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1 # Reducing αENaC expression in kidney connecting tubule

2 # induces pseudohypoaldosteronism type 1 symptoms during K$^+$

3 # loading

4

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12 Running head: Important role of ENaC in CNT

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ABSTRACT

Genetic inactivation of the epithelial Na$^+$ channel α-subunit (αENaC) in the renal collecting duct (CD) does not interfere with Na$^+$ and K$^+$ 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 αENaC in the CNT. Knock-out mice with αENaC 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$^+$] and [K$^+$], and urine Na$^+$ and K$^+$ output were unaffected. Seven days of Na$^+$ restriction (0.01% Na$^+$) led to a higher urine Na$^+$ output only on day 3–5, and after 7 days plasma [Na$^+$] and [K$^+$] were unaffected. By contrast, the CNT-KO mice were highly susceptible to a 2-day 5% K$^+$ diet and showed lower food intake and relative body weight, lower plasma [Na$^+$], higher fractional excretion (FE) of Na$^+$, higher plasma [K$^+$], and lower FE of K$^+$. The higher FE of Na$^+$ coincided with lower abundance and phosphorylation of the Na$^+$-Cl$^-$ cotransporter, NCC. In conclusion, reducing ENaC expression in CNT induces clear PHA-1 symptoms during high dietary K$^+$ loading.

KEYWORDS

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

73   the CNT cells (20). This correlates with weak V-ATPase B1-subunit expression in

74   some CNT cells (1, 21). We performed a thorough characterization of the

75   *Atp6v1b1*::*Cre* mouse line by crossing it with an enhanced green fluorescent protein

76   (EGFP) reporter mouse line. The CNT-KO mice and the control littermates were

77   examined on standard and challenging diets.

78

79   **METHODS**

80

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

82

83   The Cre/loxP recombination system was utilized to investigate the role of ENaC

84   in the CNT for Na^+ and K^+ balance. We used a transgenic mouse line (genetic

85   background: C57BL/6J) in which exon 1 of the gene encoding the α-subunit of ENaC

86   (*Scnn1a*) was flanked by loxP sites [*Scnn1a*lox/lox (13)]. The *Scnn1a*lox/lox mouse line

87   was crossed with a mouse line (genetic background: C57Bl6/CBA) expressing Cre

88   recombinase under the regulatory elements of the *Atp6v1b1* gene (*Atp6v1b1*::*Cre*)

89   encoding the V-ATPase B1-subunit (20). The *Atp6v1b1*::*Cre* mouse line was also

90   recently utilized to inactivate aquaporin-2 genetically in the CNT (16). Interbreeding

91   of *Scnn1a*lox/lox and heterozygous *Atp6v1b1*::*Cre* mice generated *Scnn1a*lox/+;

92   *Atp6v1b1*::*Cre* mice, which were further crossed to generate *Scnn1a*lox/lox;

93   *Atp6v1b1*::*Cre* mice. Finally, the *Scnn1a*lox/lox; *Atp6v1b1*::*Cre* mice were crossed with

94   *Scnn1a*lox/lox mice to generate CNT-KO mice (*Scnn1a*lox/lox; *Atp6v1b1*::*Cre*) and control

95   littermates (*Scnn1a*lox/lox). The mice were kept on a pelleted mouse chow standard

96   diet (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) in regular cages at 20°C.

97   Genotyping was carried out by running PCR analyses of tail biopsies using the

98   primers: *Scnn1a* (forward) 5'-CTC AAT CAG AAG GAC CCT GG-3', *Scnn1a* (forward):

99   5'-GTC ACT GTG TGC ACC CTT AA-3', and *Scnn1a* (reverse): 5'-GCA CAA AGA TCT TAT

100   CCA CC-3', *Cre* (forward): 5'-GTT CGC AAG AAC CTG ATG GAC-3', and *Cre* (reverse)5'-

101   CTA GAG CCT GTT TTG CAC GTT-3' (13).

4

102   In order to examine Cre recombinase activity in the *Atp6v1b1*::*Cre* line, these
103   mice were crossed with a dsRed/Cre-inducible EGFP reporter line [B6.Cg-Tg(CAG-
104   DsRed,-EGFP)5Gae/J; The Jackson Laboratory, Bar Harbor, Maine, USA].

