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Sodium and potassium balance is dependent on αENaC expression in mouse connecting tubule

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Running title: ENaC-mediated Na^+ transport in CNT

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ABSTRACT

Mutations in α (or β or γ) epithelial sodium channel (ENaC) subunits that downregulate ENaC activity cause a severe salt losing syndrome with hyperkalemia and metabolic acidosis, characteristic for the human pseudohypoaldosteronism type 1 (PHA-1) syndrome. Mice in which αENaC is selectively inactivated in the collecting duct (CD) are able to maintain sodium and potassium balance suggesting that the late distal convoluted tubule (DCT2) and/or the connecting tubule (CNT) are involved in sodium homeostasis. To investigate the relative importance of ENaC-mediated sodium absorption in the CNT, we bred mice carrying a conditional allele of αENaC (*Scnn1a*$^{lox/lox}$) to *Aqp2::iCre* mice. Western blot analysis on microdissected cortical CD (CCD) and CNT revealed absence of αENaC in the CCD and weak αENaC expression in the CNT. These mice exhibit a significant higher urinary sodium excretion accompanied by a lower osmolality and an increased volume. Serum sodium was lowered while potassium levels were increased. Upon sodium-deficient diet, significant weight loss, higher urinary sodium excretion and hyperkalemia were observed. Plasma aldosterone levels were significantly elevated both under standard and under sodium-deficient diet. In summary, αENaC expression within the CNT/CD is crucial for sodium and potassium homeostasis and if missing, causes symptoms of PHA-1.

INTRODUCTION

Sodium reabsorption in the kidney is essential for maintaining fluid and electrolyte homeostasis as well as regulation of blood pressure. Renal sodium reabsorption is under tight control of aldosterone in the DCT2, the CNT and the CD.[1] Sodium enters the aldosterone-sensitive epithelial cell through ENaC at the apical plasma membrane and sodium is extruded to the interstitial fluid via the basolateral Na^+-K^+-ATPase in exchange for potassium. In the DCT2, sodium is also absorbed through the thiazide-sensitive NaCl co-transporter (TSC).[2] The critical role of ENaC in sodium homeostasis has been emphasized by identification of gain-of-function mutations in the C-terminus of the β or the γ subunit in patients suffering from Liddle's syndrome, a severe form of hypertension caused by sodium retention.[3,4] PHA-1 on the other hand is a severe salt-wasting syndrome characterized by urinary loss of sodium and reduced potassium excretion despite elevated levels of aldosterone. In humans, a life-threatening form of the disease is inherited as an autosomal recessive trait and is caused by loss-of-function mutations in any of the three ENaC subunits.[5] Clinical symptoms of the disease are weight loss and dehydration, hypovolemia and hypotension, hyponatremia, hyperkalemia, metabolic acidosis accompanied with elevated plasma aldosterone levels.[6] Complete knockout of each of the ENaC subunits resulted in an early and lethal PHA-1 phenotype.[7-9] Previously, a CD-specific conditional knockout for αENaC has been generated and surprisingly these mice were able to maintain water, sodium and potassium balance even after one week of salt restriction, 23 h water deprivation or 4 days potassium loading.[10] In the present study, we have investigated the implication of the CNT and CD for the ENaC-mediated sodium reabsorption using mice that express the Cre recombinase from the Aqp2 promoter (Aqp2::iCre) and conditional alleles of αENaC ($Scnn1a^{loxlox}$).[11,12] Aquaporin-2 (AQP2) is a water channel expressed along the CNT and CD.[13,14] Our data indicate that αENaC expression within the CNT is important for sodium and potassium balance.

