Reducing αENaC expression in the kidney connecting tubule induces pseudohypoaldosteronism type 1 symptoms during K+ loading.

**Authors:** Poulsen SB, Praetorius J, Damkier HH, Miller L, Nelson RD, Hummler E, Christensen BM

**Journal:** American journal of physiology. Renal physiology

**Year:** 2016 Feb 15

**Volume:** 310

**Issue:** 4

**Pages:** F300-10

**DOI:** 10.1152/ajprenal.00258.2015
Reducing αENaC expression in kidney connecting tubule induces pseudohypoaldosteronism type 1 symptoms during K+ loading

Søren Brandt Poulsen1, Jeppe Praetorius1, Helle H. Damkier1,2, Lance Miller3, Raoul D. Nelson3, Edith Hummler4 and Birgitte Mønster Christensen1

1Department of Biomedicine, Aarhus University, Denmark; 2Department of Cellular and Molecular Medicine, University of Copenhagen, Denmark 3Department of Pediatrics, University of Utah School of Medicine, United States of America; and 4Department of Pharmacology and Toxicology, University of Lausanne, Switzerland

Running head: Important role of ENaC in CNT

Correspondence: Birgitte Mønster Christensen, Department of Biomedicine, Aarhus University, Wilhelm Meyers Allé 3, DK-8000 Aarhus C, Denmark (e-mail: bmc@biomed.au.dk; phone: +45 87167629; fax: +45 87167102).
ABSTRACT

Genetic inactivation of the epithelial Na\textsuperscript{+} channel $\alpha$-subunit ($\alpha$ENaC) in the renal collecting duct (CD) does not interfere with Na\textsuperscript{+} and K\textsuperscript{+} homeostasis in mice. However, inactivation in the CD and a part of the connecting tubule (CNT) induces autosomal recessive pseudohypoaldosteronism type 1 (PHA-1) symptoms already on a standard diet. In the present study, we further examined the importance of $\alpha$ENaC in the CNT. Knock-out mice with $\alpha$ENaC deleted primarily in a part of the CNT (CNT-KO) were generated using Scnn1a$^{lox/lox}$ mice and $Atp6v1b1::Cre$ mice. On a standard diet, plasma [Na\textsuperscript{+}] and [K\textsuperscript{+}], and urine Na\textsuperscript{+} and K\textsuperscript{+} output were unaffected. Seven days of Na\textsuperscript{+} restriction (0.01% Na\textsuperscript{+}) led to a higher urine Na\textsuperscript{+} output only on day 3-5, and after 7 days plasma [Na\textsuperscript{+}] and [K\textsuperscript{+}] were unaffected. By contrast, the CNT-KO mice were highly susceptible to a 2-day 5% K\textsuperscript{+} diet and showed lower food intake and relative body weight, lower plasma [Na\textsuperscript{+}], higher fractional excretion (FE) of Na\textsuperscript{+}, higher plasma [K\textsuperscript{+}], and lower FE of K\textsuperscript{+}. The higher FE of Na\textsuperscript{+} coincided with lower abundance and phosphorylation of the Na\textsuperscript{+}-Cl$^-$ cotransporter, NCC. In conclusion, reducing ENaC expression in CNT induces clear PHA-1 symptoms during high dietary K\textsuperscript{+} loading.

KEYWORDS

$\alpha$ENaC, aldosterone, kidney connecting tubule, sodium, potassium
INTRODUCTION

The functional epithelial Na\(^+\) channel (ENaC) consists of the 3 homologous subunits, α-, β-, and γ (3). Renal ENaC mediates Na\(^+\) reabsorption across the apical plasma membrane of late distal convoluted tubule (DCT2) cells, connecting tubule (CNT) cells, and collecting duct (CD) principal cells (17). Furthermore, ENaC-facilitated K\(^+\) secretion through apical K\(^+\) channels in the CNT and the cortical CD (CCD) may be crucial for maintaining K\(^+\) homeostasis (10). During conditions of hypotension and hyperkalemia, high angiotensin II or K\(^+\) plasma levels stimulate adrenal glomerulosa cells to release the steroid hormone, aldosterone (33). The DCT2/early CNT are largely insensitive to aldosterone, which is in contrast to the late CNT/CCD, where ENaC activity is markedly increased by aldosterone (18, 23).

Loss-of-function mutations in any ENaC subunit may lead to the life-threatening disease, autosomal recessive pseudohypoaldosteronism type 1 (PHA-1). PHA-1 is characterized by, e.g., hyponatremia and hyperkalemia due to impaired ability of the kidney to reabsorb Na\(^+\) and excrete K\(^+\) (4). We have in previous studies generated various αENaC knock-out (KO) mouse lines contributing to an improved understanding of the PHA-1 disease and the physiological importance of the ENaC complex. Global αENaC inactivation is highly critical, leading to neonatal death (12). Inactivation of αENaC in the CD and a part of the CNT (CNT/CD-KO) induces mild hyponatremia on a standard diet and in addition serious weight loss during Na\(^+\) restriction (5). By contrast, CD-specific αENaC KO (CD-KO) mice are unaffected both during Na\(^+\) restriction and high dietary K\(^+\) loading (28). Finally, conditional inactivation of αENaC in the colon (colon-KO) induces fecal Na\(^+\) wasting but this is compensated by the kidney and Na\(^+\) homeostasis is therefore not impaired (19). Hence, these studies collectively point towards a critical role of αENaC particularly in the DCT2 and CNT.

