na⁺ homeostasis, likely because it is partly compensated by increased NaCl uptake via the NCC. To test this hypothesis, we deleted Ncc in Ndcbe-deficient mice. This resulted in pronounced hypovolemia, despite a marked upregulation of ENaC. In addition, the double-knockout (dKO) mice developed hypokalemia.

**RESULTS**

**Ndcbe Knockout Mice Compensate for NaCl Loss by Activation of the Renin-Angiotensin-Aldosterone System**

Our previous work identified NDCBE as a component of the thiazide-sensitive electroneutral NaCl reabsorption pathway in CCDs. We first tested whether NDCBE is modulated by primary or secondary hyperaldosteronism, two conditions known to increase NaCl absorption in the collecting duct. Wild-type mice were challenged by either injection of the aldosterone analog deoxycorticosterone pivalate or an NaCl-free diet for 2 weeks, and NDCBE protein level was assessed by Western blot of membrane proteins extracted from the renal cortex. The protein expression of NDCBE was increased in both conditions (Supplemental Figure 1), supporting its role in maintaining sodium balance. We next assessed the effect of NDCBE inactivation on renal salt handling, intravascular volume, and BP by studying mice with either Ndcbe gene disruption (Ndcbe⁻/⁻ mice) or their wild-type counterparts (Ndcbe⁺/+ mice). Blood and urine parameters were measured in each group pair fed either a standard (0.3% Na⁺) or an Na⁺-free diet (Supplemental Tables 1 and 2). Urinary excretions of Na⁺ and Cl⁻ were similar in both groups of mice maintained on a standard diet (Supplemental Table 2). During the first 24 hours of NaCl restriction, all mice decreased urinary Na⁺ and Cl⁻ excretion to very low levels, and no significant difference was observed in Na⁺ and Cl⁻ excretion between Ndcbe⁻/⁻ and Ndcbe⁺/+ mice (Supplemental Figure 2). Within 2 days, mice achieved a new steady state with identical urinary Na⁺ and Cl⁻ excretions (Supplemental Figure 2, Supplemental Table 2). Ndcbe⁻/⁻ mice fed a normal salt diet exhibited similar plasma renin activities and urinary aldosterone levels as control mice (Figure 1, A and B). However, salt depletion increased plasma renin activity to a greater extent in mutant than in control animals (Figure 1A). Salt depletion resulted in increased aldosterone levels in both Ndcbe⁻/⁻ and Ndcbe⁺/+ mice; urinary aldosterone excretion was, however, significantly higher in Ndcbe⁻/⁻ compared with Ndcbe⁺/+ mice (Figure 1B). Because intravascular volume is a primary determinant of BP, we determined the effect of Ndcbe gene disruption on BP. Systolic BP, however, did not differ between genotypes (Figure 1C). Taken together, these results show that Ndcbe⁻/⁻ mice have a mild compensated perturbation of salt and body fluid balance.

**NCC Compensates for the Inactivation of NDCBE**

We recently reported that CCDs from Ncc-deficient mice displayed enhanced NaCl absorption through the pendrin/N DCBE transport system in the CCD, which likely compensated for the loss of NCC function in the distal convoluted tubule. Consistently, pendrin expression was dramatically upregulated in Ncc-deficient mice. We, therefore, postulated that NCC may also compensate for the loss of NDCBE function. Immunoblot analyses showed that both NCC and phospho-NCC protein abundances were increased in Ndcbe⁻/⁻ mice compared with Ndcbe⁺/+ mice, although protein abundance of the α- and γ-subunits of ENaC was unchanged (Figure 2). Injection of hydrochlorothiazide induced natriuresis in both groups; however, urinary Na⁺ excretion in Ndcbe⁻/⁻ mice was 160% that of Ndcbe⁺/+ mice, consistent with increased NCC activity in Ndcbe⁻/⁻ mice (Figure 3A). Increase in urinary Na⁺ excretion in response to amiloride, a blocker of ENaC activity, was slightly but not significantly higher in Ndcbe⁻/⁻ than in Ndcbe⁺/+ mice (Figure 3B). Of note, the protein abundances of the Na⁺/H⁺ exchanger (NHE3), the Na⁺⁻K⁺⁻2Cl⁻ cotransporter (NKCC2), and pendrin did not differ between Ndcbe⁺/+ and Ndcbe⁻/⁻ mice (Supplemental Figure 3). These results infer that NCC specifically compensates for the inactivation of NDCBE, thereby masking most of the effects of Ndcbe gene disruption.