RESULTS

Inactivation of αENaC in the collecting duct and the connecting tubule

Knockout and control mice were born consistent with Mendelian inheritance (*Scnn1a*$^{lox/-}$*/Aqp2::iCre*, 25.9%; *Scnn1a*$^{lox/+}$*/Aqp2::iCre,* 29.4%; *Scnn1a*$^{lox/+}$, 21.8% and *Scnn1a*$^{lox/-}$, 22.8%, n=197). To verify the deletion of αENaC expression in these CNT/CD-specific KO mice (*Scnn1a*$^{lox/-}$*/Aqp2::iCre*), the CNT and the CCD were dissected and analysed by western blot (Fig 1). The previously described CD-specific KO mice (*Scnn1a*$^{lox/lox}$*/HoxB7::Cre*) were used as positive (and negative) control.[10] In the CNT and the CCD of all control groups a band at ~95 kDa corresponding to full length αENaC protein was observed (Fig 1, panel A; lane 1-2 and panel B; lane 1-2 and 5-6). This band was absent in the CCD of the CD-specific KO mice (Fig 1, panel A; lane 4)[10] and in the CCD of the CNT/CD-specific KO mice (Fig 1, panel B; lane 4 and 8). We observed a faint band corresponding to full length αENaC protein in the CNT of the CNT/CD-specific KO mice (Fig 1, panel B; lane 3 and 7) suggesting that the inactivation of αENaC in the CNT was not complete. We further observed a band just above 26 kDa in the CNT of the CD-specific KO mice (Fig 1, panel A; lane 3), and in the CNT of the control littermates of the CNT/CD-specific KO mice (Fig 1, panel B; lane 1 and 5) that most likely corresponds to a cleavage product of the α subunit.[15,16] It was not observed in the CNT of the CNT/CD-specific KO mice (Fig 1, panel B; lane 3 and 7).

Western blots of microdissected tubules from sodium-restricted CNT/CD-specific KO mice and littermate control mice showed that the ~95 kDa band corresponding to full length αENaC protein was absent in the CCD of the CNT/CD-specific KO mice (Fig 2, panel A-D). In the CNT of the CNT/CD-specific KO mice this band was weakly expressed or absent (Fig 2, panel A-D). Thus, on a standard or upon salt-deprived diet (Fig 1 and 2) αENaC protein is barely detectable in the CNT, and not detected in the CCD of knockout mice.

To further investigate the recombination efficiency, we performed immunohistochemistry on kidneys from sodium-deprived CNT/CD-specific KO mice and control mice. Double-labeling showed almost complete co-localisation of AQP2 and Cre recombinase in the principal cells along the CD (Fig 3A). Cellular counting in the CCD revealed that 96% of the AQP2 positive cells showed co-staining with the Cre recombinase. In the CNT, cellular counting showed that about 70% of AQP2-positive cells were co-labeled with Cre recombinase (Fig 3B). Double-labeling of Cre recombinase and H^+-ATPase (marker for intercalated cells) revealed no Cre expression in the intercalated cells (Fig 3C).

Within the CNT, immunolabeling was performed with antibodies recognizing αENAC, AQP2 and TSC. The anti-TSC antibody was used as a marker of the DCT.[2] In control mice, αENaC was expressed along the early and late CNT (Fig 3, D-F). The early CNT, which was identified at the transition from the TSC-positive DCT to the TSC-negative CNT, contained none or only a few AQP2-positive cells in both control and knockout mice (Fig 3, D-I). Consistent with this, αENaC was also detected in such tubules, which were both AQP2 and TSC negative and likely represent early parts of the CNT with undetectable AQP2 expression. In knockout mice, αENaC was partly absent in the CNT (Fig 3, G-I) with some expression remaining in both late and early CNT. About 70% of all early CNT cells (both CNT and intercalated cells) in the control were αENaC positive, in comparison to only 50% in the knockout situation. In the late CNT, the percentage of αENaC positive CNT cells (control: 63%, knockout: 18%, identified as TSC negative and AQP2 positive) was reduced by about 70% in the knockout. In summary, the CNT/CD-specific αENaC knockout mice have reduced numbers of αENaC expressing cells in both the late and early CNT, with a more severe reduction (up to 70%) in the late CNT, and corresponding to an increase in AQP2 expressing cells in the late CNT.

