Phenotype Resembling Gitelman’s Syndrome in Mice Lacking the Apical Na⁺-Cl⁻ Cotransporter of the Distal Convoluted Tubule*

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Mutations in the gene encoding the thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC) of the distal convoluted tubule cause Gitelman’s syndrome, an inherited hypokalemic alkalosis with hypomagnesemia and hypocalciuria. These metabolic abnormalities are secondary to the deficit in NaCl reabsorption, but the underlying mechanisms are unclear. To gain a better understanding of the role of NCC in sodium and fluid volume homeostasis and in the pathogenesis of Gitelman’s syndrome, we used gene targeting to prepare an NCC-deficient mouse. Null mutant (Ncc⁻/⁻) mice appear healthy and are normal with respect to acid-base balance, plasma electrolyte concentrations, serum aldosterone levels, and blood pressure. Ncc⁻/⁻ mice retain Na⁺ as well as wild-type mice when fed a Na⁺-depleted diet; however, after 2 weeks of Na⁺ depletion the mean arterial blood pressure of Ncc⁻/⁻ mice was significantly lower than that of wild-type mice. In addition, Ncc⁻/⁻ mice exhibited increased renin mRNA levels in kidney, hypomagnesemia and hypocalciuria, and morphological changes in the distal convoluted tubule. These data indicate that the loss of NCC activity in the mouse causes only subtle perturbations of sodium and fluid volume homeostasis, but renal handling of Mg²⁺ and Ca²⁺ are altered, as observed in Gitelman’s syndrome.

The thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC) is expressed on the apical membranes of distal convoluted tubule epithelial cells (1, 2) and is closely related to the apical Na⁺-K⁺-2Cl⁻ cotransporter of the thick ascending limb (3, 4). Both transporters play major roles in the reabsorption of NaCl in the kidney (5). In humans, mutations in the genes encoding NCC and the apical Na⁺-K⁺-2Cl⁻ cotransporter have been shown to cause Gitelman’s (6, 7) and Bartter’s (8) syndromes, respectively. Hypokalemic alkalosis is a common feature of both diseases but there are a number of significant differences. Patients with Bartter’s syndrome present clinically at an early age, have severe salt wasting and volume depletion (9, 10) and exhibit increased urinary Ca²⁺ and relatively normal serum Mg²⁺ levels. In contrast, patients with Gitelman’s syndrome are frequently asymptomatic at younger ages, display only limited evidence of salt wasting or hypovolemia, and exhibit hypocalciuria and hypomagnesemia (9, 10).

Identification of mutations in NCC as the molecular basis of Gitelman’s syndrome was a major breakthrough in the elucidation of the mechanisms underlying the metabolic abnormalities seen in affected patients (6, 7). Given the established biochemical function of NCC, it was apparent that the loss of NCC or a reduction in its activity would impair NaCl reabsorption in the DCT, and that the metabolic disturbances must be secondary to this defect. Despite this advance in our understanding of Gitelman’s syndrome, the role of NCC in the maintenance of sodium and fluid volume homeostasis is unclear, and the mechanisms by which NCC deficiency causes perturbations of acid-base homeostasis and renal handling of K⁺, Na⁺, Ca²⁺, and Mg²⁺ have not been determined. To begin addressing these issues we have prepared and analyzed a mouse model with a null mutation in the Ncc gene (locus Slc12a3).

EXPERIMENTAL PROCEDURES

Generation of Mutant Mice—The strain 129/SvJ phage library from which Ncc genomic clones were isolated and the targeting vector, MJK KO, were described previously (11). A 2.5-kb PstI genomic fragment, ending with codon 485 in exon 12, was subcloned into a cloning site 5’ of the neo gene in the vector, and the 5’ homologous arm. The 3’ arm (1.8 kb) was PCR amplified from a genomic clone using primers corresponding to codons 494–506 in exon 12 and codons 528–539 in exon 13. XhoI sites were included in the primers to facilitate subcloning into the vector. The 3’ outside probe used for Southern blot analysis began at codon 546 in exon 13 and included 3.7 kb of genomic sequence. Electroporation of embryonic stem cells, selection of cells in the presence of G418 and gancyclovir, Southern blot analysis of DNA samples from cells and mice, and blastocyst-mediated transgenesis were carried out as described previously (11).