**Ndcbe/Ncc dKO Mice Develop Hypokalemia**

We next generated dKO mice with a targeted disruption of both Ndcbe and Ncc by mating double-heterozygous mice (Ndcbe⁺/⁻;Ncc⁺/⁻). Western blot analyses confirmed the
absence of NDCBE and NCC proteins in kidneys of dKO mice (Supplemental Figure 4). On a normal NaCl diet, plasma renin activity in dKO mice was twice that of wild-type mice (Figure 4A). A similar increase was seen in Ncc−/− (NCC KO) mice. Compared with wild-type mice, urinary aldosterone levels were only slightly higher in dKO and NCC KO mice. Again, no significant difference was seen between dKO and NCC KO mice. Neither hematocrit (41.3±0.4% [n=24], 41.0±0.5% [n=14], and 42.3±0.6% [n=13] for wild-type, dKO, and NCC KO mice, respectively; NS by one-way ANOVA) nor urine output

Figure 1. The renin-angiotensin-aldosterone system is activated in Ndcbe knockout mice. Renin-angiotensin-aldosterone system status and systolic BP in Ndcbe−/− mice. (A) Plasma renin activity in Ndcbe+/+ (black bars) and Ndcbe−/− (white bars) mice fed a normal NaCl diet or after 7 days of an NaCl-depleted diet. Data are presented as the means±SEM; n=49 and n=45 for Ndcbe−/− mice fed a 0.3% Na+ or a 0% Na+ diet, respectively, and n=40 and n=28 for Ndcbe−/− mice fed a 0.3% Na+ or a 0% Na+ diet, respectively. Statistical significance was assessed by two-way ANOVA. **P<0.005, Ndcbe−/− versus Ndcbe+/+ mice during NaCl depletion; ***P<0.001 versus normal NaCl diet; ****P<0.001 versus normal NaCl diet. (B) Aldosterone excretion was measured in Ndcbe+/+ (black bars) and Ndcbe−/− (white bars) mice fed on a normal NaCl diet or after 7 days of an NaCl-depleted diet. Data are presented as the means±SEM; n=7 for mice on a normal NaCl diet, and n=9 for mice on an NaCl-depleted diet. Statistical significance was assessed by two-way ANOVA. **P<0.001, Ndcbe−/− versus Ndcbe+/+ mice during NaCl depletion; ****P<0.001 versus normal NaCl diet. (C) Effect of dietary NaCl content on systolic BP of Ndcbe−/− mice. Systolic BP was measured by tail cuff under normal salt diet (0.3% Na+/0.8% NaCl) and after 2 weeks on an NaCl-depleted diet (0% Na+). Data are presented as the means±SEM; n=8 for each genotype. Statistical significance was assessed by unpaired t test. Angio, angiotensin; SBP, systolic BP.

Figure 2. NCC protein expression is increased in Ndcbe knockout mice. Effect of Ndcbe disruption on NCC and ENaC expression. (A) NCC, phospho-T53 NCC (pNCC), α–ENaC, and γ–ENaC protein abundance was assessed by Western blot of plasma membrane–enriched preparations from the renal cortex of Ndcbe−/− and Ndcbe+/+ mice fed a normal NaCl diet. Each lane was loaded with a protein sample from a different mouse; 15 μg proteins were loaded per gel lane. Equal loading was confirmed by parallel Coomassie–stained gels. The α–ENaC antibody recognized two bands at 90 and 30 kD. The γ–ENaC antibody recognized a double band at 85–80 kD and a large band centered around 70 kD (bracket). (B) Bar graphs show a summary of densitometric analyses. For γ–ENaC, the sum of the double band at 85 kD and the broad band at 70 kD was used. Statistical significance was assessed by unpaired t test. *P<0.05 versus Ndcbe+/+ mice.