In the present study, we further examined the importance of αENaC in the CNT. KO mice with αENaC deleted primarily in the CNT (CNT-KO) were generated using Scnn1a\(^{lox/lox}\) mice (13) and Atp6v1b1::Cre mice. The latter mice have previously been shown to express Cre recombinase in intercalated cells and in approximately 50% of
the CNT cells (20). This correlates with weak V-ATPase B1-subunit expression in some CNT cells (1, 21). We performed a thorough characterization of the Atp6v1b1::Cre mouse line by crossing it with an enhanced green fluorescent protein (EGFP) reporter mouse line. The CNT-KO mice and the control littermates were examined on standard and challenging diets.

**METHODS**

**Breeding of CNT-KO mice and control littermates, and Cre/EGFP reporter mice**

The Cre/loxP recombination system was utilized to investigate the role of ENaC in the CNT for Na+ and K+ balance. We used a transgenic mouse line (genetic background: C57BL/6J) in which exon 1 of the gene encoding the α-subunit of ENaC (Scnn1a) was flanked by loxP sites [Scnn1a<sup>lox/lox</sup> (13)]. The Scnn1a<sup>lox/lox</sup> mouse line was crossed with a mouse line (genetic background: C57Bl6/CBA) expressing Cre recombinase under the regulatory elements of the Atp6v1b1 gene (Atp6v1b1::Cre) encoding the V-ATPase B1-subunit (20). The Atp6v1b1::Cre mouse line was also recently utilized to inactivate aquaporin-2 genetically in the CNT (16). Interbreeding of Scnn1a<sup>lox/lox</sup> and heterozygous Atp6v1b1::Cre mice generated Scnn1a<sup>lox/</sup>/+; Atp6v1b1::Cre mice, which were further crossed to generate Scnn1a<sup>lox/lox</sup>; Atp6v1b1::Cre mice. Finally, the Scnn1a<sup>lox/lox</sup>; Atp6v1b1::Cre mice were crossed with Scnn1a<sup>lox/lox</sup> mice to generate CNT-KO mice (Scnn1a<sup>lox/lox</sup>; Atp6v1b1::Cre) and control littermates (Scnn1a<sup>lox/lox</sup>). The mice were kept on a pelleted mouse chow standard diet (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) in regular cages at 20°C. Genotyping was carried out by running PCR analyses of tail biopsies using the primers: Scnn1a (forward) 5'-CTC AAT CAG AAG GAC CCT GG-3', Scnn1a (forward): 5'-GTC ACT GTG TGC ACC CTT AA-3', and Scnn1a (reverse): 5'-GCA CAA AGA TCT TAT CCA CC-3', Cre (forward): 5'-GTT CGC AAG AAC CTG ATG GAC-3', and Cre (reverse)5'-CTA GAG CCT GTT TTG CAC GTT-3' (13).
In order to examine Cre recombinase activity in the Atp6v1b1::Cre line, these mice were crossed with a dsRed/Cre-inducible EGFP reporter line [B6.Cg-Tg(CAG-DsRed,-EGFP)5Gae/J; The Jackson Laboratory, Bar Harbor, Maine, USA].

**Experimental protocols**

A mixed population of male and female mice was studied [body weight (BW) CNT-KO mice: 22.9 ± 0.4 g, n = 64; BW control mice: 22.6 ± 0.4 g, n = 60; P = 0.383]. For metabolic experiments, mice were kept in individual metabolic cages (Techniplast, Buguggiate, Italy) at 27°C and initially fed a 3-day specialized standard diet (0.25% Na+/0.7% K+; Altromin Spezialfutter GmbH & Co. KG, Lage, Germany). On day 3, baseline parameters were recorded (BW, food intake, water intake, and urine output) after which the mice were put on either a 4-day 0.01% Na+ diet (age: 10-15 weeks; for immunolabeling only), 7-day 0.01% Na+ diet (age: 13–28 weeks, two experiments pooled), 4-day 2% K+ diet (age: 12–25 weeks), or a 2-day 5% K+ diet (age: 10–37 weeks; two experiments pooled) or a or a 5% K+/0.01% Na+ diet (age: 5–25 weeks; two experiments pooled). The diets were given as a mixture of food and water [37.5% water (w/w)]. The added water was included in the total water intake. For experiments in regular cages (blood sampled on standard diet; two experiments were pooled for aldosterone measurements), mice were kept in individual cages at 20°C and fed a pelleted mouse chow diet for 7 days (Altromin Spezialfutter GmbH & Co. KG). Mice had free access to food and water for the duration of all experiments. On the last experimental day, mice were anesthetized by isoflurane inhalation, and blood and tissue were collected. All experimental protocols complied with the European Community guidelines for the use of experimental animals and were performed in agreement with a license issued by the Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food Administration.
Collection and analyses of urine and blood

Urine was collected in metabolic cages and cleared by centrifugation at 1000 g for 4 min, and concentrations of Na⁺ and K⁺ were measured using an IL943™ flame photometer (Instrumentation Laboratory, Bedford, MA, USA; range Na⁺ and K⁺: 0–200 mM; QC: standards). Blood was collected through the portal vein using a 0.6 x 25 mm needle containing 5 µl Li⁺ heparin solution and transferred to heparin-coated centrifuge tubes (PST™ LH Tubes; BD, Franklin Lakes, NJ, USA) and immediately centrifuged at 12,000 g for 4 min. Plasma concentrations of Na⁺ and K⁺ were measured by MRC Harwell (Oxfordshire, UK) and determined using an ion selective electrode (AU680; Beckman Coulter, Brea, CA, USA; range Na⁺: 50–200 mM; range K⁺: 1.0–10.0 mM, QC: standards). Plasma aldosterone concentrations were determined using an enzyme immunoassay kit (EIA-5298; DRG International Inc., Springfield, NJ, USA; range: 20–1000 pg/ml; QC: standards). Osmolality of urine and plasma was measured using a freezing point depression osmometer (Advanced® Model 3320 Micro-Osmometer; Advanced Instruments, Inc., Norwood, MA, USA; range: 0–2000 mOsm/kg; QC: standards). Urinary NGAL [neutrophil gelatinase-associated lipocalin; a biomarker for acute kidney injury (AKI) (7, 15)] concentrations were measured using a mouse NGAL ELISA kit (Kit 042, Bioporto Diagnostics, Hellerup, Denmark). Samples exceeding the upper limits of the test procedures were diluted according to the manufacturers’ protocols.