CNT/CD-specific αENaC knockout mice exhibit a PHA-1 phenotype under standard salt diet

Following a standard salt diet, mice did not show a reduced body weight. In contrary, the urinary sodium excretion was significantly higher in the knockout mice ($p<0.05$, Table 1), whereas the urinary potassium excretion was unchanged. Food, and thus sodium intake, were not altered (Table 1). The increased urine excretion ($p<0.01$, Table 1) was accompanied by lower urinary osmolality ($p<0.01$, Table 1) and higher water intake ($p<0.05$, Table 1). The CNT/CD-specific αENaC knockout mice presented with significantly lower serum sodium concentrations ($p<0.05$, Table 1) and hyperkalemia revealed by significantly higher blood potassium concentrations ($p<0.05$, Table 1). Plasma aldosterone were measured in CNT/CD-specific αENaC knockout and control mice which were homozygous for a specific renin allele (*Ren-2*$^{-/-}$)[17] and the CNT/CD-specific αENaC knockout presented with significantly higher plasma aldosterone levels ($p<0.05$, Table 2). Blood pressure was slightly reduced in the knockout mice, without any significant difference (Table 1). No significant changes were observed in the heart rate (Table 1).

Sodium-deficient diet induces severe renal sodium loss

The CNT/CD-specific αENaC knockout and control mice were challenged with a sodium-deficient diet for 4 consecutive days. After one day, the knockout mice lost significant body weight, while the control mice kept or even gained body weight ($p<0.001$, Fig 4a). No difference was observed in food intake (ko, n=8: 0.15 ± 0.01 vs ctr, n=10: 0.14 ± 0.01 g/g BW), and loss of body weight was paralleled by a severe urinary sodium loss (Fig 4b). The cumulative sodium balance showed that the control mice were able to retain their sodium, whereas the knockout mice continued to excrete sodium (ko: 0.25 ± 0.014 mmol, ctr: 0.07 ± 0.005 mmol, $p<0.001$, day 4, Fig 3b). After one day of sodium-deficient diet, the

urinary potassium excretion was significantly increased in the knockout mice ($p<0.05$), a difference that vanished during the following days (Fig 4c). The knockout mice continued to have a significant higher urinary excretion during the first 3 days on the sodium-deficient diet (Fig 6a). The urine osmolality was significantly lower in the knockout mice upon the sodium-deficient diet ($p<0.001$, day 4, Fig 6b). Water intake was not different (Fig 6c). After 4 days, a significant increase was found in blood potassium levels (ko, n=8: 6.4 ± 0.2 vs ctr, n=10: 5.8 ± 0.2 mM, $p<0.05$, day 4) and plasma aldosterone levels ($p<0.01$, Table 2).

When the mice were followed over a period of 15 days upon salt-deprivation in standard cages, the knockout mice lost continuously weight. The weight lost reached more than 10% of their initial weight ($p<0.001$, Fig 5). Moreover, plasma aldosterone levels were significantly elevated ($p<0.001$, Table 2).

CNT/CD-specific αENaC knockout mice are not able to eliminate a potassium load

The ability of the knockout mice to eliminate a potassium load was tested by challenging the animals with a diet containing 5% potassium for 2 consecutive days. The knockout mice had a significant lower urinary potassium excretion ($p<0.01$, day 2, Fig 7a) and significantly higher levels of potassium in the blood ($p<0.05$, Table 3), whereas the serum sodium concentration was significantly reduced ($p<0.05$, Table 3). After 1 day of high potassium diet, urinary sodium excretion was not affected, but significantly reduced after day 2 ($p<0.05$, day 2, Fig 7b). Fractional excretion of sodium FE(Na^+) was not different (Table 3), but knockout mice showed significantly lower urinary chloride excretion (ko, n=11: 1.1 ± 0.2 vs ctr, n=11: 2.5 ± 0.4 mmol, $p<0.05$, day 2), whereas no difference in serum chloride concentration was observed (Table 3). The concentration of bicarbonate was significantly lower in the knockout animals (Table 3) indicating metabolic acidosis. Serum osmolality (Table 3), urine osmolality (ko, n=11: 1198 ± 54, ctr, n=11: 1306 ± 62 mmol/kg H_2O), urine

output (ko, n=11: 0.14 ± 0.03, ctr, n=11: 0.21 ± 0.04 ml/g BW/24 hr) and water intake (ko, n=11: 0.28 ± 0.05, ctr, n=11: 0.37 ± 0.04 ml/g BW/24 hr) did not reveal significant differences after 2 days of high potassium diet.