105

106   *Experimental protocols*

107

108   A mixed population of male and female mice was studied [body weight (BW)
109   CNT-KO mice: 22.9 ± 0.4 g, $n = 64$; BW control mice: 22.6 ± 0.4 g, $n = 60$; $P = 0.383$].
110   For metabolic experiments, mice were kept in individual metabolic cages
111   (Techniplast, Buguggiate, Italy) at 27°C and initially fed a 3-day specialized standard
112   diet (0.25% Na^+/0.7% K^+; Altromin Spezialfutter GmbH & Co. KG, Lage, Germany). On
113   day 3, baseline parameters were recorded (BW, food intake, water intake, and urine
114   output) after which the mice were put on either a 4-day 0.01% Na^+ diet (age: 10-15
115   weeks; for immunolabeling only), 7-day 0.01% Na^+ diet (age: 13–28 weeks, two
116   experiments pooled), 4-day 2% K^+ diet (age: 12–25 weeks), or a 2-day 5% K^+ diet
117   (age: 10–37 weeks; two experiments pooled) or a or a 5% K^+/0.01% Na^+ diet (age: 5–
118   25 weeks; two experiments pooled). The diets were given as a mixture of food and
119   water [37.5% water (w/w)]. The added water was included in the total water intake.
120   For experiments in regular cages (blood sampled on standard diet; two experiments
121   were pooled for aldosterone measurements), mice were kept in individual cages at
122   20°C and fed a pelleted mouse chow diet for 7 days (Altromin Spezialfutter GmbH &
123   Co. KG). Mice had free access to food and water for the duration of all experiments. On
124   the last experimental day, mice were anesthetized by isoflurane inhalation, and blood
125   and tissue were collected. All experimental protocols complied with the European
126   Community guidelines for the use of experimental animals and were performed in
127   agreement with a license issued by the Animal Experiments Inspectorate, Ministry of
128   Food, Agriculture and Fisheries, Danish Veterinary and Food Administration.

Collection and analyses of urine and blood

130

131   Urine was collected in metabolic cages and cleared by centrifugation at 1000 g for

132   4 min, and concentrations of Na^+ and K^+ were measured using an IL943™ flame

133   photometer (Instrumentation Laboratory, Bedford, MA, USA; range Na^+ and K^+: 0–200

134   mM; QC: standards). Blood was collected through the portal vein using a 0.6 x 25 mm

135   needle containing 5 µl Li^+ heparin solution and transferred to heparin-coated

136   centrifuge tubes (PST™ LH Tubes; BD, Franklin Lakes, NJ, USA) and immediately

137   centrifuged at 12,000 g for 4 min. Plasma concentrations of Na^+ and K^+ were

138   measured by MRC Harwell (Oxfordshire, UK) and determined using an ion selective

139   electrode (AU680; Beckman Coulter, Brea, CA, USA; range Na^+: 50–200 mM; range K^+:

140   1.0–10.0 mM, QC: standards). Plasma aldosterone concentrations were determined

141   using an enzyme immunoassay kit (EIA-5298; DRG International Inc., Springfield, NJ,

142   USA; range: 20–1000 pg/ml; QC: standards). Osmolality of urine and plasma was

143   measured using a freezing point depression osmometer (Advanced® Model 3320

144   Micro-Osmometer; Advanced Instruments, Inc., Norwood, MA, USA; range: 0–2000

145   mOsm/kg; QC: standards). Urinary NGAL [neutrophil gelatinase-associated lipocalin;

146   a biomarker for acute kidney injury (AKI) (7, 15)] concentrations were measured

147   using a mouse NGAL ELISA kit (Kit 042, Bioporto Diagnostics, Hellerup, Denmark).

148   Samples exceeding the upper limits of the test procedures were diluted according to

149   the manufacturers' protocols.

150

151   *Immunolabeling*

152

153   Mice were perfusion fixed via the left ventricle with 3% (v/v) paraformaldehyde

154   in PBS (pH 7.4), where after tissue was post fixed for 1 hour at 4°C. Subsequently, the

155   tissue was gradually dehydrated in ethanol, incubated in xylene, and embedded in

156   paraffin. Using a previously described standard protocol (26), paraffin embedded

157   kidney and colon sections (2 µm) from CNT-KO mice and control mice were labeled

158   with primary αENaC rabbit antibody (28), dilution 1:800). Paraffin-embedded kidney

159   sections from the Cre/EGFP reporter mice were labeled with NCC SPC-402D rabbit

160   primary antibody (StressMarq Biosciences Inc. Victoria, BC, Canada; dilution 1:200),

161   calbindin (D28K – 10R-C106A) mouse primary antibody (Fitzgerald Industries

162   International, Concord, MA, USA; dilution 1:20.000), AQP2 7661 rabbit primary

163   antibody ((24), dilution 1:1000), and EGFP goat primary antibody (ab6673, Abcam,

164   Cambridge, UK; dilution 1:1000). For light microscopy, immunolabeling was

165   visualized using peroxidase-conjugated goat anti-rabbit secondary antibody (p448;

166   Dako, Glostrup, Denmark; dilution 1:200) and 3,3'-diaminobenzidine (Kem-EN-Tec

167   Diagnostics A/S, Tåstrup, Danmark). For fluorescence microscopy, immunolabeling

168   was visualized using the secondary antibodies (dilution 1:600) donkey anti-goat 488