PCR Genotyping—DNA from tail biopsies was analyzed by PCR. Forward (5’-AGGCTCAAGGACGCAGTTGCG-3’) and reverse (5’-GGAAGGAAAGCGGTCGAGG-3’) primers corresponding to intron sequences flanking the disrupted exon amplified a 265-base pair product from the wild-type gene. A reverse primer (5’-GCATGCTCCAGACTGCG-3’) complementary to sequences in the phosphoglycerate kinase promoter, which drives the neo gene, and the forward primer described above amplified a 188-base pair product from the targeted allele.

Northern Blot Analysis—Total RNA was isolated from the kidneys of adult mice using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the supplier’s protocol. Blots were prepared and hybridized with GAPDH, rat renin, and rat NCC cDNA probes as described previously (11). mRNA expression was quantified by PhosphorImager analysis.

Analysis of Blood—Blood (~50 μl) was collected into heparin-treated capillary tubes from the femoral artery of anesthetized mice or from the
tail vein of awake mice and immediately analyzed for gases, electrolytes, and pH using a Chiron diagnostics model 348 pH/blood gas analyzer (Chiron, Norwood, MA). Serum Mg²⁺ and Ca²⁺ concentrations were determined colorimetrically (calmagite and arsenazo III assays, Sigma). An 115I radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA) was used to measure serum aldosterone concentrations.

Na⁺ Depletion Protocols and Analysis of Urine—Custom research diets were purchased from Harlan-Teklad (Madison, WI). Adult Ncc⁺/+ and Ncc⁻/⁻ mice were housed in metabolic cages as described previously (12). The mice were given deionized water ad libitum and fed either Na⁺-replete (TD 90229, 1% NaCl) or Na⁺-depleted diets (TD 94268, 0.1% NaCl, TD 90228, 0.01% NaCl). The volume of urine collected during each 24-h period was measured and Na⁺ and K⁺ content was assayed by flame photometry (Corning model 480). Urinary Ca²⁺ concentrations were determined colorimetrically (arsenazo III assay, Sigma).

Blood Pressure—Mean arterial blood pressure of mice that were maintained on a 1% NaCl diet or fed a 0.01% NaCl diet for 2 weeks was measured using a femoral artery catheter (13). The mice were anesthetized with intraperitoneal injections of inactin (100 μg/g body weight) and ketamine (50 μg/g body weight).

Microscopy and Morphometric Analyses of DCT Cells—Kidney sections from 10–15-week-old Ncc⁺/+ and Ncc⁻/⁻ mice (n = 5 for each genotype) were prepared for light and transmission electron microscopy as described previously (11). Both objective and subjective criteria were used in the analysis of DCT and other distal tubule cells, and morphometric analyses were performed without prior knowledge of the genotype. Features used to distinguish DCT cells from other cortical cell types (14, 15) included the absence of a brush border, apicallv placed nuclei, epithelial thickness, and the shape and placement of mitochondria relative to invaginations of the basolateral membrane. The transition point between DCT and connecting tubule cannot be clearly delineated in some species (15), and there is evidence that the morphology of DCT cells is dependent on the transport activity of the cell (16–18). Therefore, objective measurements were used to distinguish DCT and connecting tubule cells, and to identify alterations in the DCT. Measurements were made on Ncc⁺/+ and Ncc⁻/⁻ DCT cells which had been classified based on both appearance and, as an objective criterion, the position of their nuclei. At 1250 magnification, the distances from the basement membrane to the inferior pole of the nucleus and the apical membrane were measured. Distal tubule cells containing apically (within the upper two-thirds of the cell) and basally (within or penetrating into the lower one-third of the cell) positioned nuclei were classified as DCT and “other” distal tubule cells, respectively. While this method cannot be used to show definitively that DCT cells are altered in number, rather than having an altered morphology that more closely resembles connecting tubule cells, it does indicate that the features used to classify them as DCT have changed. To determine the volume density of the DCT a 32-point grid was projected onto sequentially encountered fields of kidney cortex at 1250 magnification, with 25 fields surveyed for kidneys of each mouse. The height of DCT and other distal tubule cells was obtained by digitizing a line that extended from the basement membrane to the tubule lumen.

Statistical Analysis—Values represent the mean ± S.E. A mixed factorial analysis of variance with repeated measures was used to assess statistical significance, and post hoc analysis of individual means was accomplished by contrast analysis.