DISCUSSION

Constitutive knockout of either β or γENaC causes an early lethal phenotype due to disturbances in the electrolyte balance.[7,9] αENaC deficiency also induces death shortly after birth with a lung, skin and kidney phenotype.[8,18] Mice in which αENaC was deleted specifically in the CD are viable and do not show disturbances in sodium and potassium balance even when subjected to challenging diets.[10] This suggested that the CNT is important in controlling ENaC-mediated sodium reabsorption in the kidney. To investigate this further we generated mice in which the αENaC gene was deleted in the CD and partly in the CNT. These mice are viable until adulthood, and exhibit normal blood pressure, although they show increased urinary sodium excretion, urine output, and plasma aldosterone, leading to hyponatremia and hyperkalemia already under standard diet. Following sodium-restriction, the mice become severe salt-losing and show a continuous life-threatening reduction of body weight.

Partial inactivation of αENaC in the CNT is sufficient to induce a severe salt-losing syndrome

While αENaC was deleted efficiently in the CCD principal cells, about 30% of the late CNT cells are not targeted and still express αENaC protein. This may explain the remaining αENaC expression in microdissected CNT of some animals under normal and sodium-deprived diet. Similarly to our findings, CNT/CD-specific mineralocorticoid receptor (MR) knockout mice (using the same Aqp2::iCre transgene) show complete deletion of MR in the

CD, whereas deletion of the MR protein in the early and late CNT was equally partial thus following the AQP2 expression pattern in these segments.[11]

The absence of AQP2 expression in the early CNT is therefore consistent with previous observations. In vasopressin-deficient Brattleboro rats, the initial portion of the CNT lacks detectable levels of AQP2, whereas chronic vasopressin treatment induced its expression throughout the CNT.[19] Moreover, in *TRPV5::EGFP* transgenic mice, some tubule segments were EGFP and calbindin-positive, but negative for AQP2 and TSC thus likely representing early CNT.[20]

We observed that the Cre recombinase protein was expressed along the CD consistent with CNT/CD-specific MR knockout mice.[11] Immunohistochemistry also showed that αENaC was not expressed anymore in the early part of the CCD in the CNT/CD-specific αENaC knockout mice (Supp Fig 1).

The CNT is critical for the ENaC-mediated sodium reabsorption

The increased urinary sodium excretion, hyponatremia and hyperkalemia observed in the knockout mice demonstrates impaired sodium reabsorption in the CNT and is therefore consistent with the absence of ENaC in this segment. Moreover, the knockout mice show a decreased urine osmolality and increased urine output/water intake. The changes in water balance could be explained by a reduced renal urine-concentrating ability leading to increased urine output and causing increased water intake. The renal effect can be due to impaired ENaC-mediated sodium reabsorption in the CNT and CD which leads to a reduced driving force for osmotic reabsorption of water in these segments. Apparently, the urine-concentrating ability would only be affected when both the CNT and the CD are targeted, since no change in water balance was seen in the CD-specific αENaC knockout mice.[10] An impaired urine-concentrating ability has previously been described in aldosterone synthase-

deficient mice.[21] The changes in water balance could also be due to the activation of the renin-angiotensin-aldosterone system as shown by elevated plasma aldosterone concentrations, which could lead to a stimulation of thirst resulting in increased water intake and subsequently increased urine output.