169   (Molecular Probes, Life Technologies), donkey anti-rabbit 555 (Molecular Probes, Life

170   Technologies), and donkey anti-mouse 633 (Molecular Probes, Life Technologies).

171

172   *Microscopy*

173

174   Counting of αENaC-positive cells in CNT/DCT2 (in cortical labyrinth), and CCD (in

175   medullary arrays) was performed on kidney sections from CNT-KO mice and control

176   mice directly in the microscope (400x magnification). Only cells with a distinct

177   nucleus and apical αENaC labeling were counted. Furthermore, counting was only

178   performed on cells situated in tubules with a clear visible luminal space and with at

179   least one αENaC-positive cell in the tubule. The total number of cells counted in the

180   cortical labyrinth were 345 in the CNT-KO mice ($n = 5$) and 402 in the control mice (n

181    $= 5$). The total number of cells counted in medullary arrays were 86 in the CNT-KO

182   mice ($n = 5$) and 70 in the control mice ($n = 5$). The fraction of αENaC-positive cells

183   was calculated from the number of αENaC-positive cells divided by the total number

184   of cells counted in each animal. Imaging of kidney and distal colon sections was

185   carried out using a Leica DMRE light microscope equipped with a digital camera

186   (Leica, Wetzlar, Germany). Imaging of kidney and distal colon sections from

187   Cre/EGFP reporter mice (n = 2) were performed using a Leica TCS SL laser scanning

188   confocal microscope and Leica confocal software (Leica). Images were merged using

189   Image J software (Image J, Bethesda, MD, USA). Counting of EGFP-positive cells in the

190   CNT and DCT2 was performed on confocal images taken from Cre/EGFP mice

191   reporter mice, which were labeled for EGFP, calbindin and NCC (n = 2 animals, one
192   slide from each animal). The images were taken with a 63x objective. The fraction of
193   EGFP-positive CNT cells (strongly calbindin-positive/NCC-negative) was calculated
194   from the number of EGFP-positive/strongly calbindin-positive/NCC-negative cells
195   divided by the total number of strongly calbindin-positive/NCC-negative cells. The
196   fraction of EGFP-positive DCT2 cells (calbindin- and NCC-positive) was calculated
197   from the number of EGFP-positive/calbindin- and NCC-positive cells divided by the
198   total number of calbindin- and NCC-positive cells. The total number of cells counted
199   in the CNT were 132 (n = 2) and 64 in the DCT2 (n = 2).
200
201   *Semi-quantitative immunoblotting*
202
203   Tissue was collected, dissected on ice, and immediately homogenized at 4°C in
204   dissection buffer containing protease and phosphatase inhibitors. The homogenates
205   were centrifuged at 1000 g for 10 min at 4°C. The supernatants were supplemented
206   with sample buffer to a final concentration of 0.1 M SDS and heated to 65° C for 15
207   min. Samples were run on Criterion™ TGX™ Precast Gels (4–15% or Any kD; Bio-Rad
208   Laboratories, Hercules, CA, USA) and transferred by electroelution to PVDF
209   membranes (Bio-Rad Laboratories, Hercules, CA, USA) or Hybond-P PVDF
210   membranes (GE Healthcare, Little Chalfont, UK). Subsequently, the membranes were
211   blocked and incubated overnight at 4°C with primary rabbit antibodies [αENaC (30),
212   dilution 1:1000; NKCC2 1495 (9, 14), dilution 1:50; pT96-T101-NKCC2 9934, (6)
213   dilution 1:250]; NCC SPC-402D, dilution 1:1000; pT53-NCC 1246 (25), dilution 1:250;
214   pT58-NCC 1251 (25), dilution 1:1000; or Anti-Kir1.1 (ROMK1) (Alomone, Jerusalem,
215   Israel; dilution 1:400)]. Labeling was visualized using the Enhanced
216   Chemiluminescence system (GE Healthcare, Little Chalfont, UK) or SuperSignal West
217   Femto Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Because
218   aldosterone might regulate housekeeping genes such as actin, Coomassie-stained gels
219   were used to correct quantification for deviations in protein loading. The maximal
220   deviations in total protein concentration between samples on individual blots were ±
221   10%.

Statistical analyses

223

224   Data meeting statistical assumptions of normality and variance homogeneity were

225   analyzed using Students two-sided *t*-test, while data only meeting assumptions of

226   normality were analyzed using Satterthwaite's two-sided unequal variance *t*-test.