RESULTS

Generation of NCC-deficient Mice—Embryonic stem cells carrying an Ncc null allele were generated by inserting the neo gene into exon 12 (Fig. 1, A and B) and used to establish a null mutant mouse line. PCR analysis of tail DNAs from offspring of heterozygous mating pairs (Fig. 1C) showed that mice of all genotypes were born at the expected Mendelian ratios (37 +/+ , 88 +/− , and 46 −/−). Northern blot analysis of kidney RNA showed that Ncc mRNA expression was reduced in Ncc⁻/− mice and absent in Ncc⁻/⁻ mice, demonstrating that the Ncc gene was inactivated. Ncc⁻/− mice grew normally, were indistinguishable from Ncc⁺/+ littermates in appearance and behavior, and were fertile.

Acid-Base Status and Plasma Electrolytes in Mice Maintained on Normal and Na⁺-depleted Diets—Blood gases, blood pH, and plasma electrolytes were measured in both awake and anesthetized mice that were maintained on either a normal diet or on a Na⁺-depleted diet for 14 or 20 days. As shown in Table 1, there were no significant differences in blood gases, blood pH, or HCO₃⁻ concentrations between Ncc⁺/+ and Ncc⁻/⁻ mice when maintained on either diet, nor were there significant differences between the two genotypes in plasma Na⁺, Cl⁻, K⁺, or Ca²⁺ concentrations. Interestingly, serum Ca²⁺ concentrations of both Ncc⁺/+ and Ncc⁻/⁻ mice maintained on the Na⁺-depleted diet were significantly reduced when compared with the levels observed under Na⁺-replete conditions.

Urinary Na⁺ and K⁺ Excretion in Mice Maintained on Normal and Na⁺-depleted Diets—Ncc⁺/+ and Ncc⁻/⁻ mice were fed a Na⁺-replete diet (1% NaCl) for 9 days followed by a Na⁺-depleted diet (0.01% NaCl) for 3 weeks, and the amounts of Na⁺ and K⁺ excreted in the urine were measured (Fig. 2A and B). Urinary Na⁺ excretion was similar in Ncc⁺/+ (0.50 ± 0.02 mmol/day) and Ncc⁻/⁻ (0.46 ± 0.03 mmol/day) mice during the Na⁺-replete period, and after only 2 days of Na⁺ restriction (Fig. 2A) was reduced to very low levels (~0.5% of the excretion rate during the Na⁺-replete period) in mice of both genotypes. During the Na⁺ depletion period, Na⁺ excretion averaged 2.8 ± 0.45 and 1.9 ± 0.39 mmol/day in Ncc⁺/+ and Ncc⁻/⁻ mice, respectively; the differences were not statistically significant.

No significant difference in daily urinary K⁺ excretion was observed between Ncc⁺/+ and Ncc⁻/⁻ mice during the Na⁺-replete (0.63 ± 0.03 mmol/day in Ncc⁺/+ mice; 0.57 ± 0.03 mmol/day in Ncc⁻/⁻ mice) or Na⁺ depletion (0.50 ± 0.02 mmol/day in Ncc⁺/+ mice, 0.51 ± 0.02 mmol/day in Ncc⁻/⁻ mice) periods (Fig. 2B).

Intra-arterial Blood Pressure Measurements—When the mice were fed a normal diet, mean arterial pressures, measured using a femoral artery catheter, were slightly lower in Ncc⁻/− (84.7 ± 5.6 mm Hg) than in Ncc⁺/+ (87.4 ± 6.6 mm Hg) mice (Fig. 3), but the difference was not statistically significant.
TABLE I
Blood gases, acid-base status, and plasma electrolytes

All values (mm Hg for gases and mm for ions) are means ± S.E.

| Parameter | Na⁺-replete diet | Na⁺-depleted diet |
|-----------|------------------|------------------|
|           | Anesthetized<sup>a</sup> | Awake<sup>b</sup> | Anesthetized<sup>c</sup> | Awake<sup>d</sup> |
| pCO₂      |                   |                  |                   |                  |
| 47.4 ± 2.4 | 38.6 ± 1.2       | 52.8 ± 3.0       | 72.0 ± 3.2       |
| 45.9 ± 2.9 | 39.9 ± 1.7       | 51.2 ± 1.8       | 71.8 ± 2.1       |
| pH        | 7.36 ± 0.01       | 7.43 ± 0.01      | 7.31 ± 0.01      |
| HCO<sub>3</sub>⁻ | 7.39 ± 0.01  | 7.29 ± 0.05      | 7.45 ± 0.01      |
| Na⁺       | 150.5 ± 1.9      | 150.0 ± 1.7      | 151.3 ± 0.7     |
| K⁺        | 4.4 ± 0.35       | 5.7 ± 0.29       | 4.5 ± 0.08      |
| Ca<sup>2+</sup> | ND                | ND               | ND               |
| Cl⁻       | 6.1 ± 0.18       | 116.9 ± 0.9      | ND               |
| Na<sup>2+</sup> | ND               | 3.2 ± 0.1       | ND               |
| Mg<sup>2+</sup> | ND               | 3.1 ± 0.1       | ND               |