It has previously been shown that CD-specific αENaC knockout mice do not exhibit a phenotype even after challenging diets.[10] In contrast we observed that sodium-restriction caused a continuous loss of body weight and sodium in the knockout mice. The body weight of the knockout mice did not stabilize after 15 days of sodium restriction. A similar weight loss in response to low-sodium diet was reported in transgenic mice expressing low levels of βENaC.[22] Moreover the severe salt loss in humans with the recessive form of PHA-1 does not improve with age.[6] The CNT/CD-specific αENaC knockout mice were hyperkalemic in contrast to the CD-specific αENaC knockout mice. Both CNT/CD-specific and CD-specific αENaC knockout mice decreased their urine osmolality upon sodium-restriction, but only the CNT/CD-specific αENaC knockout mice showed a significant lower urine osmolality compared to their littermate controls. Challenging the CD-specific αENaC knockout mice with a potassium-rich diet for 4 days resulted in increased sodium and potassium in both plasma and urine, but not different from the control.[10] In contrast the CNT/CD-specific αENaC knockout mice were not able to excrete a potassium load as shown by significantly higher potassium concentration in blood and decreased urinary potassium excretion. The potassium-loaded CNT/CD-specific αENaC knockout mice also showed indications of metabolic acidosis. Thus, in contrast to CD-specific αENaC knockout mice, the CNT/CD-specific αENaC knockout mice exhibit symptoms of PHA-1 supporting that the CNT is critical for the ENaC-mediated sodium reabsorption. This is consistent with previous suggestions that the late DCT and CNT, rather than the CD, are the main regulators of ENaC-mediated sodium and potassium homeostasis.[23,24] The majority (90%) of the sodium

delivered to aldosterone-sensitive distal segments is reabsorbed in the CNT and DCT.[25] Moreover, the CNT exhibit a higher ENaC activity compared to the CD of aldosterone-infused rats.[26] The apical ENaC expression has also been shown to be more pronounced in the CNT compared to the CD in mice on a moderately low-sodium diet (0.05%).[27]

The fact that a partial gene deletion in the CNT was sufficient to induce a severe salt losing syndrome also shows that the functional reserve in the CNT is limited since the remaining αENaC positive CNT cells were unable to fully compensate for the loss of αENaC in the other cells.

CNT/CD-specific deletion of αENaC is more severe than inactivation of the mineralocorticoid receptor in the same segments

The actions of aldosterone are mediated through the MR. Following binding of aldosterone to the MR, the hormone is translocated to the nucleus where it controls the transcription of ENaC and other genes. In contrast to the CNT/CD-specific αENaC knockout mice no disturbance in electrolyte and water balance was observed in CNT/CD-specific MR knockout mice subjected to a normal salt diet.[11] Thus, partial deletion of αENaC in the CNT induces a more severe phenotype than inactivation of MR in the same segments. This may not be surprising since the MR protein is an upstream effector on sodium absorption compared to ENaC as an effector. Upon sodium restriction, the CNT/CD-specific MR knockout mice also showed loss of body weight, increased sodium and urinary excretion, and significant higher plasma aldosterone levels [11] similar to CNT/CD-specific αENaC knockout mice.

In summary, gradual gene deletion in the CNT was sufficient to induce a severe salt-losing syndrome confirming that the CNT is crucial for maintaining sodium and potassium balance.

CONCISE METHODS

Generation of transgenic mice

To inactivate the *Scnn1a* gene in the CNT cells, we used mice expressing Cre recombinase under the control of the regulatory elements of the mouse *Aqp2* gene[11] and *Scnn1a*^{loxlox} conditional knockout mice and the *Scnn1a*$^{+/-}$ mice.[8,12] CNT/CD-specific αENaC knockout mice (*Scnn1a*$^{lox/-}$*/Aqp2::iCre*, knockout group) and heterozygous (*Scnn1a*$^{lox/+}$*/±Aqp2::iCre* and *Scnn1a*$^{lox/-}$, control group) littermates were obtained by interbreeding *Scnn1a*$^{+/-}$*/Aqp2::iCre* mice with *Scnn1a*^{loxlox} mice. Genotyping of the mice was performed at the age of weaning by DNA-based PCR analysis as described previously.[12]