227   Data not meeting assumptions of normality were ln-transformed or square-root

228   transformed in accordance with Sokal and Rohlf (29), and analyzed using the

229   appropriate *t*-tests. If data did not fulfill assumptions of normality after

230   transformation, untransformed data were analyzed using Mann-Whitneys U-test. For

231   parameters where the CNT-KO mice and control mice were compared at multiple

232   time points, *P*-values were adjusted using FDR correction (22). Tests were carried out

233   using Stata 12.0 (StataCorp, College Station, TX, USA) for Windows. All values are

234   presented as mean ± SE.

235

236   **RESULTS**

237

238   *Evaluation of CNT-KO mice*

239

240   Breeding of CNT-KO mice and control mice followed Mendelian inheritance (50%

241   *Scnn1a*$^{lox/lox}$; *Atp6v1b1*::*Cre* and 50% *Scnn1a*$^{lox/lox}$, *n* = 204). Using single

242   immunolabeling, counting of αENaC-positive cells in the cortical labyrinth (no

243   discrimination was made between CNT and DCT2) identified αENaC expression in

244   approximately 45% of the cells in the CNT-KO mice, whereas in the control mice the

245   fraction was approximately 70% (Fig. 1*A, B and E*). This corresponded to

246   approximately 40% fewer αENaC-positive cells in the CNT-KO mice (*P* < 0.001). In the

247   CCD (medullary arrays), the CNT-KO mice showed a weak tendency towards fewer

248   αENaC-positive cells (9%; *P* = 0.217; Fig. 1*C, D,* and E). The overall lower fraction of

249   αENaC-positive cells coincided with immunoblotting of cortical/outer medullary

250   (OM) tissue showing 55% lower total protein intensity of αENaC in the CNT-KO mice

251   [both cleaved (30 kD) and full length (90 kD), *P* < 0.001, Fig. 1*F* and *G*]. Cre

252   recombinase activity in the DCT2 was examined by crossing the *Atp6v1b1*::*Cre* mice

253   with an dsRed/inducible enhanced green fluorescent protein (EGFP) reporter line

254   (Fig. 2 and 3). The progeny expressed EGFP in only a minor portion of the DCT2 cells

255   (approximately 6% [5% and 7%, respectively, in the 2 mice], Fig. 2*A–C*), whereas the

256   majority of DCT2 cells were EGFP-negative (Fig. 3*A–C*). This indicated that a potential

257   deletion of αENaC in the DCT2 was minimal. Consistent with the lower number of

258   αENaC-positive cells in the CNT-KO, the EGFP reporter mouse expressed EGFP in a

259   high number of the CNT cells (Fig. 2*D–I*), while EGFP was absent in the majority of

260   CCD principal cells (Fig. 2*J–L* and 3*D–F*). Cellular counting revealed that

261   approximately 36% of the CNT cells ([32% and 39%, respectively, in the 2 mice],

262   identified as strongly calbindin-positive and NCC-negative) expressed EGFP. Thus,

263   characterization of the mice indicated that αENaC was inactive primarily in a part of

264   the CNT cells.

265

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

267

268   ENaC is an important mediator of Na^+ reabsorption in the surface epithelial layer

269   of the distal colon (8, 19). It was previously reported that the *Atp6v1b1*::*Cre* mice

270   show unspecific Cre recombinase activity in the colon (20). This could potentially

271   cause inactivation of αENaC in the distal colon of the CNT-KO mice. The Cre/EGFP

272   reporter mice showed no detectable EGFP expression in the surface epithelial layer or

273   in the crypts (Fig. 4*A*). However, some cells in the connective tissue expressed EGFP

274   (Fig. 4*A*, arrows), indicating Cre recombinase activity. We further tested whether

275   αENaC expression was impaired in the distal colon of the CNT-KO mice by using

276   immunolabeling and immunoblotting. Immunolabeling detected αENaC in the surface

277   epithelial layer of both CNT-KO mice and control mice kept on a 4-day 0.01% Na^+ diet

278   (Fig. 4*B* and *C*). Immunoblotting demonstrated that the abundance of total αENaC

279   [(cleaved (30 kD) + full length (90 kD)] did not differ between CNT-KO mice and

280   control mice kept on the 7-day 0.01% Na^+ diet [*P* = 0.512; Fig.4 and *E*]. However, the

281   30 kD/90 kD ratio was higher in the CNT KO mice [*P* = 0.017, Fig. 4*D* and *E*],

282   suggesting greater cleavage of αENaC and thus, increased activity [reviewed in (27)].

283   A non-regulated band was found at 55 kD [CNT-KO mice: 1.00 ± 0.08 (*n* = 9), control

284 mice: 1.00 ± 0.22 ($n = 9$), $P = 0.986$, Fig. 4*D* and *E*], but whether this was a cleaved

285 form of αENaC was not further examined. Collectively, data demonstrated that αENaC

286 expression was not impaired in the distal colon of the CNT-KO mice.