Immunolabeling

Mice were perfusion fixed via the left ventricle with 3% (v/v) paraformaldehyde in PBS (pH 7.4), where after tissue was post fixed for 1 hour at 4°C. Subsequently, the tissue was gradually dehydrated in ethanol, incubated in xylene, and embedded in paraffin. Using a previously described standard protocol (26), paraffin embedded kidney and colon sections (2 µm) from CNT-KO mice and control mice were labeled with primary αENaC rabbit antibody (28), dilution 1:800). Paraffin-embedded kidney sections from the Cre/EGFP reporter mice were labeled with NCC SPC-402D rabbit
primary antibody (StressMarq Biosciences Inc. Victoria, BC, Canada; dilution 1:200),
calbindin (D28K – 10R-C106A) mouse primary antibody (Fitzgerald Industries
International, Concord, MA, USA; dilution 1:20,000), AQP2 7661 rabbit primary
antibody ((24), dilution 1:1000), and EGFP goat primary antibody (ab6673, Abcam,
Cambridge, UK; dilution 1:1000). For light microscopy, immunolabeling was
visualized using peroxidase-conjugated goat anti-rabbit secondary antibody (p448;
Dako, Glostrup, Denmark; dilution 1:200) and 3,3’-diaminobenzidine (Kem-EN-Tec
Diagnostics A/S, Tåstrup, Denmark). For fluorescence microscopy, immunolabeling
was visualized using the secondary antibodies (dilution 1:600) donkey anti-goat 488
(Molecular Probes, Life Technologies), donkey anti-rabbit 555 (Molecular Probes, Life
Technologies), and donkey anti-mouse 633 (Molecular Probes, Life Technologies).

Microscopy

Counting of αENaC-positive cells in CNT/DCT2 (in cortical labyrinth), and CCD (in
medullary arrays) was performed on kidney sections from CNT-KO mice and control
mice directly in the microscope (400x magnification). Only cells with a distinct
nucleus and apical αENaC labeling were counted. Furthermore, counting was only
performed on cells situated in tubules with a clear visible luminal space and with at
least one αENaC-positive cell in the tubule. The total number of cells counted in the
cortical labyrinth were 345 in the CNT-KO mice (n = 5) and 402 in the control mice (n
= 5). The total number of cells counted in medullary arrays were 86 in the CNT-KO
mice (n = 5) and 70 in the control mice (n = 5). The fraction of αENaC-positive cells
was calculated from the number of αENaC-positive cells divided by the total number
of cells counted in each animal. Imaging of kidney and distal colon sections was
carried out using a Leica DMRE light microscope equipped with a digital camera
(Leica, Wetzlar, Germany). Imaging of kidney and distal colon sections from
Cre/EGFP reporter mice (n = 2) were performed using a Leica TCS SL laser scanning
confocal microscope and Leica confocal software (Leica). Images were merged using
Image J software (Image J, Bethesda, MD, USA). Counting of EGFP-positive cells in the
CNT and DCT2 was performed on confocal images taken from Cre/EGFP mice
reporter mice, which were labeled for EGFP, calbindin and NCC \( (n = 2 \) animals, one slide from each animal). The images were taken with a 63x objective. The fraction of EGFP-positive CNT cells (strongly calbindin-positive/NCC-negative) was calculated from the number of EGFP-positive/strongly calbindin-positive/NCC-negative cells divided by the total number of strongly calbindin-positive/NCC-negative cells. The fraction of EGFP-positive DCT2 cells (calbindin- and NCC-positive) was calculated from the number of EGFP-positive/calbindin- and NCC-positive cells divided by the total number of calbindin- and NCC-positive cells. The total number of cells counted in the CNT were 132 \( (n = 2) \) and 64 in the DCT2 \( (n = 2) \).

Semi-quantitative immunoblotting

Tissue was collected, dissected on ice, and immediately homogenized at 4°C in dissection buffer containing protease and phosphatase inhibitors. The homogenates were centrifuged at 1000 \( g \) for 10 min at 4°C. The supernatants were supplemented with sample buffer to a final concentration of 0.1 M SDS and heated to 65°C for 15 min. Samples were run on Criterion™ TGX™ Precast Gels (4–15% or Any kD; Bio-Rad Laboratories, Hercules, CA, USA) and transferred by electroelution to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) or Hybond-P PVDF membranes (GE Healthcare, Little Chalfont, UK). Subsequently, the membranes were blocked and incubated overnight at 4°C with primary rabbit antibodies [\( \alpha \)ENaC (30), dilution 1:1000; NKCC2 1495 (9, 14), dilution 1:50; pT96-T101-NKCC2 9934, (6) dilution 1:250]; NCC SPC-402D, dilution 1:1000; pT53-NCC 1246 (25), dilution 1:250; pT58-NCC 1251 (25), dilution 1:1000; or Anti-Kir1.1 (ROMK1) (Alomone, Jerusalem, Israel; dilution 1:400)]. Labeling was visualized using the Enhanced Chemiluminescence system (GE Healthcare, Little Chalfont, UK) or SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Because aldosterone might regulate housekeeping genes such as actin, Coomassie-stained gels were used to correct quantification for deviations in protein loading. The maximal deviations in total protein concentration between samples on individual blots were ±10%.
Statistical analyses