ENaC and Ca2+-Activated BK Channel Are Upregulated in Ndcbe/Ncc dKO Mice

To test the effect of the inactivation of NCC in Ndcbe−/− mice on the expression of other major renal Na+ and Cl− transporters, we performed immunoblot analyses of renal plasma membrane preparations. We quantified the relative protein abundance of the α- and γ-subunits of ENaC, pendrin, the NKCC2, and the NHE3 in wild-type, dKO, and NCC KO mice, respectively; NS by one-way ANOVA) nor urine output

(1.34±0.11 [n=18], 1.47±0.14 [n=11], and 1.54±0.17 [n=14] for wild-type, dKO, and NCC KO mice, respectively; NS by one-way ANOVA) differed between genotypes. Notably, dKO mice exhibited a significant decrease in blood K+ concentration (Figure 4C).
form of γ-ENaC which represents the active form of γ-ENaC,10,11 was markedly increased, whereas the full length of γ-ENaC was unchanged, consistent with increased expression and activation of γ-ENaC through its proteolytic cleavage. In NCC KO, the full length of γ-ENaC was decreased, whereas its cleaved form was increased, indicating similar total expression levels but activation of γ-ENaC by proteolytic cleavage. The immunoblot profile of γ-ENaC suggests a greater activation of γ-ENaC in dKO than in NCC KO mice. The cleaved N-terminal 30-kD fragment but not the full-length 90-kD form of α-ENaC was markedly upregulated in both dKO and NCC KO mice. Pendrin was also markedly upregulated in both dKO and NCC KO mice. No significant change was observed for NHE3, NKCC2, and phospho-NKCC2 protein abundance (Figure 5, A and B). Consistent with the immunoblotting data, urinary Na⁺ excretion in response to amiloride was 1.9-fold in dKO and 1.6-fold in NCC KO mice compared with in wild-type mice (Figure 5C). Na⁺
Excretion was significantly higher in dKO mice relative to NCC KO mice, indicating that ENaC activity was higher in dKO than in NCC KO mice.

To evaluate whether there was a concomitant alteration of K⁺ channels, we performed immunoblot experiments for renal outer medullary K⁺ (ROMK) and the α-subunit of the Ca²⁺-activated BK channel (α-BKCa). The specificity of the antibody raised against α-BKCa is shown in Supplemental Figure 5. Both α-BKCa and the mature 50-kD form of ROMK were increased in dKO but not in NCC KO mice (Figure 6).

**Na⁺-Depleted Ndcbe/Ncc dKO Mice Develop Marked Volume Contraction**

During NaCl depletion, dKO mice developed increased plasma renin activity (Figure 4A) and a higher hematocrit (Table 1), suggesting a marked dehydration. Plasma renin activity was less increased in NCC KO mice than in dKO mice (Figure 4A), and no change in hematocrit was noticed in NCC KO mice (Table 1). Plasma aldosterone levels were not proportionately increased in dKO and NCC KO mice (Figure 4B). During NaCl depletion, blood K⁺ concentration decreased in NCC KO mice to almost the same extent as in dKO mice (Figure 4C, Table 1).