287

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

289

290 To examine the effect of genetic αENaC inactivation in the CNT on Na^+ and K^+

291 homeostasis, CNT-KO mice and control mice were examined on a standard diet

292 (baseline), 7-day 0.01% Na^+ diet, 4-day 2% K^+ diet, a 2-day 5% K^+ diet, or a 5%

293 K^+/0.01% Na^+ diet.

294

295 *Standard diet.* When fed a standard diet in regular cages, no differences were found in

296 plasma $[Na^+]$, $[K^+]$, creatinine concentration, or osmolality between CNT-KO mice and

297 control mice (Fig. 5*A*, *B*, *D* and *E*). However, the plasma aldosterone concentration

298 was higher in the CNT-KO mice ($P < 0.01$, Fig. 5*C*). Baseline measurements in

299 metabolic cages did not show any differences in the tested parameters [relative body

300 weight (BW), food intake, water intake, urine output, urine Na^+ and K^+ output, and

301 urine osmolality; Fig. 6*A–U*]. Similarly, no significant differences in these parameters

302 were observed when pooling baseline data (collected over the last 24 h prior to diet

303 manipulation) from the metabolic cage experiments (data not shown).

304

305 *Seven-day 0.01% Na^+ diet.* Challenging the CNT-KO mice with a 7-day 0.01% Na^+ diet

306 did not affect plasma $[Na^+]$, $[K^+]$, creatinine concentration, or osmolality (Fig. 5*A*, *B*, *D*

307 and *E*), however, the plasma aldosterone concentration was still higher compared to

308 the control mice ($P < 0.01$, day 7, Fig. 5*C*). On day 3–5, the CNT-KO mice excreted

309 more Na^+ in the urine than the control mice (P between 0.01 and 0.05; Fig. 6*E*),

310 however, the difference diminished on day 6 and eventually disappeared on day 7

311 [not significant (NS), Fig. 6*E*]. The urine osmolality was higher in the CNT-KO mice on

312 day 4 and 7 ($P < 0.05$, Fig. 6*G*) compared to the control mice, whereas no differences

313 were found in relative BW, food intake, water intake, urine output, and urine K^+

314 excretion (Fig. 6*A–D* and *F*).

315

Four-day 2% K+ diet. When challenged with a 4-day 2% K+ diet, plasma [Na+], [K+], creatinine concentration, and osmolality (Fig. 5*A*, *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. 5*C*). By contrast, no differences were found in relative BW, food and water intake, urine output, urine Na+ and K+ output, and urine osmolality (Fig. 6*H*–*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. 6*O*), which was accompanied by lower plasma [Na+] (day 2: *P* < 0.001, Fig. 5*A*). The total urine Na+ output was unaffected (NS, Fig. 6*S*), 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. 6*P*). 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. 5*B*) and lower urine K+ output (day 1: *P* < 0.001, day 2: *P* < 0.05, Fig. 6*T*) 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. 5*C*), 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. 6*Q*), and urine output (day 1: *P* < 0.05, Fig. 6*R*) 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].

346   Mice were, furthermore, tested on a 2-day 5% K^+/0.01% Na^+ diet. The control

347   mice showed lower relative BW both on day 1 ($P < 0.001$) and day 2 ($P < 0.001$)

348   compared to baseline, suggesting that BW loss during K^+ loading was not specific for

349   the CNT-KO mice, but could be induced in control mice when combining K^+ loading

350   and Na^+ restriction. However, the weight loss was less severe in the control mice

351   compared to the CNT-KO both on day 1 (control: 0.96 ± 0.01, n = 10; KO: 0.91 ± 0.00, n

352   = 10; $P < 0.001$) and day 2 (control: 0.94 ± 0.01, n = 10; KO: 0.86 ± 0.01, n = 10; $P <$

353   0.001).

354

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

356   *diet*

357

358   Hyperkalemia is connected with reduced Na^+ reabsorption in the thick ascending

359   limb (TAL) and the distal convoluted tubule (DCT), leading to increased Na^+ delivery

360   to ENaC-expressing tubular segments thereby favoring K^+ secretion (30, 31, 34, 35).