<sup>a</sup> n = 4 Ncc<sup>+/+</sup> and 4 Ncc<sup>−/−</sup> samples.
<sup>b</sup> n = 8 Ncc<sup>+/−</sup> and 9 Ncc<sup>−/−</sup> samples except for Ca<sup>2+</sup> (Ncc<sup>+/+</sup>, n = 5; Ncc<sup>−/−</sup>, n = 6).
<sup>c</sup> n = 8 Ncc<sup>+/−</sup> and 9 Ncc<sup>−/−</sup> samples.
<sup>d</sup> n = 5 Ncc<sup>+/+</sup> and 5 Ncc<sup>−/−</sup> samples.
<sup>e</sup> ND, not determined.

<sup>f</sup> Significantly different from mice of the same genotype fed a Na⁺-replete diet (p < 0.0001).

**Fig. 3. Intra-arterial blood pressure measurements**. Mean arterial blood pressure was measured in anesthetized Ncc<sup>+/+</sup> and Ncc<sup>−/−</sup>-mice maintained on a Na⁺-replete diet and after being fed a Na⁺-depleted diet (0.01% NaCl) for 2 weeks. The number of animals analyzed for each group is shown in parentheses. *Significantly different from wild-type as determined by single factor analysis of variance (p < 0.05). Values for all analyses are means ± S.E.

**Fig. 2. Urinary Na⁺ and K⁺ excretion in response to Na⁺-replete and Na⁺-depleted diets**. Mice were fed a diet containing 1% NaCl for nine days followed by a Na⁺-depleted diet (0.01% NaCl) for 3 weeks (shaded area). The amount of Na⁺ (A) and K⁺ (B) excreted in urine of Ncc<sup>+/+</sup> (filled circles) and Ncc<sup>−/−</sup> (open squares) mice was determined by flame photometry. Results are expressed as mmoles of Na⁺ or K⁺ excreted per day. No significant difference in Na⁺ or K⁺ excretion was observed between Ncc<sup>+/+</sup> (n = 5) and Ncc<sup>−/−</sup> (n = 5) mice during the Na⁺-replete or Na⁺-depletion periods. Average Ncc<sup>+/+</sup> and Ncc<sup>−/−</sup> body weights at the end of the Na⁺-replete (34.8 ± 1.4 g in Ncc<sup>+/+</sup>, 31.3 ± 2.0 g in Ncc<sup>−/−</sup>) and Na⁺-depletion (35.2 ± 2.2 g in Ncc<sup>+/+</sup> vs 34.2 ± 2.4 g in Ncc<sup>−/−</sup>) periods were not significantly different.

However, after both groups of mice were fed a Na⁺-depleted diet for 2 weeks (Fig. 3), the mean arterial pressure of Ncc<sup>−/−</sup>-mice (72.3 ± 5.0 mm Hg) was significantly lower than that of wild-type mice (86.1 ± 3.9 mm Hg).

**Renin mRNA in Kidney and Serum Aldosterone**—To assess whether the renin-angiotensin-aldosterone axis was activated in Ncc<sup>−/−</sup> mice, renin mRNA in kidney and serum aldosterone levels were measured in mice that were fed a Na⁺-replete diet. In an initial experiment we analyzed renin mRNA in pooled kidney samples (two mice of each genotype) and found that renin mRNA, when normalized against GAPDH mRNA, was 1.9-fold higher in kidneys of Ncc<sup>−/−</sup> mice (data not shown). An additional experiment was performed in which kidney RNA from three mice of each genotype was analyzed. As shown in Fig. 4A, renin mRNA levels were higher in Ncc<sup>−/−</sup> kidneys than in Ncc<sup>+/+</sup> kidneys (averaging 1.7-fold when normalized against GAPDH mRNA). Serum aldosterone levels (Fig. 4B) were similar in Ncc<sup>−/−</sup> (0.67 ± 0.16 ng/ml) and Ncc<sup>+/+</sup> (0.65 ± 0.13 ng/ml) mice that were fed a normal diet (1% NaCl). After 2 weeks of Na⁺ restriction, aldosterone levels were higher in Ncc<sup>−/−</sup> mice (2.73 ± 0.22 ng/ml) than in Ncc<sup>+/+</sup> mice (2.18 ± 0.27 mg/ml), but the difference was not statistically significant.