Microdissection of nephron segments

Microdissection of kidneys was performed from knockout mice (n=2) and control littermates (*Scnn1a*$^{lox/+}$*and Scnn1a*$^{lox/+}$*/Aqp2::iCre,* n=2, about 3 months of age). As additional controls, we included CD-specific KO mice (*Scnn1a*^{loxlox}*/HoxB7::iCre*, n=2) and their littermate controls (*Scnn1a*^{loxlox}, n=2, about 11 months of age).[10] Microdissection was also performed of kidneys from knockout mice (n=4) and control littermates (*Scnn1a*$^{lox/+}$, n=4, 2-2 ½ months of age) subjected to a sodium-deficient diet in standard cages from 6 to 17 days. The experiment was performed 4 times and each time with one knockout mouse and one control mouse in parallel. The kidney was perfused with DMEM/F-12 (1:1) medium (21041 medium, Invitrogen) completed with 40µg/ml liberase blendzyme 2 or 30µg/ml blendzyme TM (Roche Applied Science). Thin pyramids cut along the corticomedullary axis were incubated at 37°C or at 30°C for 40 min in the perfusion medium. The action of enzyme was stopped by washing the pyramids with ice-cold DMEM/F-12 (1:1) medium without blendzyme. Then, the medulla was removed under microscope and the CNT and CCD were microdissected in ice cold DMEM/F-12 (1:1) medium without blendzyme. Pools of 10–20 microdissected

tubules within 5µl of DMEM/F12 (1:1) were the transferred to 5µl of 2x concentrated protein sample buffer (9.6% (w/v) SDS, 13.8% (w/v) sucrose, 0.026% (w/v) bromphenol blue, 4.2% (v/v) β-mercaptoethanol).

Western blot analysis of microdissected nephron segments

Samples were heated at 95°C for 5 min and loaded and electrophoresed on a 8% SDS-PAGE. Proteins were then transferred to Protran nitrocellulose membrane (Schleicher & Schuell) or Amersham Hybond-ECL nitrocellulose membrane (Amersham), and western blots were performed according to standard procedures. The membrane was first probed with affinity-purified αENaC antibody (1:100, 1:500 or 1:2000 dilution[10]) and following stripping, anti-actin antibody (1:200 dilution, Sigma) was used. Blots were revealed with SuperSignal reagent (Pierce) or Amersham ECL Western blotting Detection Reagents (Amersham).

Immunohistochemistry

Kidneys from mice sodium-restricted for 4 consecutive days (*Scnn1a*$^{lox/-}$*/Aqp2::iCre,* n=2 and *Scnn1a*$^{lox/+}$*/Aqp2::iCre,* n=3) were fixed by intravascular perfusion of 3% paraformaldehyde and subjected to paraffin embedding and sectioning (2 µm thick sections).

Double labeling: Double-labeling experiments were performed using 1) rabbit polyclonal Cre recombinase antibody (1:8000 dilution, Covance) and biotinylated rabbit polyclonal AQP2 antibody (7661AP, 1:250 dilution), and 2) Cre recombinase antibody (1:8,000 dilution) and biotinylated rabbit polyclonal H^+-ATPase antibody (H7659AP, 1:100 dilution[28]). Sections were incubated overnight at 4ºC with Cre antibody before undergoing incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody and visualization by 3,3'-diaminobenzidine (DAB, brown color). Sections were then incubated in 3.5% H_2O_2 in methanol to remove any remaining peroxidase from the first staining.

Following blocking for endogenous biotin (Biotin Blocking System, DakoCytomation) sections were then incubated overnight at 4ºC with either biotinylated AQP2 or H^+-ATPase antibodies. Labeling was visualized by use of Strepavidin HRP (Sigma) and Vector SG substrate (Vector Laboratories, blue-gray color).