361   Therefore, we tested whether the higher FE of Na^+ in the CNT-KO mice compared to

362   the control mice during K^+ loading occurred in parallel with lower protein abundance

363   and phosphorylation of the Na^+-K^+-$2Cl^-$ cotransporter, NKCC2 (expressed in TAL), and

364   the Na^+-Cl^- cotransporter, NCC (expressed in DCT). Immunoblotting of cortical/OM

365   tissue (collected on day 2) showed no differences in the protein abundances of

366   NKCC2, pT96-T101-NKCC2, or the pT96-T101-NKCC2/NKCC2 ratio (NS, Fig. 7). By

367   contrast, we found a lower abundance of total NCC ($P < 0.05$, Fig. 7), pT53-NCC ($P <$

368   0.01, Fig. 7), and pT58-NCC ($P < 0.001$, Fig. 7). Furthermore, the pT53-NCC/NCC ($P <$

369   0.05, Fig. 7) and the pT58-NCC/NCC ratios ($P < 0.001$, Fig. 7) were lower in the CNT-

370   KO mice, suggesting that the lower phosphorylation of NCC was not a result of lower

371   total NCC protein abundance *per se*. Finally, no effect was found on ROMK abundance

372   (Fig. 7). Collectively, the higher FE of Na^+ in the CNT-KO mice fed a 5% K^+ diet

373   coincided with lower abundance and phosphorylation of NCC in the DCT and lower

374   αENaC expression in the CNT.

13

DISCUSSION

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).

14

406   The CNT-KO mice presented higher aldosterone levels on the low-Na$^+$ diet and a

407   mild phenotype as evidenced by Na$^+$ wasting on day 3–5. However, this effect

408   eventually disappeared after 7 days, after which no effects were found on plasma

409   [Na$^+$] and [K$^+$]. Data indicated higher cleavage of ENaC in the distal colon and this

410   potential activation of ENaC could, at least partially, have compensated for reduced

411   renal Na$^+$ reabsorption in the CNT.

412   We have previously shown that genetic inactivation of αENaC in the CD did not

413   interfere with Na$^+$ and K$^+$ balance in mice kept on a standard diet, low-Na$^+$ diet, or a

414   6% K$^+$ diet (28). This suggests that αENaC in the CD is apparently not a prerequisite

415   for maintaining Na$^+$ and K$^+$ homeostasis. By contrast, combined αENaC inactivation in

416   a part of the CNT (approximately 30% fewer αENaC-positive cells in early CNT, and

417   70% fewer αENaC-positive cells in late CNT) and CD induced PHA-1 symptoms

418   already on a standard diet as evidenced by natriuresis, hyponatremia, and

419   hyperkalemia (5). During Na$^+$ restriction, the CNT/CD-KO mice showed lower relative

420   BW, severe urine Na$^+$ loss, hyponatremia, and hyperkalemia (5). Thus, the phenotype

421   was stronger in the CNT/CD-KO mice than in the CNT-KO mice on a standard diet or a

422   low-Na$^+$ diet. Besides a potential compensation in the colon, it is possible that the CD

423   in the CNT-KO mice could partially compensate for the lack of αENaC in the CNT.

424   Because αENaC was deleted in the CD of the CNT/CD-KO mice, such compensation

425   was not possible in these mice. However, it cannot be ruled out either that the

426   deletion of αENaC in the CNT-KO was less efficient than in the CNT/CD-KO mice

427   thereby explaining the milder phenotype. Thus, it is possible that the remaining CNT

428   cells still expressing αENaC in the CNT-KO were sufficient to maintain proper Na$^+$

429   balance.

430   A 5% K$^+$ diet induced a weaker phenotype in the CNT/CD-KO mice than in the

431   CNT-KO mice. Both mouse lines showed lower plasma [Na$^+$] and higher plasma [K$^+$]

432   on the 5% K$^+$ diet, however, the CNT-KO mice additionally presented lower food

433   intake and lower relative BW, whereas these parameters were unaffected in the

434   CNT/CD-KO mice. Impaired ENaC-facilitated K$^+$ secretion in the colon (11, 32), was

435   not likely to cause the stronger phenotype in the CNT-KO mice, because the colonic

436   αENaC expression was intact. Although the role of ENaC in the DCT2 for K$^+$ secretion

15

437   is unknown, it could be speculated that deletion of αENaC only in a few DCT2 cells

438   could become critical during K^+ loading.

439   In summary, we examined the importance of αENaC in the CNT by generating KO

440   mice in which αENaC was deleted primarily in the CNT. The mice showed no obvious

441   phenotype on a standard diet, 0.01% Na^+ diet, or a 2% K^+ diet. The elevated

442   aldosterone levels in the CNT-KO mice may stimulate ENaC activity in the late CNT (in

443   remaining ENaC expressing cells), in the CD, or in the colon and potentially

444   compensate for the reduced Na^+ reabsorption in the CNT. On a 5% K^+ diet, however,

445   the mice presented clear PHA-1 symptoms. Our data provide an unprecedented

446   insight into compensatory mechanisms taking place in a conditional model of the

447   PHA-1 syndrome. Even when αENaC is deleted only in a part of the CNT, several

448   compensatory mechanisms occur. During 5% K^+ loading, this includes higher plasma

449   aldosterone level, lower renal NCC activity, and lower GFR. Clinically, the results may

450   contribute to a deeper understanding of how the body copes with the physiological

451   challenges that are taking place in PHA-1 patients.