**Urinary Ca<sup>2+</sup> Excretion and Serum Mg<sup>2+</sup> Levels**—Because hypocalcemia and hypomagnesemia are commonly observed in Gitelman’s syndrome, we analyzed urinary Ca<sup>2+</sup> and serum Mg<sup>2+</sup> concentrations in the Ncc<sup>−/−</sup> and Ncc<sup>+/+</sup> mice that were used in the Na⁺-depletion study. Urinary Ca<sup>2+</sup> excretion was significantly lower in Ncc<sup>−/−</sup> (0.045 ± 0.004 mg/day) than in Ncc<sup>+/+</sup> (0.208 ± 0.014 mg/day) mice when they were fed a Na⁺-replete diet (Fig. 5A). However, urinary Ca<sup>2+</sup> excretion in...
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**Fig. 4. Renin mRNA and serum aldosterone.** A, Northern blot analysis of total kidney RNA (10 µg) isolated from individual adult Ncc<sup>+/+</sup>, Ncc<sup>-/-</sup>, and Ncc<sup>-/-</sup> mice maintained on a Na<sup>-</sup>-replete diet. The blots were hybridized with a renin cDNA probe and then stripped and hybridized with a GAPDH probe. After autoradiography, the blots were analyzed by PhosphorImager analysis and the renin/GAPDH signal intensity ratios (1.00 ± 0.02 for Ncc<sup>+/+</sup> mice; 0.58 ± 0.01 for Ncc<sup>-/-</sup> mice; p = 0.05) were calculated. B, serum aldosterone levels in adult Ncc<sup>+/+</sup> and Ncc<sup>-/-</sup> mice after being fed a Na<sup>-</sup>-replete diet and then a Na<sup>-</sup>-depleted diet (0.01% NaCl) for 2 weeks. The number of animals analyzed for each group is shown in parentheses. Serum aldosterone increased in response to Na<sup>-</sup> depletion in mice of both genotypes (p ≤ 0.0005) but there were no significant differences between Ncc<sup>+/+</sup> and Ncc<sup>-/-</sup> mice maintained on the same diet.

Ncc<sup>+/+</sup> mice dropped by ~60% after 24 h of Na<sup>-</sup> depletion and remained at this level (0.084 ± 0.008 mg/day) throughout the 3-week Na<sup>-</sup> depletion period. In contrast, there was no significant change in urinary Ca<sup>2+</sup> excretion in Ncc<sup>-/-</sup> mice when they were fed the Na<sup>-</sup>-depleted diet. Serum Mg<sup>2+</sup> was significantly lower in Ncc<sup>+-/-</sup> than in Ncc<sup>+/+</sup> mice when the animals were fed either normal (0.51 ± 0.03 and 0.87 ± 0.03 mmol/l, respectively) or Na<sup>-</sup>–depleted (0.76 ± 0.02 and 1.17 ± 0.07 mmol/l, respectively) diets (Fig. 5B). For both Ncc<sup>+-/-</sup> and Ncc<sup>-/-</sup> mice, serum Mg<sup>2+</sup> was significantly higher (p < 0.002) after being fed the Na<sup>-</sup>-depleted diet for 3 weeks.

**Fig. 5. Urinary Ca<sup>2+</sup> excretion and serum Mg<sup>2+</sup>**. A, urine samples assayed for Ca<sup>2+</sup> content were the same as those analyzed in Fig. 2. Results were calculated for the Na<sup>-</sup>-replete (days 1–9) and Na<sup>-</sup>-depletion (days 11–30) periods and are expressed as milligrams of Ca<sup>2+</sup> excreted per day. B, Mg<sup>2+</sup> concentration in serum samples collected at the beginning of the Na<sup>-</sup>-replete diet period and at the end of the Na<sup>-</sup> depletion period. Values for all analyses are means ± S.E. *Significantly different from levels in wild-type mice fed the same diet (p ≤ 0.0003).