Single labeling of serial kidney sections: Serial paraffin sections were incubated with 1) rabbit polyclonal αENaC antibody (1:800 dilution[10]), rabbit polyclonal AQP2 antibody (1:3,000 dilution) and mouse monoclonal Calbindin D-28K antibody (1:20,000 dilution, Research Diagnostics Inc); 2) rabbit polyclonal TSC antibody (1:1,000 dilution[29]), rabbit polyclonal αENaC antibody (1:800 dilution[10] and rabbit polyclonal AQP2 antibody (1:3,000 dilution). Labeling was visualized by use of peroxidase-conjugated secondary antibody and 3,3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin. Light microscopy was carried out using a Leica DMRE microscope.

Quantification of αENaC positive cells in the CNT

Cell counting was performed on kidney sections from *Scnn1a$^{lox/-}$/Aqp2::iCre* mice and *Scnn1a$^{lox/+}$/Aqp2::iCre* mice that were labeled with rabbit polyclonal αENaC antibodies and peroxidase-conjugated secondary antibodies. Counting was performed on electronic images taken with an x25 objective. The number of αENaC positive (labeled) and αENaC negative (unlabeled) cells with a distinct nucleus was counted in the early CNT, adjacent to the transition from DCT to CNT (the transition was identified on serial sections labeled with TSC antibodies). The total number of cells counted were 65 in the *Scnn1a$^{lox/-}$/Aqp2::iCre* mice (n=2) and 81 in the *Scnn1a$^{lox/+}$/Aqp2::iCre* mice (n=2). Cellular counting was also performed in the late CNT (tubules were identified on serial sections labeled with AQP2 and TSC antibodies). The number of cells counted were 330 in the *Scnn1a$^{lox/-}$/Aqp2::iCre* mice (n=2)

and 339 in the *Scnn1a*$^{lox/+}$*/Aqp2::iCre* mice (n=3). The fraction of ENaC positive cells was calculated from the number of positive cells divided by the total number of cells counted for each animal.

Quantification of Cre recombinase positive cells in the CCD and the CNT

The cellular counting was performed on kidney sections from *Scnn1a*$^{lox/-}$*/Aqp2::iCre* mice and *Scnn1a*$^{lox/+}$*/Aqp2::iCre* mice that were double-labeled with rabbit polyclonal Cre recombinase antibody and biotinylated rabbit polyclonal AQP2 antibody. Counting was performed on electronic images taken with an x63 objective. The number of AQP2 positive/Cre recombinase positive and AQP2 positive/Cre recombinase negative cells with a distinct nucleus were counted in the CCD (313 cells, n=5 mice) and in the CNT (465 cells, n=5 mice). The fraction of Cre recombinase positive cells was calculated from the number of AQP2 positive/Cre recombinase positive cells divided by the total number of AQP2 positive cells counted for each animal.

Experimental protocols

Sodium-deficient diet in metabolic cages: For each metabolic cage study, experimental mice and controls from the same litter were used. 6-12 weeks old mice were placed in individual metabolic cages (Tecniplast, Italy) and fed a standard salt diet (0.23% sodium, Institut National de la Recherche Agronomique, Unité de Préparation des Aliments Expérimentaux, Jouy en Josas, France) for 2 days, followed by 4 consecutive days on a sodium-deficient diet (0% sodium, Institut National de la Recherche Agronomique, Unité de Préparation des Aliments Expérimentaux, Jouy en Josas, France). During the experiment, the animals had free access to food and water. The diet was given as a mixture of food in gelatin and water (100 g food/60 ml water). Blood was collected from the tail vein of conscious mice at the end

of the diet. The experiment was also performed with 8-12 weeks old mice fed a standard salt diet (0.17% sodium, Ssniff Spezialdiäten GmbH, Soest, Germany, given as powder food) for 2 days followed by 3 days on a sodium-deficient diet (<0.01% sodium, Ssniff Spezialdiäten GmbH, Soest, Germany).