Data meeting statistical assumptions of normality and variance homogeneity were analyzed using Students two-sided t-test, while data only meeting assumptions of normality were analyzed using Satterthwaite's two-sided unequal variance t-test. Data not meeting assumptions of normality were ln-transformed or square-root transformed in accordance with Sokal and Rohlf (29), and analyzed using the appropriate t-tests. If data did not fulfill assumptions of normality after transformation, untransformed data were analyzed using Mann-Whitney's U-test. For parameters where the CNT-KO mice and control mice were compared at multiple time points, P-values were adjusted using FDR correction (22). Tests were carried out using Stata 12.0 (StataCorp, College Station, TX, USA) for Windows. All values are presented as mean ± SE.

RESULTS

Evaluation of CNT-KO mice

Breeding of CNT-KO mice and control mice followed Mendelian inheritance (50% Scnn1a\textsuperscript{lox/lox}; Atp6v1b1::Cre and 50% Scnn1a\textsuperscript{lox/lox}, n = 204). Using single immunolabeling, counting of αENaC-positive cells in the cortical labyrinth (no discrimination was made between CNT and DCT2) identified αENaC expression in approximately 45% of the cells in the CNT-KO mice, whereas in the control mice the fraction was approximately 70% (Fig. 1A, B and E). This corresponded to approximately 40% fewer αENaC-positive cells in the CNT-KO mice (P < 0.001). In the CCD (medullary arrays), the CNT-KO mice showed a weak tendency towards fewer αENaC-positive cells (9%; P = 0.217; Fig. 1C, D, and E). The overall lower fraction of αENaC-positive cells coincided with immunoblotting of cortical/outer medullary (OM) tissue showing 55% lower total protein intensity of αENaC in the CNT-KO mice [both cleaved (30 kD) and full length (90 kD), P < 0.001, Fig. 1F and G]. Cre recombinase activity in the DCT2 was examined by crossing the Atp6v1b1::Cre mice
with an dsRed/inducible enhanced green fluorescent protein (EGFP) reporter line (Fig. 2 and 3). The progeny expressed EGFP in only a minor portion of the DCT2 cells (approximately 6% [5% and 7%, respectively, in the 2 mice], Fig. 2A–C), whereas the majority of DCT2 cells were EGFP-negative (Fig. 3A–C). This indicated that a potential deletion of αENaC in the DCT2 was minimal. Consistent with the lower number of αENaC-positive cells in the CNT-KO, the EGFP reporter mouse expressed EGFP in a high number of the CNT cells (Fig. 2D–I), while EGFP was absent in the majority of CCD principal cells (Fig. 2J–L and 3D–F). Cellular counting revealed that approximately 36% of the CNT cells ([32% and 39%, respectively, in the 2 mice], identified as strongly calbindin-positive and NCC-negative) expressed EGFP. Thus, characterization of the mice indicated that αENaC was inactive primarily in a part of the CNT cells.

**Distal colonic αENaC expression was not impaired in the CNT-KO mice.**

ENaC is an important mediator of Na⁺ reabsorption in the surface epithelial layer of the distal colon (8, 19). It was previously reported that the Atp6v1b1::Cre mice show unspecific Cre recombinase activity in the colon (20). This could potentially cause inactivation of αENaC in the distal colon of the CNT-KO mice. The Cre/EGFP reporter mice showed no detectable EGFP expression in the surface epithelial layer or in the crypts (Fig. 4A). However, some cells in the connective tissue expressed EGFP (Fig. 4A, arrows), indicating Cre recombinase activity. We further tested whether αENaC expression was impaired in the distal colon of the CNT-KO mice by using immunolabeling and immunoblotting. Immunolabeling detected αENaC in the surface epithelial layer of both CNT-KO mice and control mice kept on a 4-day 0.01% Na⁺ diet (Fig. 4B and C). Immunoblotting demonstrated that the abundance of total αENaC [(cleaved (30 kD) + full length (90 kD))] did not differ between CNT-KO mice and control mice kept on the 7-day 0.01% Na⁺ diet [P = 0.512; Fig.4 and E]. However, the 30 kD/90 kD ratio was higher in the CNT KO mice [P = 0.017, Fig. 4D and E], suggesting greater cleavage of αENaC and thus, increased activity [reviewed in (27)]. A non-regulated band was found at 55 kD [CNT-KO mice: 1.00 ± 0.08 (n = 9), control
mice: 1.00 ± 0.22 (n = 9), P = 0.986, Fig. 4D and E], but whether this was a cleaved form of αENaC was not further examined. Collectively, data demonstrated that αENaC expression was not impaired in the distal colon of the CNT-KO mice.

Phenotyping of CNT-KO mice on standard and challenging diets.

To examine the effect of genetic αENaC inactivation in the CNT on Na⁺ and K⁺ homeostasis, CNT-KO mice and control mice were examined on a standard diet (baseline), 7-day 0.01% Na⁺ diet, 4-day 2% K⁺ diet, a 2-day 5% K⁺ diet, or a 5% K⁺/0.01% Na⁺ diet.