The Western blot profile of renal samples from wild-type, dKO, and NCC KO mice fed an NaCl-depleted diet was very similar to that observed on a normal salt diet, with a higher expression of pendrin and the cleaved forms of α-ENaC and γ-ENaC in both dKO and NCC KO mice compared with in wild-type mice (Supplemental Figure 6). As shown in Supplemental Figure 7, the abundance of the full-length 85-kD form of Ndcbe/Ncc 2/2 mice on Na⁺ and Cl⁻ transporters. (A) α-ENaC, γ-ENaC, pendrin, NKCC2, and NHE3 protein abundance was assessed by Western blot of proteins extracted from the kidneys of Ndcbe+/+;Ncc+/+ (WT), Ndcbe−/−;Ncc−/− (dKO), and Ndcbe+/+;Ncc−/− (NCCKO) mice on a normal NaCl diet. Each lane was loaded with a protein sample from a different mouse; 15 μg proteins were loaded per gel lane. Equal loading was confirmed by parallel Coomassie-stained gels. The α-ENaC antibody recognized two bands at 90 and 30 kD. The γ-ENaC antibody recognized two bands at 85 and 70 kD. The phosphospecific R5 antibody recognized both phospho-NKCC2 (pNKCC2) and phospho-NCC (pNCC). (B) Bar graphs show summary of densitometric analyses in NCCKO (gray bars) and dKO (white bars) mice. Statistical significance was assessed by one-way ANOVA. *P<0.05 versus Ndcbe−/−;Ncc−/− mice; ***P<0.01 versus Ndcbe−/−;Ncc−/− mice; ****P<0.001 versus Ndcbe−/−;Ncc−/− mice. (C) Effect of amiloride injection on urinary Na⁺ excretion in WT, dKO, and NCC KO mice. Mice kept on a normal salt diet were subcutaneously injected with either vehicle or amiloride (1.45 mg/kg body wt). Urines from WT, dKO, and NCCKO mice were collected over 2 consecutive days starting at 10:00 a.m. and ending at 4:00 p.m. Data are presented as the means±SEMs from 24, 19, and 11 determinations in Ndcbe+/+;Ncc+/+, Ndcbe−/−;Ncc−/−, and Ndcbe+/−;Ncc−/− groups, respectively. Statistical significance was assessed by two-way ANOVA. *P<0.05, NCCKO versus dKO mice after amiloride injection; ****P<0.001 versus WT mice after amiloride injection; #P<0.001 versus vehicle.
higher in dKO and NCC KO mice after NaCl depletion, although the difference did not reach statistical significance. As indicated by the shift of the 85-kD band toward the 70-kD band, cleavage of γ-ENaC still occurs in both dKO and NCC KO mice in response to NaCl depletion. The cleaved 30-kD fragment of α-ENaC further increased in both groups of mice. No further increase in pendrin was seen in dKO and NCC KO mice during NaCl depletion. NaCl depletion induced a significant increase in NHE3 expression in both dKO and NCC KO mice. The strong increase in NDCBE expression in NCC KO mice likely explains why these mice do not develop dehydration in contrast to dKO mice. During NaCl depletion, α-BKCa expression became significantly higher in NCC KO than in wild-type mice (Supplemental Figure 8). ROMK protein abundance was not significantly different between groups during NaCl depletion.

**DISCUSSION**

We previously showed that Ndcb expression abolishes electroneutral thiazide-sensitive NaCl absorption in the CCD and proposed that it works in parallel with pendrin.4 In this study, we addressed the physiologic role of NDCBE in renal Na+ handling. Mice with targeted disruption of Ndcb displayed mild but largely compensated perturbations of salt and body fluid balance. We subsequently assessed to what extent the loss of NDCBE may be compensated by other sodium transporters to maintain overall sodium balance. We found an increase in the expression and activity of NCC in Ndcb knockout mice. To further characterize the role of NCC in Ndcb knockout mice, we generated a dKO model of both transporters. Supporting our assumption that NCC may compensate for NDCBE loss, these mice displayed volume contraction as evidenced by a stronger activation of the renin-angiotensin system and an increase in hematocrit after NaCl depletion.

Although single Ndcb knockout mice have a greater activation of the renin-angiotensin-aldosterone system than control mice during NaCl depletion, hematocrit and BP are unchanged, likely because angiotensin II has repeatedly been shown to upregulate NCC.12,13 In contrast, hematocrit was increased and arterial BP was decreased in pendrin knockout mice during severe NaCl restriction, despite an increase in NCC expression.6,14 Reduced ENaC expression was proposed to contribute to the lower BP of pendrin knockout mice.6

This study confirms that inactivation of NCC in mice results in only subtle perturbations of sodium and fluid homeostasis.15,16 Upregulation of both ENaC and the NDCBE/pendrin pathway in the CCD compensates for the loss of NCC,9,17 Grimm et al.18 recently evaluated compensatory mechanisms in SPAK kinase-deficient mice, which have a very low NCC activity, and showed coordinate induction of a multigene transport system involving pendrin, NDCBE, the anion exchanger AE4, carbonic anhydrase isoform 15, and the B1 subunit of the V-type H+-ATPase in pendrin-positive intercalated...
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Table 1. Physiologic parameters of Ndcbe/Ncc dKO, Ncc knockout, and wild-type mice fed an NaCl-depleted diet for 6 days

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ndcbe+/+;Ncc+/+</th>
<th>Ndcbe−/−;Ncc+/−</th>
<th>Ndcbe−/−;Ncc−/−</th>
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</thead>
<tbody>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>[K+] mmol/L</td>
<td>4.14±0.07</td>
<td>3.49±0.08a</td>
<td>3.61±0.09a</td>
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<tr>
<td>Hematocrit, %</td>
<td>42.4±0.5</td>
<td>44.5±0.4b</td>
<td>42.9±0.6</td>
</tr>
<tr>
<td>Creatinine, μM</td>
<td>11.5±0.9</td>
<td>10.6±1.2</td>
<td>9.7±0.8</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
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<tr>
<td>Urine output, ml/24 h</td>
<td>1.16±0.10</td>
<td>1.56±0.23</td>
<td>1.56±0.18</td>
</tr>
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</table>