**DISCUSSION**

In order to examine the role of NCC in sodium and fluid volume homeostasis and in the pathogenesis of Gitelman’s syndrome we generated an NCC-deficient mouse line. Insertion of the neo gene into exon 12 of the Ncc gene clearly produced a null mutation, as Ncc mRNA could not be detected in the kidneys of homozygous mutant mice. Ncc<sup>-/-</sup> mice were born at the expected Mendelian ratios, exhibited no overt disease phenotype, and were fertile. Subsequent analyses provided evidence for a mild but largely compensated perturbation of sodium and fluid volume homeostasis, revealed morphological changes in the DCT, and demonstrated that Ncc<sup>-/-</sup> mice have hypocalciuria and hypomagnesemia, which are characteristic of Gitelman’s syndrome.

Although hypokalemic alkalosis is recognized as a major feature of Gitelman’s syndrome in humans, analysis of blood samples from Ncc<sup>-/-</sup> mice revealed no apparent disturbances of potassium or acid-base homeostasis. The absence of hypokalemic alkalosis in Ncc<sup>-/-</sup> mice may indicate a major species difference in the effects of NCC dysfunction; however, Gitelman’s syndrome is often quite mild and many patients are not identified until adulthood. In many of the case histories that have been reported, as well as in the studies demonstrating that mutations in the NCC gene cause Gitelman’s syndrome (6, 7), hypokalemic alkalosis was among the criteria used in selecting patients to be studied. Thus, it is possible that the patients that have been reported in the literature represent...
Fig. 6. Electron micrographs of Ncc<sup>−/−</sup> and Ncc<sup>+/−</sup> DCT cells. Relative to the mitochondria in wild-type cells (A), mitochondria in Ncc<sup>−/−</sup> cells (B) appear to be reduced in number and less elongated in shape. Cells of both genotypes have extensive basolateral membrane folds, but the membrane folds are more closely associated with the mitochondria in Ncc<sup>−/−</sup> cells than in Ncc<sup>+/−</sup> cells. Scale bar, 5 μm.

a more strongly affected subset of individuals with mutations in the NCC gene, and that the physiological consequences of such mutations differ depending on genetic background and environmental factors.

Serious impairments of sodium and fluid volume homeostasis are observed in humans and/or mice with null mutations in the epithelial sodium channel of the collecting duct (21, 22), the apical Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup> cotransporter of the thick ascending limb (8), and the NHE3 Na<sup>+</sup>/H<sup>+</sup> exchanger of the proximal tubule (23). Given the role of NCC in NaCl reabsorption in the distal tubule, one might anticipate that NCC null mutations would also perturb sodium and fluid volume homeostasis. However, when cases of Gitelman’s syndrome were distinguished from Bartter’s syndrome by relatively rigorous diagnostic criteria, Gitelman’s syndrome patients were typically normotensive and exhibited little direct evidence of hypovolemia (9). Nevertheless, elevated serum aldosterone levels (9) indicated that some of the mechanisms that compensate for a perturbation of sodium and fluid volume homeostasis were activated in these patients. The results of the current study suggest that NCC-deficient mice also have a mild, compensated perturbation of sodium and fluid volume homeostasis. Serum aldosterone was not elevated in Ncc<sup>−/−</sup> mice, indicating that the systemic renin-angiotensin-aldosterone axis was not activated. However, the increased renin mRNA levels in Ncc<sup>−/−</sup> kidneys is consistent with the possibility that compensation might be mediated by the intra-renal renin-angiotensin system (24–26). There was no significant difference in mean arterial blood pressure between Ncc<sup>−/−</sup> and Ncc<sup>+/−</sup> mice when maintained on a Na<sup>+</sup>-replete diet, but anesthetized Ncc<sup>−/−</sup> mice exhibited a lower blood pressure than wild-type mice when fed a Na<sup>+</sup>-depleted diet for 2 weeks. This was surprising because Ncc<sup>−/−</sup> mice were able to retain Na<sup>+</sup> as well as wild-type mice during the Na<sup>+</sup>-depletion period. A possible explanation for this result is that Ncc<sup>−/−</sup> mice are mildly hypovolemic, and that gradual Na<sup>+</sup> loss during Na<sup>+</sup> restriction reduces intravascular volume to a point where the mechanisms that regulate blood pressure can no longer fully compensate. If this interpretation is correct, it implies that Ncc<sup>−/−</sup> mice have an altered set point for Na<sup>+</sup>-fluid volume balance in which the reduction in intravascular volume due to the loss of Na<sup>+</sup> reabsorption via NCC is largely, but not completely, corrected by compensatory mechanisms.