452

468 **DISCLOSURES**

469

470   None.

471

472 **AUTHOR CONTRIBUTIONS**

473

474 S.B.P.: conception and design of the experiments; collection, analysis, and
475 interpretation of data; drafting the article or revising it critically for important
476 intellectual content; J.P.: drafting the article or revising it critically for important
477 intellectual content; H.H.D.: collection of data; drafting the article or revising it
478 critically for important intellectual content; L.M.: drafting the article or revising it
479 critically for important intellectual content; R.D.N.: drafting the article or revising it
480 critically for important intellectual content; E.H.: drafting the article or revising it
481 critically for important intellectual content; B.M.C.: conception and design of the
482 experiments; collection, analysis, and interpretation of data; drafting the article or
483 revising it critically for important intellectual content; All authors approved the final
484 version of the manuscript.

485

486

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FIGURE LEGENDS

604

605   Fig. 1. αENaC expression was lower in the CNT-KO mice. *A–D*, in mice kept on a 4-day

606   0.01% Na⁺ diet, immunolabeling showed more αENaC-positive cells in the CNT/DCT2

607   (in the cortical labyrinth) of controls (*A*, arrows indicate αENaC-positive cells) than in

608   the CNT-KO mice (*B*, arrow heads indicate αENaC-negative cells). *E*, the fraction of

609   αENaC-positive cells in the CNT/DCT2 was approximately 40% lower in the CNT-KO

610   mice, whereas no significant difference was found in the CCD (*C–E*, arrows indicate

611   αENaC-positive cells, in medullary arrays). *F* and *G*, on a standard diet, the CNT-KO

612   mice showed 55% lower total αENaC protein intensity in cortical/outer medullary

613   tissue homogenate [cleaved (30 kD) + full length (90 kD)]. Each bar indicates mean ±

614   SE. **$P < 0.01$, ***$P < 0.001$.

615

616   Fig. 2. *Atp6v1b1*::*Cre*/enhanced green fluorescent protein (EGFP) reporter mice

617   expressed EGFP in AQP2 and calbindin-positive cells in the CNT, whereas only a few

618   NCC-positive cells in the DCT2 and a few AQP2-positive cells in the CCD were EGFP

619   positive. Calbindin was used a marker for CNT/DCT2. A–C, arrow: NCC-

620   positive/EGFP-positive cell. D–F, arrow: calbindin-positive/EGFP-positive cell;

621   arrowhead: calbindin-negative/EGFP-positive cell; asterisk: calbindin-positive/EGFP-

622   negative cell. G–I, arrow: calbindin-positive/AQP2-positive/EGFP-positive cell;

623   arrowhead: AQP2-negative/EGFP-positive cell. J–L, arrow: AQP2-positive/EGFP-

624   positive cell; arrowhead: AQP2-negative/EGFP-positive cell; asterisk: AQP2-

625   positive/EGFP-negative cell. Glo: glomerulus.

626   Fig. 3. The majority of NCC-positive cells in the DCT2 and AQP2-positive cells in the

627   CCD did not express EGFP in the *Atp6v1b1*::*Cre*/EGFP reporter mice. A–C, arrowhead:

628   NCC-negative/EGFP-positive cell; asterisks: NCC-positive/EGFP-negative cells. D–F,

629   arrowhead: AQP2-negative/EGFP-positive cell; asterisks: AQP2-positive/EGFP-

630   negative cells.

631

632 Fig. 4. Distal colonic αENaC expression was not impaired in the CNT-KO mice. *A*,
633 *Atp6v1b1*::*Cre*/EGFP reporter mice showed EGFP expression in some cells of the
634 connective tissue in the distal colon (arrows), whereas EGFP was not detectable in
635 the surface epithelial layer (*n* = 2). *B*, in the control mice and *C*, the CNT-KO mice kept
636 on a 4-day 0.01% Na$^+$ diet, immunolabeling revealed clear apical labelling in the distal
637 colonic surface epithelial layer. *D* and *E*, immunoblotting and corresponding
638 densitometric analyses of distal colonic homogenates showed that total αENaC
639 abundance (30+90 kD bands, arrows) did not differ between the control mice and the
640 CNT-KO mice kept on a 7-day 0.01% Na$^+$ diet. The 30/90 kD-ratio was, however,
641 higher in the CNT-KO mice than in the control mice. Each bar indicates mean ± SE. *P
642 < 0.05. LU: lumen, CR: crypt.