**Standard diet.** When fed a standard diet in regular cages, no differences were found in plasma [Na⁺], [K⁺], creatinine concentration, or osmolality between CNT-KO mice and control mice (Fig. 5A, B, D and E). However, the plasma aldosterone concentration was higher in the CNT-KO mice (P < 0.01, Fig. 5C). Baseline measurements in metabolic cages did not show any differences in the tested parameters [relative body weight (BW), food intake, water intake, urine output, urine Na⁺ and K⁺ output, and urine osmolality; Fig. 6A–U]. Similarly, no significant differences in these parameters were observed when pooling baseline data (collected over the last 24 h prior to diet manipulation) from the metabolic cage experiments (data not shown).

**Seven-day 0.01% Na⁺ diet.** Challenging the CNT-KO mice with a 7-day 0.01% Na⁺ diet did not affect plasma [Na⁺], [K⁺], creatinine concentration, or osmolality (Fig. 5A, B, D and E), however, the plasma aldosterone concentration was still higher compared to the control mice (P < 0.01, day 7, Fig. 5C). On day 3–5, the CNT-KO mice excreted more Na⁺ in the urine than the control mice (P between 0.01 and 0.05; Fig. 6E), however, the difference diminished on day 6 and eventually disappeared on day 7 [not significant (NS), Fig. 6E]. The urine osmolality was higher in the CNT-KO mice on day 4 and 7 (P < 0.05, Fig. 6G) compared to the control mice, whereas no differences were found in relative BW, food intake, water intake, urine output, and urine K⁺ excretion (Fig. 6A–D and F).
Four-day 2% $K^+$ diet. When challenged with a 4-day 2% $K^+$ diet, plasma [Na$^+$], [K$^+$], creatinine concentration, and osmolality (Fig. 5A, B, and D) did not differ between the CNT-KO mice and the control mice, however, the plasma aldosterone concentration was clearly higher (day 4: $P < 0.001$, Fig. 5C). By contrast, no differences were found in relative BW, food and water intake, urine output, urine Na$^+$ and K$^+$ output, and urine osmolality (Fig. 6H–N).

Two-day 5% $K^+$ diet. The 2-day 5% $K^+$ diet induced a lower relative BW in the CNT-KO mice compared to the control mice (day 1: $P < 0.001$; day 2: $P < 0.001$; Fig. 6O), which was accompanied by lower plasma [Na$^+$] (day 2: $P < 0.001$, Fig. 5A). The total urine Na$^+$ output was unaffected (NS, Fig. 6S), however, the CNT-KO mice consumed less food (and thereby less Na$^+$) than the control mice (day 1: $P < 0.001$; day 2: $P < 0.01$; Fig. 6P). This was taken into account by calculating the Na$^+$ output (urine)/intake (food) ratio. The Na$^+$ output/intake ratio was higher in the CNT-KO mice compared to the control mice on both day 1 ($P < 0.001$, Table 1) and day 2 ($P < 0.01$, Table 1). Furthermore, the fractional excretion (FE) of Na$^+$ was higher in the CNT-KO mice when measured on day 2 ($P < 0.01$, Table 1). In terms of K$^+$ homeostasis, the CNT-KO mice presented higher plasma [K$^+$] (day 2: $P < 0.001$, Fig. 5B) and lower urine K$^+$ output (day 1: $P < 0.001$, day 2: $P < 0.05$, Fig. 6T) compared to the control mice, but the K$^+$ output/intake ratio was not different, neither on day 1 nor day 2 (NS, Table 1). However, the FE of K$^+$ was lower in CNT-KO mice when measured on day 2 ($P < 0.05$, Table 1), indicating impaired K$^+$ excretion. Finally, the CNT-KO mice showed higher plasma aldosterone concentration (day 2: $P < 0.001$, Fig. 5C), creatinine concentration ($P < 0.01$, Table 1), and lower glomerular filtration rate (GFR; $P < 0.05$, Table 1), water intake (day 1: $P < 0.01$, Fig. 6Q), and urine output (day 1: $P < 0.05$, Fig. 6R) than the control mice. NGAL concentrations were measured in urine samples collected on day 2. There were no significant differences in urinary NGAL concentrations [CNT-KO mice: 150.8 ± 52.4 ng/ml ($n = 10$), control mice: 85.8 ± 35.4 ng/ml ($n = 8$), $P = 0.131$] or in urinary NGAL excretion [CNT-KO mice: 32.0 ± 11.6 ng/g BW/24 h ($n = 10$), control mice: 16.6 ± 6.8 ng/g BW/24 h ($n = 8$), $P = 0.248$].
Mice were, furthermore, tested on a 2-day 5% K+/0.01% Na+ diet. The control mice showed lower relative BW both on day 1 ($P < 0.001$) and day 2 ($P < 0.001$) compared to baseline, suggesting that BW loss during K+ loading was not specific for the CNT-KO mice, but could be induced in control mice when combining K+ loading and Na+ restriction. However, the weight loss was less severe in the control mice compared to the CNT-KO both on day 1 (control: 0.96 ± 0.01, $n = 10$; KO: 0.91 ± 0.00, $n = 10$; $P < 0.001$) and day 2 (control: 0.94 ± 0.01, $n = 10$; KO: 0.86 ± 0.01, $n = 10$; $P < 0.001$).