Like patients with Gitelman’s syndrome, NCC-deficient mice had low levels of both urinary Ca<sup>2+</sup> and serum Mg<sup>2+</sup>, which are thought to be due to increased Ca<sup>2+</sup> and decreased Mg<sup>2+</sup> reabsorption in the DCT (27, 28). The mechanisms by which NCC dysfunction perturbs renal Ca<sup>2+</sup> and Mg<sup>2+</sup> handling in Gitelman’s patients are not well understood. It has been suggested that a reduction in intracellular Na<sup>+</sup> levels as a result of the loss of NCC activity could increase the rate of Ca<sup>2+</sup> efflux via basolateral Na<sup>+</sup>/Ca<sup>2+</sup> exchange (27). Also, inhibition of NaCl uptake by chlorothiazide has been shown to decrease intracellular Cl<sup>−</sup> activity, causing hyperpolarization that in turn causes increased Ca<sup>2+</sup> uptake via apical Ca<sup>2+</sup> channels (29). With regard to Mg<sup>2+</sup> handling, there are data indicating that the hypomagnesemia might be secondary to hypokalemia (30). Our results tend to negate this hypothesis as Ncc<sup>−/−</sup> mice develop hypomagnesemia in the absence of hypokalemia. Also, clinical studies of patients with hypomagnesemia and hypokalemia show that correction of hypomagnesemia by dietary Mg<sup>2+</sup> supplements often leads to correction of the hypokalemia (31–33), thereby suggesting that the hypokalemia is secondary to hypomagnesemia.

We observed significant histological changes in the distal
tubule of NCC-deficient mice, but saw no evidence of apoptosis in this segment, as observed following acute treatment with high doses of thiazides (19). In Ncc−/− mice, there was a sharp reduction in the number of readily identifiable DCT cells, the height of DCT cells was decreased, their mitochondria were less elongated, and their basolateral membrane folds were not as closely associated with the mitochondria. These changes are the opposite of those observed in rats treated with furosemide (16) or maintained on a high salt diet (17), in which Na+ reabsorption is increased. Thus, it seems likely that the alterations in the DCT of Ncc−/− mice are due to the reduced transcellular Na+ transport capacity. These data support the view (16–18, 20) that the structure of DCT cells is regulated by the level of Na+ reabsorption, and suggest that the alterations in renal Ca2+ and Mg2+ handling could be due, at least in part, to the altered structure of the DCT.

In summary, our studies show that Ncc−/− mice have hypercalciuria and hypomagnesemia, but not hypokalemic alkalosis. This suggests that the renal defects in Ca2+ and Mg2+ reabsorption are a consequence of functional and/or structural alterations in the DCT caused by the loss of NCC activity rather than being secondary to systemic metabolic disturbances such as hypokalemia or alkalosis. Our results also indicate that NCC plays a relatively subtle role in the maintenance of sodium and fluid volume homeostasis, compared with that of the NHE3 Na+/H+ exchanger (23), the apical Na+/K+-2Cl− cotransporter (8), and the epithelial Na+ channel (21, 22). In future studies the NCC-deficient mouse should serve as a valuable model for deciphering the mechanisms by which NCC handling and for further investigations of the role of NCC in electrolyte, fluid volume, and acid-base homeostasis.