643

644 Fig. 5. Blood parameters: the CNT-KO mice presented a clear phenotype on a 5% K$^+$
645 diet. Effects of various diets on, *A*, plasma [Na$^+$], *B*, plasma [K$^+$], *C*, plasma aldosterone
646 concentration, *D*, plasma creatinine concentration, and, *E*, plasma osmolality in the
647 control mice and the CNT-KO mice. The mice were kept on either a standard diet in
648 regular cages ([Na$^+$], [K$^+$], creatinine and osmolality: control *n* = 7, KO *n* = 9;
649 aldosterone: control *n* = 14, KO *n* = 17), 7-day 0.01% Na$^+$ diet in metabolic cages
650 ([Na$^+$], [K$^+$], aldosterone, and osmolality: control *n* = 17, KO *n* = 18; creatinine: control
651 *n* = 15, KO *n* = 15), 4-day 2% K$^+$ diet in metabolic cages (control *n* = 9, KO *n* = 9), or a
652 2-day 5% K$^+$ diet in metabolic cages (control *n* = 8, KO *n* = 10). Each bar indicates
653 mean ± SE. **P < 0.01, ***P < 0.001.

654

655 Fig. 6. Metabolic parameters: the CNT-KO mice presented a clear phenotype on a 5%
656 K$^+$ diet. The control mice and the CNT-KO mice were kept in metabolic cages on a
657 standard diet (baseline) followed by either a, *A–G*, 7-day 0.01% Na$^+$ diet (control *n* =
658 17–18, KO *n* = 17–18), *H–N*, 4-day 2% K$^+$ diet (control *n* = 9, KO *n* = 9), or, *O–U*, a 2-
659 day 5% K$^+$ diet (*O–R*, control *n* = 17, KO *n* = 18; *S–U*, control *n* = 9, KO *n* = 8). Each
660 circle indicates mean ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.

661

662   Fig. 7. The CNT-KO mice presented lower abundance and phosphorylation of NCC on

663   a 5% K^+ diet. Presented are protein abundances of NKCC2, p-NKCC2 (pT96/T101),

664   NCC, pT53-NCC, pT58-NCC, and ROMK in control mice (n = 8) and CNT-KO mice (n =

665   10) as determined by immunoblotting of cortical/outer medullary tissue

666   homogenates. Furthermore, presented are the ratios of pT96-T101-NKCC2/NKCC2,

667   pT53-NCC/NCC, and pT58-NCC/NCC. Each bar indicates mean ± SE. *P < 0.05, **P <

668   0.01, ***P < 0.001.

669

670 **TABLES**

Table 1. Urine and plasma parameters in control mice and CNT-KO mice kept on a 2-day 5% K^+ diet

Parameter		Control			KO			P
		Mean	SE	n	Mean	SE	n	
Na^+ output/intake ratio	Baseline	0.73	0.07	9	0.71	0.06	8	NS
	day 1	0.98	0.05	9	1.47	0.07	8	< 0.001
	day 2	0.72	0.04	9	0.95	0.05	8	< 0.01
K^+ output/intake ratio	Baseline	0.72	0.05	9	0.66	0.06	8	NS
	day 1	0.73	0.03	9	0.60	0.04	8	NS
	day 2	0.69	0.03	9	0.70	0.04	8	NS
FE Na^+ (%)	day 2	0.42	0.03	8	0.56	0.03	10	< 0.01
FE K^+ (%)	day 2	71	7	8	51	5	10	< 0.05
Plasma creatinine (mM)	day 2	7.6	0.4	8	11.0	0.7	10	< 0.01
GFR (µl/min)	day 2	290	30	8	219	17	10	< 0.05

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679

680

Con   **KO**

A   CNT/DCT2   B

C   CCD   D

25 µm   25 µm

E

αENaC-positive cells

□ Con ■ KO

Fraction of con

CNT/DCT2   CCD

*** NS **

F

kD
150
100
90 kD
75
50
37
30 kD
25

Con   KO

G

□ Con ■ KO

Fraction of con

30 kD   90 kD   30+90 kD

*** *** ***

Cre/EGFP reporter mouse

Cre/EGFP reporter mouse

Cre/EGFP reporter mouse **Con** **KO**

A LU

CR

20 µm

B LU

CR

20 µm 20 µm

C LU

CR

20 µm 20 µm

D

kD
150
100
75

50

37

25

90 kD

30 kD

Con KO

E

□ Con ■ KO

Fraction of con

4.0
3.0
2.0
1.0
0

*

30 kD 90 kD 30+90 kD 30/90 kD

	0.01% Na⁺ diet	2% K⁺ diet	5% K⁺ diet

Relative BW
A — Con — KO
H
O
*** ***

Food intake (g/g BW)
B
I
P
*** **

Water intake (g/g BW)
C
J
Q
**

Urine output (g/g BW)
D
K
R
*

Urine Na⁺ output (nmol/g BW)
E
* ** *
L
S

Urine K⁺ output (nmol/g BW)
F
M
T
*** *

Urine Osmolality (mOsm/kg)
G
* *
N
U

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

5% K⁺ diet