Values are the means±SEMs. For K+, n=24, n=11, and n=19 for wild-type (Ndcbe+/+;Ncc+/+), Ndcbe−/−;Ncc+/−, and Ndcbe−/−;Ncc−/− mice, respectively. For hematocrit, n=23, n=11, and n=18 for wild-type (Ndcbe+/+;Ncc+/+), Ndcbe−/−;Ncc+/−, and Ndcbe−/−;Ncc−/− mice, respectively. For creatinine and urine output, n=18, n=11, and n=14 for wild-type (Ndcbe+/+;Ncc+/+), Ndcbe−/−;Ncc+/−, and Ndcbe−/−;Ncc−/− mice, respectively. Statistical significance between groups was assessed by ANOVA following Tukey multiple comparison tests when appropriated. [K+], potassium concentration. *P<0.001 versus wild-type mice. **P<0.05 versus wild-type mice.

Changes in α-BKCa expression parallel those of blood potassium concentration, consistent with a role for BKCa in mediating kaliuresis. Recent studies suggest that With No Lysine (K) (WNK) kinase activity can be activated by hypokalemia,20 and the kinase active form of WNK1 is a known BK activator.21 ROMK may also facilitate K+ secretion in dKO mice on a normal NaCl diet. However, ROMK is strongly expressed in thick ascending limbs, whereas expression levels in collecting ducts are quite low. Therefore, an increase in ROMK expression in whole-kidney lysates may rather reflect an increase in thick ascending limbs than an increase in collecting ducts.

The WNK kinase pathway is considered to fine tune the balance between electroneutral NaCl absorption by NCC in the distal convoluted tubule and electrogenic Na+/K+ exchange promoted by ENaC in the downstream CNT/CD. This provides a means to regulate Na+ balance without disturbing K+ secretion during hypovolemia or increase K+ secretion without impairing Na+ balance when K+ intake is high22 and vice versa. In Ncc-deficient mice, electroneutral NaCl transport was the dominant mechanism mediating Na+ absorption from the CDD.23 By favoring the electroneutral NaCl transport pathway in the CD, Ncc-deficient mice are able to maintain NaCl balance while minimizing K+ loss, which was shown by the absence of hypokalemia in this model when dietary K+ intake is maintained at a normal level.23

Thiazides are commonly used to treat patients with hypertension or hypercalciuria. When introduced in medicine, relatively high doses of thiazides were used, and hypokalemia was common and severe. It is now recognized that the best balance between effectiveness and adverse effects is obtained with lower doses.3 This study gives an explanation for these observations. Other than its canonical target NCC, thiazides at high doses (100 μM) also block the NDCBE/pendrin system in β-intercalated cells4 (lower doses [10 μM] are sufficient to inhibit NCC24,25). Low doses of thiazides likely inhibit just NCC, whereas high doses block NCC and NDCBE/pendrin and thereby, cause hypokalemia. However, thiazide inhibition of just NCC may induce upregulation of NaCl uptake by NDCBE/pendrin in the CCD and lead to thiazide resistance. Our results also suggest a mechanism for the clinically relevant effect, whereby a low-salt diet promotes to thiazide-induced hypokalemia.26 One can speculate that, because the NDCBE/pendrin system is expressed in β-intercalated cells, an alkaline diet (low-protein diet), which is known to increase the number of β-intercalated cells, might be protective against hypokalemia during thiazide treatment.

In conclusion, increased activity of NCC in Ndcbe knock-out mice helps to maintain normal intravascular volume status. As a consequence, NCC and NDCBE dKO mice display volume contraction and hypokalemia, despite a dramatic...
increase in renin activity. Together, these results provide significant insight into potassium-conserving NaCl transport in the distal nephron.