REFERENCES

1. Ellison, D. H., Biemesderfer, D., Morrissey, J., Lauring, J., and Desir, G. V. (1995) Am. J. Physiol. 264, F141–F148
2. Pletkin, M. D., Kaplan, M. R., Verlander, J. W., Lee, W.-S., Brown, D., Poch, E., Gullans, S. R., and Hebert, S. C. (1996) Kidney Int. 50, 174–183
3. Gamba, G, Miyano-shita, A., Lombardi, M., Lytton, J., Lee, W.-S., Hediger, M. A., and Hebert, S. C. (1994) J. Biol. Chem. 269, 17713–17722
4. Payne, J. A., and Forbush, B., III (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4544–4548
5. Kaplan, M. R., Mount, D. B., Delpere, E., Gamba, G., and Hebert, S. C. (1996) Annu. Rev. Physiol. 58, 649–668
6. Simon, D. B., Nelson-Williams, C., Bia, M. J., Ellison, D., Karet, F. E., Molina, A. M., Varaan, I., Iwata, F., Cuscher, H. M., Koolen, M., Gainza, F. J., Mastroianni, N., Bettinelli, A., Bianchetti, M., Colussi, G., De Fusco, M., Sereni, F., Ballabio, A., and Casari, G. (1996) Am. J. Hum. Genet. 59, 1019–1026
7. Simon, D. B., Karet, F. E., Hamdan, J. M., Di Pietro, A., Sanjad, S. A., and Lifton, R. P. (1996) Nat. Genet. 13, 183–188
8. Bettinelli, A., Bianchetti, M. G., Girardin, E., Caringella, A., Cecconi, M., Appiani, A. C., Pavanello, L., Gastaldi, R., Ismailli, C., Lama, G., Marcheson, C., Matteucci, C., Patriarca, P., Di Natale, B., Setza, C., and Vitucci, P. (1992) J. Pediatr. 120, 38–43
9. Simon, D. B., and Lifton, R. P. (1996) Am. J. Physiol. 271, F961–F966
10. Schultheiss, P. J., Clarke, L. L., Meneton, P., Harline, M., Boivin, G. P., Stemermann, G., Duffy, J. J., Doetschman, T., Miller, M. L., and Shull, G. E. (1998) J. Clin. Invest. 101, 1243–1253
11. Meneton, P., Schultheiss, P. J., Greeb, J., Nieman, M. L., Liu, H. H., Clarke, L. L., Duffy, J. J., Doetschman, T., Lorenz, J. N., and Shull, G. E. (1998) J. Clin. Invest. 101, 536–542
12. Lorenz, J. N., and Robbins, J. (1997) Am. J. Physiol. 272, H1137–H1146
13. Dorup, J. (1995) J. Ultrastruct. Res. 92, 101–118
14. Madsen, K. M., and Tisher, C. C. (1986) Am J. Physiol. 250, F1–F15
15. Kaisling, B., Bachmann, S., and Kriz, W. (1985) Am. J. Physiol. 248, F574–F581
16. Kaisling, B., and Stanton, B. A. (1988) Am. J. Physiol. 255, F1256–F1268
17. Ellison, D. H., Velazquez, H., and Wright, F. S. (1989) J. Clin. Invest. 83, 113–126
18. Loffing, J., Loffing-Cueni, D., Hegyi, I., Kaplan, M. R., Hebert, S. C., Le Hir, M., and Kaisling, B. (1996) Kidney Int. 50, 1180–1190
19. Stanton, B. A., and Kaissling, B. (1988) Am. J. Physiol. 255, F1269–F1275
20. Chang, S. S., Grunder, S., Hanukoglu, S., Rosler, A., Mathew, P. M., Hanukoglu, I., Schild, L., Lu, Y., Shimkets, R. A., Nelson-Williams, C., Rossier, B. C., and Lifton, R. P. (1996) Nat. Genet. 12, 248–253
21. Hummler, E., Barker, P., Talbot, C., Wang, Q., Verduno, C., Grubb, B., Gaty, J., Burnier, M., Hornberger, J.-D., Beermann, F., Boucher, R., and Rossett, R. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11710–11715
22. Schultheiss, P. J., Clarke, L. L., Meneton, P., Miller, M. L., Soleimani, M., Gawenis, L. R., Ridge, T. M., Duffy, J. J., Doetschman, T., Wang, T., Giebish, G., Aronson, P. S., Lorenz, J. N., and Shull, G. E. (1998) Nat. Genet. 19, 282–285
23. Yangawa, N. (1991) Kidney Int. 39, S33–S36
24. Navar, L. G., Imig, J. D., Zou, L., and Wang, C. T. (1997) Semin. Nephrol. 17, 412–422
25. Quan, A., and Baum, M. (1997) Semin. Nephrol. 17, 423–430
26. Costanzo, L. S., and Whitingger, E. E. (1992) in The Kidney: Physiology (Seldin, D. W., and Giebish, G., eds) pp. 2375–2393, Raven Press, New York
27. Quamme, G. A. (1997) Kidney Int. 52, 1180–1195
28. Gesek, F. A., and Friedman, P. A. (1992) J. Clin. Invest. 90, 429–438
29. Dai, L.-J., Friedman, P. A., and Quamme, G. A. (1997) Kidney. Int. 51, 1098–1017
30. Whang, R., Flink, E. B., Dyckner, T., Wester, P. O., Aikawa, J. K., and Ryan, M. P. (1985) Arch. Intern. Med. 145, 1686–1689
31. Gullner, H.-G., Gill, J. R. Jr., and Bartter, F. C. (1981) Am. J. Med. 71, 578–582
32. Cushner, H. M., Peller, T. P., Fried, T., and Delea, C. S. (1990) Pediatr. Nephrol. 4, 33–38
33. Cushner, H. M., Peller, T. P., Fried, T., and Delea, C. S. (1990) Am. J. Kidney Dis. 16, 495–500