The CNT-KO mice presented lower abundance and phosphorylation of NCC on a 5% K+ diet

Hyperkalemia is connected with reduced Na+ reabsorption in the thick ascending limb (TAL) and the distal convoluted tubule (DCT), leading to increased Na+ delivery to ENaC-expressing tubular segments thereby favoring K+ secretion (30, 31, 34, 35). Therefore, we tested whether the higher FE of Na+ in the CNT-KO mice compared to the control mice during K+ loading occurred in parallel with lower protein abundance and phosphorylation of the Na+-K+-2Cl- cotransporter, NKCC2 (expressed in TAL), and the Na+-Cl- cotransporter, NCC (expressed in DCT). Immunoblotting of cortical/OM tissue (collected on day 2) showed no differences in the protein abundances of NKCC2, pT96-T101-NKCC2, or the pT96-T101-NKCC2/NKCC2 ratio (NS, Fig. 7). By contrast, we found a lower abundance of total NCC ($P < 0.05$, Fig. 7), pT53-NCC ($P < 0.01$, Fig. 7), and pT58-NCC ($P < 0.001$, Fig. 7). Furthermore, the pT53-NCC/NCC ($P < 0.05$, Fig. 7) and the pT58-NCC/NCC ratios ($P < 0.001$, Fig. 7) were lower in the CNT-KO mice, suggesting that the lower phosphorylation of NCC was not a result of lower total NCC protein abundance per se. Finally, no effect was found on ROMK abundance (Fig. 7). Collectively, the higher FE of Na+ in the CNT-KO mice fed a 5% K+ diet coincided with lower abundance and phosphorylation of NCC in the DCT and lower αENaC expression in the CNT.
This study provides novel insight into the physiological role of αENaC in the kidney and the activation of compensatory mechanisms in a conditional model of the PHA-1 syndrome. CNT-KO mice showed a severe phenotype when examined on a 5% K+ diet as evidenced by e.g. higher plasma [K+] and lower FE of K+. This could be a result of impaired ENaC-mediated K+ secretion in CNT through apical K+ channels such as ROMK (2). Although the CNT-KO mice were unable to maintain K+ balance to the same extent as the controls, data indicated that compensatory mechanisms did take place. The mice presented higher plasma aldosterone level and lower abundance and phosphorylation of the DCT-specific Na+ transporter, NCC, indicating lower Na+ reabsorption in the DCT. Inhibition of Na+ reabsorption in the TAL and the DCT during hyperkalemia may lead to higher tubular flow rate and Na+ delivery to ENaC-expressing tubule segments (30, 31, 34, 35), producing a favorable gradient for K+ secretion in the CNT and CCD. Thus, a compensatory target in our model could be NCC in the DCT. By contrast, we did not observe a significantly lower abundance or phosphorylation of NKCC2, which is vital for Na+ reabsorption in the TAL and macula densa. Another potential compensatory target is ROMK, but our data did not show any changes in ROMK abundance either.

On the 5% K+ diet, lower NCC activity, together with inactivation of αENaC in the CNT, coincided with higher FE of Na+ and Na+ output/intake ratio in the CNT-KO mice. Importantly, this suggests that in the CNT-KO mice, ENaC in the CD was not sufficient to effectively reabsorb a higher Na+ delivery. Collectively, these events may have induced the lower plasma [Na+] in the CNT-KO mice. To exclude that the phenotype could be a result of AKI, urinary NGAL concentrations were determined on day 2. The lack of differences in both urinary NGAL concentration and excretion as well as the lack of differences in urine output and water intake between the CNT-KO mice and the control mice on day 2 indicated that the CNT-KO mice had not developed AKI. We did not observe any difference in total urinary Na+ output after 1 and 2 days of 5% K+ loading, however, it cannot be excluded that a potential difference in Na+ output could be detected at earlier time points (i.e. after minutes) (30).
The CNT-KO mice presented higher aldosterone levels on the low-Na\(^+\) diet and a mild phenotype as evidenced by Na\(^+\) wasting on day 3–5. However, this effect eventually disappeared after 7 days, after which no effects were found on plasma [Na\(^+\)] and [K\(^+\)]. Data indicated higher cleavage of ENaC in the distal colon and this potential activation of ENaC could, at least partially, have compensated for reduced renal Na\(^+\) reabsorption in the CNT.

We have previously shown that genetic inactivation of αENaC in the CD did not interfere with Na\(^+\) and K\(^+\) balance in mice kept on a standard diet, low-Na\(^+\) diet, or a 6% K\(^+\) diet (28). This suggests that αENaC in the CD is apparently not a prerequisite for maintaining Na\(^+\) and K\(^+\) homeostasis. By contrast, combined αENaC inactivation in a part of the CNT (approximately 30% fewer αENaC-positive cells in early CNT, and 70% fewer αENaC-positive cells in late CNT) and CD induced PHA-1 symptoms already on a standard diet as evidenced by natriuresis, hyponatremia, and hyperkalemia (5). During Na\(^+\) restriction, the CNT/CD-KO mice showed lower relative BW, severe urine Na\(^+\) loss, hyponatremia, and hyperkalemia (5). Thus, the phenotype was stronger in the CNT/CD-KO mice than in the CNT-KO mice on a standard diet or a low-Na\(^+\) diet. Besides a potential compensation in the colon, it is possible that the CD in the CNT-KO mice could partially compensate for the lack of αENaC in the CNT. Because αENaC was deleted in the CD of the CNT/CD-KO mice, such compensation was not possible in these mice. However, it cannot be ruled out either that the deletion of αENaC in the CNT-KO was less efficient than in the CNT/CD-KO mice thereby explaining the milder phenotype. Thus, it is possible that the remaining CNT cells still expressing αENaC in the CNT-KO were sufficient to maintain proper Na\(^+\) balance.