**CONCISE METHODS**

**Animals**

Studies were performed in a pure C57BL/6 background. Mice heterozygous for Ndcbe gene disruption were crossed, and wild-type (Ndcbe+/+) and homozygous knockout mice (Ndcbe−/−) were genotyped in their offspring by PCR of tail biopsies as described previously. The Ncc/Ndcbe dKO mice were obtained by crossing double-heterozygous Ndcbe+/−;Ncc+/− mice with each other. Ndcbe knockout (Ndcbe−/−;Ncc+/−), Ncc knockout (Ndcbe+/+;Ncc−/−), Ndcbe/Ncc dKO (Ndcbe−/−;Ncc−/−), and wild-type (Ndcbe+/+;Ncc+/+) mice were genotyped in their offspring by PCR of tail biopsies. In all experiments, controls consisted of wild-type littermates.

**Physiologic Studies**

All experiments were performed using age- and sex-matched Ndcbe+/+ and Ndcbe−/− littermate mice (3–5 months old). For urine collection, mice were housed in metabolic cages (Techniplast). Mice were given deionized water ad libitum and pair fed with standard laboratory powdered chow containing 0.3% sodium (INRA, Jouy-en-Josas, France). They were first allowed to adapt for 3–5 days to the cages. At steady state, urine collection was performed daily under mineral oil in the urine collector for electrolyte measurements. Mice were then switched to an NaCl-free diet (INRA). After the switch, urines were collected every 24 hours for 7 days. Urine creatinine (modified kinetic Jaffé colorimetric method) was measured with a Konelab 20i Autoanalyzer (Thermo Electron Corp., Waltham, MA). Urinary chloride was measured with a DL 55 Titrator (Mettler Toledo, Viroflay, France). Urinary Na+ and K+ were measured by flame photometry (IL943; Instruments Laboratory, Lexington, MA). Plasma renin activity was determined by RIA of angiotensin I generated by incubation of the plasma at pH 8.5 in the presence of an excess of rat angiotensinogen (GammaCoat RIA; Diasorin). Urine aldosterone was measured by RIA (DPC Dade Behring, La Défense, France). Blood collection by tail incision on mice anesthetized by peritoneal injection of a mixture (0.1 ml/g body wt) of ketamine (Imalgene; 10%; Rhône Mérieux) and xylazine (Rompun; 5%; Bayer HealthCare, Whippany, NJ) was performed for K+ measurement with an ABL 77 pH/Blood-Gas Analyzer (Radiometer, Copenhagen, Denmark). Plasma renin concentration was determined on plasma with a Konelab 20i Autoanalyzer (Thermo Electron Corp.). Blood gas analyses were performed by retro-orbital puncture, and pH, PCO2, PO2, Na+, and Cl− were measured with an ABL 77 pH/Blood-Gas Analyzer (Radiometer). Blood bicarbonate concentration was calculated from the measured values using the Henderson–Hasselbach equation.

For acute hydrochlorothiazide and amiloride injections, mice placed in metabolic cages and fed an NaCl-free diet were subcutaneously injected with HCTZ (Sigma-Aldrich, St. Louis, MO) at a dose of 50 mg/kg body wt, amiloride (HCTZ; Sigma-Aldrich) at a dose of 1.45 mg/kg body wt, or vehicle. Urines were collected over 2 days from 9:00 a.m. to 3:00 p.m. The first-day injections were of vehicle only, whereas the diuretics were injected at 9:00 a.m. on the following day. The concentration of excreted Na+ (millimolar) was normalized to the urinary concentration of creatinine (millimolar) to minimize the effects of incomplete urine sampling over such short periods.

**BP Measurements in Conscious Mice**

Systolic BP was measured in conscious mice fed a normal salt diet using a computerized tail–cuff system after 1 week of daily training as described elsewhere. Then, at least 10 measurements were performed every day for at least 7 consecutive days. Only data from the last 4 days were kept for analyses. If the variability of the measurements made in a single day exceeded the SD by >20%, this day was discarded and replaced by an additional day of measurement. This method has been extensively validated and correlates well with direct measurements of intra-arterial pressure.

**Immunoblot Analyses**

Animals were euthanized with ketamine and xylazine (0.1 and 0.01 mg/g body wt, respectively). Kidneys were removed. When appropriate, kidneys were cut into 5-mm slices, and the cortex and medulla were excised under a stereoscopic microscope and placed into ice-cold isolation buffer (250 mM sucrose and 20 mM Tris-Hepes, pH 7.4) containing protease inhibitors [4 μg/ml aprotinin, 4 μg/ml leupeptin, 1.5 μg/ml pepstatin A, and 28 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride]. Mincing tissues were homogenized in 1 ml homogenizing buffer by using an ultraturrax homogenizer at maximum speed (24,000 rpm) for 30 seconds. The homogenate was centrifuged at 4000×g for 15 minutes, and the supernatant was centrifuged at 17,000×g for 30 minutes at 4°C. The pellet was resuspended in isolation buffer. Protein contents were determined using the Bradford Protein Assay (microBradford; Bio-Rad, Hercules, CA).