A 5% K\(^+\) diet induced a weaker phenotype in the CNT/CD-KO mice than in the CNT-KO mice. Both mouse lines showed lower plasma [Na\(^+\)] and higher plasma [K\(^+\)] on the 5% K\(^+\) diet, however, the CNT-KO mice additionally presented lower food intake and lower relative BW, whereas these parameters were unaffected in the CNT/CD-KO mice. Impaired ENaC-facilitated K\(^+\) secretion in the colon (11, 32), was not likely to cause the stronger phenotype in the CNT-KO mice, because the colonic αENaC expression was intact. Although the role of ENaC in the DCT2 for K\(^+\) secretion
is unknown, it could be speculated that deletion of αENaC only in a few DCT2 cells could become critical during K\(^+\) loading.

In summary, we examined the importance of αENaC in the CNT by generating KO mice in which αENaC was deleted primarily in the CNT. The mice showed no obvious phenotype on a standard diet, 0.01% Na\(^+\) diet, or a 2% K\(^+\) diet. The elevated aldosterone levels in the CNT-KO mice may stimulate ENaC activity in the late CNT (in remaining ENaC expressing cells), in the CD, or in the colon and potentially compensate for the reduced Na\(^+\) reabsorption in the CNT. On a 5% K\(^+\) diet, however, the mice presented clear PHA-1 symptoms. Our data provide an unprecedented insight into compensatory mechanisms taking place in a conditional model of the PHA-1 syndrome. Even when αENaC is deleted only in a part of the CNT, several compensatory mechanisms occur. During 5% K\(^+\) loading, this includes higher plasma aldosterone level, lower renal NCC activity, and lower GFR. Clinically, the results may contribute to a deeper understanding of how the body copes with the physiological challenges that are taking place in PHA-1 patients.

ACKNOWLEDGMENTS

The antibodies against αENaC, and pT53-NCC and pT58-NCC used for semi-quantitative immunoblotting were kindly provided by J. Loffing (Institute of Anatomy, University of Zurich, Switzerland) and R. Fenton (Department of Biomedicine, Aarhus University, Denmark), respectively. We thank J. Frøkiær (Department of Clinical Medicine, Aarhus University Hospital, Aarhus, Denmark) for help on measuring urine [Na\(^+\)] and [K\(^+\)], and I. M. S. Paulsen, H. Høyer, C. Westberg, T. Drejer, P. A. Nielsen, and M. S. Gandry for technical assistance.

GRANTS

Funding for this study was provided by the Danish Council for Independent Research (B.M.C.), the Lundbeck Foundation (B.M.C.), the Danish Heart Foundation (B.M.C.), and Health (Faculty of Health Sciences, Aarhus University; S.B.P.).
DISCLOSURES

None.

AUTHOR CONTRIBUTIONS

S.B.P.: conception and design of the experiments; collection, analysis, and interpretation of data; drafting the article or revising it critically for important intellectual content; J.P.: drafting the article or revising it critically for important intellectual content; H.H.D.: collection of data; drafting the article or revising it critically for important intellectual content; L.M.: drafting the article or revising it critically for important intellectual content; R.D.N.: drafting the article or revising it critically for important intellectual content; E.H.: drafting the article or revising it critically for important intellectual content; B.M.C.: conception and design of the experiments; collection, analysis, and interpretation of data; drafting the article or revising it critically for important intellectual content; All authors approved the final version of the manuscript.
REFERENCES


34. **Vallon V, Schroth J, Lang F, Kuhl D and Uchida S.** Expression and phosphorylation of the Na⁺-Cl⁻ cotransporter NCC in vivo is regulated by

FIGURE LEGENDS

Fig. 1. αENaC expression was lower in the CNT-KO mice. A–D, in mice kept on a 4-day 0.01% Na+ diet, immunolabeling showed more αENaC-positive cells in the CNT/DCT2 (in the cortical labyrinth) of controls (A, arrows indicate αENaC-positive cells) than in the CNT-KO mice (B, arrow heads indicate αENaC-negative cells). E, the fraction of αENaC-positive cells in the CNT/DCT2 was approximately 40% lower in the CNT-KO mice, whereas no significant difference was found in the CCD (C–E, arrows indicate αENaC-positive cells, in medullary arrays). F and G, on a standard diet, the CNT-KO mice showed 55% lower total αENaC protein intensity in cortical/outer medullary tissue homogenate [cleaved (30 kD) + full length (90 kD)]. Each bar indicates mean ± SE. **P < 0.01, ***P < 0.001.

Fig. 2. Atp6v1b1::Cre/enhanced green fluorescent protein (EGFP) reporter mice expressed EGFP in AQP2 and calbindin-positive cells in the CNT, whereas only a few NCC-positive cells in the DCT2 and a few AQP2-positive cells in the CCD were EGFP positive. Calbindin was used a marker for CNT/DCT2. A–C, arrow: NCC-positive/EGFP-positive cell. D–F, arrow: calbindin-positive/EGFP-positive cell; arrowhead: calbindin-negative/EGFP-positive cell; asterisk: calbindin-positive/EGFP-negative cell. G–I, arrow: calbindin-positive/AQP2-positive/EGFP-positive cell; arrowhead: AQP2-negative/EGFP-positive cell; asterisk: AQP2-negative/EGFP-positive cell. J–L, arrow: AQP2-positive/EGFP-negative cell; arrowhead: AQP2-negative/EGFP-positive cell; asterisk: AQP2-positive/EGFP-negative cell. Glo: glomerulus.