Membrane proteins were solubilized in SDS-loading buffer (62.5 mM Tris HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol, and bromophenol blue) and incubated at room temperature for 30 minutes. Electrophoresis was initially performed for all samples on 7.5% polyacrylamide minigels (XCell SureLock Mini-Cell; Invitrogen, Carlsbad, CA), which were stained with Coomassie blue to provide quantitative assessment of loading as previously described. For immunoblotting, proteins were transferred electrophoretically (XCell II Blot Module; Invitrogen) for 1.5 hours at 4°C from unstained gels to nitrocellulose membranes (Amersham) and then stained with 0.5% Ponceau S in acetic acid to check uniformity of protein transfer onto the nitrocellulose membrane. Membranes were first incubated in 5% nonfat dry milk in PBS (pH 7.4) for 1 hour at room temperature to block nonspecific binding of antibody followed by overnight incubation at 4°C with the primary antibody (anti-NDCBE, 1:500; anti-NCC, 1:50,000; anti–NCC-phospho—Thr 53, 1:10,000; anti–Penrin, 1:1,000; anti–α–ENaC, 1:10,000; anti–γ–ENaC, 1:30,000; anti–NKCC2, 1:10,000; anti–phospho-NKCC2, 1:5000; anti-NHE3, 1:5000; anti–ROMK, 1:2000; and anti–α–BKCa, 1:500) in PBS containing 1% nonfat dry milk. After four 5-minute washes in PBS containing 0.1% Tween-20, membranes were incubated with 1:10,000 dilution of goat anti–rabbit IgG (Bio-Rad) or horse anti–goat IgG (Vector Laboratories, Burlingame, CA) conjugated to horseradish
peroxidase in PBS containing 5% nonfat dry milk for 2 hours at room temperature. Blots were washed as above, and luminol-enhanced chemiluminescence (ECL; PerkinElmer, Waltham, MA) was used to visualize bound antibodies before exposure to Hyperfilm ECL (Amer sham). The autoradiography was digitized with the use of a laser scanner (Epson Perfection 1650; Epson Electronics America, San Jose, CA), and quantification of each band was performed by densitometry using NIH ImageJ software (National Institutes of Health, Bethesda, MD). Densitometric values were normalized to the mean for the control group that was defined as 100%, and results were expressed as means±SEMs.

Antibody against NDCBE was described elsewhere. Antibodies against α- and γ-subunits of ENaC, NKCC2, and phospho-T53 NCC were a gift from Joyannes Loffing (University of Zurich, Zurich, Switzerland). The phosphospecific R5 antibody from Beff Forbush was used to reveal phospho-NKCC2.28 NCC antibody was a gift from David Ellison (Oregon Health and Science University, Portland, OR). Antibody against pendrin was a gift from Peter Aronson (Yale University, New Haven, CT). Antibody against NHE3 was a gift from Mark Knepper (National Institutes of Health), ROMK antibody was a gift from Paul Welling (University of Maryland School of Medicine, Baltimore, MD). Antibody against the α-subunit of the BKCa channel was purchased from Alomone Labs [anti-KCa1.1 (1097–1196)].

Specificity of this antibody was tested on kidney lysates from Kcnma1–/– mice (Kcnma1−/−),30 and their wild-type counterparts (Kcnma1+/+) were provided by Peter Ruth (Universität Tübingen, Tübingen, Germany).

STATISTICAL ANALYSES

Experimental results are summarized as means±SEMs. Statistical comparisons were made using the unpaired t test or one- or two-way ANOVA followed by the appropriate multiple comparisons test. A P value <0.05 was considered significant.

Study Approval

All of the experimental procedures conformed to the Protocol of Animal Welfare (Amsterdam Treaty; www.eurocbc.org/page673.html) and were approved by the French Government Animal Welfare Policy (agreement no. A75–15–32).

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DISCLOSURES

None.

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