Fig. 3. The majority of NCC-positive cells in the DCT2 and AQP2-positive cells in the CCD did not express EGFP in the Atp6v1b1::Cre/EGFP reporter mice. A–C, arrowhead: NCC-negative/EGFP-positive cell; asterisks: NCC-positive/EGFP-negative cells. D–F, arrowhead: AQP2-negative/EGFP-positive cell; asterisks: AQP2-positive/EGFP-negative cells.
Fig. 4. Distal colonic αENaC expression was not impaired in the CNT-KO mice. A, Atp6v1b1::Cre/EGFP reporter mice showed EGFP expression in some cells of the connective tissue in the distal colon (arrows), whereas EGFP was not detectable in the surface epithelial layer (n = 2). B, in the control mice and C, the CNT-KO mice kept on a 4-day 0.01% Na+ diet, immunolabeling revealed clear apical labelling in the distal colonic surface epithelial layer. D and E, immunoblotting and corresponding densitometric analyses of distal colonic homogenates showed that total αENaC abundance (30+90 kD bands, arrows) did not differ between the control mice and the CNT-KO mice kept on a 7-day 0.01% Na+ diet. The 30/90 kD-ratio was, however, higher in the CNT-KO mice than in the control mice. Each bar indicates mean ± SE. *P < 0.05. LU: lumen, CR: crypt.

Fig. 5. Blood parameters: the CNT-KO mice presented a clear phenotype on a 5% K+ diet. Effects of various diets on, A, plasma [Na+], B, plasma [K+], C, plasma aldosterone concentration, D, plasma creatinine concentration, and, E, plasma osmolality in the control mice and the CNT-KO mice. The mice were kept on either a standard diet in regular cages ([Na+], [K+], creatinine and osmolality: control n = 7, KO n = 9; aldosterone: control n = 14, KO n = 17), 7-day 0.01% Na+ diet in metabolic cages ([Na+], [K+], aldosterone, and osmolality: control n = 17, KO n = 18; creatinine: control n = 15, KO n = 15), 4-day 2% K+ diet in metabolic cages (control n = 9, KO n = 9), or a 2-day 5% K+ diet in metabolic cages (control n = 8, KO n = 10). Each bar indicates mean ± SE. **P < 0.01, ***P < 0.001.

Fig. 6. Metabolic parameters: the CNT-KO mice presented a clear phenotype on a 5% K+ diet. The control mice and the CNT-KO mice were kept in metabolic cages on a standard diet (baseline) followed by either a, A–G, 7-day 0.01% Na+ diet (control n = 17–18, KO n = 17–18), H–N, 4-day 2% K+ diet (control n = 9, KO n = 9), or, O–U, a 2-day 5% K+ diet (O–R, control n = 17, KO n = 18; S–U, control n = 9, KO n = 8). Each circle indicates mean ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.
Fig. 7. The CNT-KO mice presented lower abundance and phosphorylation of NCC on a 5% K⁺ diet. Presented are protein abundances of NKCC2, p-NKCC2 (pT96/T101), NCC, pT53-NCC, pT58-NCC, and ROMK in control mice (n = 8) and CNT-KO mice (n = 10) as determined by immunoblotting of cortical/outer medullary tissue homogenates. Furthermore, presented are the ratios of pT96-T101-NKCC2/NKCC2, pT53-NCC/NCC, and pT58-NCC/NCC. Each bar indicates mean ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.
Table 1. Urine and plasma parameters in control mice and CNT-KO mice kept on a 2-day 5% K+ diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>KO</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>n</td>
</tr>
<tr>
<td>Na(^+) output/intake ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.73</td>
<td>0.07</td>
<td>9</td>
</tr>
<tr>
<td>day 1</td>
<td>0.98</td>
<td>0.05</td>
<td>9</td>
</tr>
<tr>
<td>day 2</td>
<td>0.72</td>
<td>0.04</td>
<td>9</td>
</tr>
<tr>
<td>K(^+) output/intake ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.72</td>
<td>0.05</td>
<td>9</td>
</tr>
<tr>
<td>day 1</td>
<td>0.73</td>
<td>0.03</td>
<td>9</td>
</tr>
<tr>
<td>day 2</td>
<td>0.69</td>
<td>0.03</td>
<td>9</td>
</tr>
<tr>
<td>FE Na(^+) (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>0.42</td>
<td>0.03</td>
<td>8</td>
</tr>
<tr>
<td>FE K(^+) (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>71</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Plasma creatinine (mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>7.6</td>
<td>0.4</td>
<td>8</td>
</tr>
<tr>
<td>GFR (µl/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>290</td>
<td>30</td>
<td>8</td>
</tr>
</tbody>
</table>
Cre/EGFP reporter mouse

AQP2
Calb
EGFP

NCC
Calb
EGFP

Glo

AQP2
Calb
EGFP

NCC
Calb
EGFP

DCT2

CNT

CNT

CCD
Cre/EGFP reporter mouse

DCT2

A

NCC
Calb
EGFP

40 μm

B

NCC

40 μm

C

EGFP

40 μm

CCD

D

AQP2
Calb
EGFP

40 μm

E

AQP2

40 μm

F

EGFP

40 μm
Cre/EGFP reporter mouse

A B C

20 μm 20 μm 20 μm 20 μm

D E

Con KO

Fraction of con

Con KO

CR CR CR

LU LU LU

30 kD 30 kD 99 kD 99 kD

**KO**
2% K+ diet

Relative BW

Food intake (g/g BW)

Water intake (g/g BW)

Urine output (g/g BW)

Urine Na+ output (mmol/g BW)

Urine K+ output (mmol/g BW)

Urine Osmolality (mOsm/kg)

0.70

0.80

0.90

1.00

1.10

Baseline 1d 2d 3d 4d 5d 6d 7d

Baseline 1d 2d 3d 4d

Baseline 1d 2d
5% K+ diet

- NKCC2
- p-NKCC2
- NCC
- pT53-NCC
- pT58-NCC
- ROMK

Comparisons between Con and KO:

- Fraction of con

Legend:
- Con
- KO

Significance:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001