Sodium-deficient diet in standard cages: Experimental (n=7) and control littermate mice (n=7, 8–12 weeks old) were fed with a standard salt diet (0.17% sodium, Ssniff Spezialdiäten GmbH, Soest, Germany) and the body weight was measured at day 0 to determine the reference weight. Then, mice were fed a sodium-deficient diet (<0.01% sodium, Ssniff Spezialdiäten GmbH, Soest, Germany) with free access to water, and their body weight was monitored daily (at the same time) for 15 consecutive days.

Blood collection for aldosterone measurements: Control and *Scnn1a*$^{lox/lox}$*/Aqp2::iCre* (knockout) mice (8-12 weeks old) which were homozygous for a specific renin allele (*Ren-2*$^{-/-}$) were kept in standard cages with free access to food and water. Thirteen mice (8 control and 5 experimental) were fed with a standard salt diet (0.17% sodium, Ssniff Spezialdiäten GmbH, Soest, Germany) and 10 mice (6 control and 4 knockout) were fed a sodium-deficient diet (<0.01% sodium, Ssniff Spezialdiäten GmbH, Soest, Germany) for 4 consecutive days. At the end of experiment, blood samples were collected after decapitation. Plasma aldosterone levels were measured according to standard procedures using a radioimmunoassay (RIA) (Coat-A-Count RIA kit, Siemens Medical Solutions Diagnostics, Ballerup, Denmark). Mouse samples with values > 1200 pg/ml were further diluted using a serum pool with a low aldosterone concentration (<50 pg/ml).

High-potassium diet: Data from two separate experiments were pooled. In the first series of experiments mice were 4 months old and in the second series, mice were 2 ½-12 months old. Experimental mice (n=5, first experiment and n=6, second experiment) and control mice (n=6, first experiment and n=5, second experiment) were placed in individual metabolic cages and fed a standard diet for 2 consecutive days (0.59% potassium in the first experiment and 0.95% potassium in the second experiment). This was followed by 2 days on a 5% potassium diet (the potassium was added as KCl). During the experiment the animals had free access to the food and water. The diets were given as a mixture of food in gelatin and water (100 g food/60 ml water). Following the diet, blood was collected from the eye.

Urine and serum/plasma analysis

Urine and serum/plasma osmolarity as well as sodium, potassium, chloride, creatinine and bicarbonate composition were analyzed at the Laboratoire Central de Chimie Clinique, Centre Hospitalier Universitaire Vaudoise (CHUV, Lausanne, Switzerland). The potassium values were corrected for the degree of hemolysis.

Blood pressure measurements

The blood pressure (BP) and heart rate (HR) were measured in knockout (n=13) and control mice (n=19). The mice were kept on a normal salt diet containing 0.23% of sodium with free access to tap water and were analyzed at the age of 4 to 6 months. Blood pressure and heart rate were recorded intra-arterially using a computerized data-acquisition system (Notocord Systems SA, Croissy, France).[17] Briefly, for placement of the intra-arterial catheter, a mouse was anesthetized via inhalation of 1 to 2% halothane with oxygen. The right carotid artery was exposed for a length of approximately 4 mm. A silicone/PE10 catheter filled with 0.9% NaCl solution containing heparin (300 IU/ml) was inserted into the artery. After ligation, the

catheter was subcutaneously tunneled to exit at the back of the neck and fixed with a piece of scotch and dental cement. The mouse was allowed 3 to 4 hours to recover from the anesthesia and placed into a Plexiglas tube for partial restriction of its movements. Thirty minutes later, the arterial line was connected to a pressure transducer, BP and HR were then monitored every 20 s for 15 to 20 min using Notocord computerized data-acquisition system at a sampling rate of 500 Hz. Once BP measurement was completed, blood was sampled from the arterial catheter for analysis of serum sodium and potassium concentrations.

Statistical analysis

Results are presented as mean ± SE. Data were analyzed by one-way ANOVA and unpaired *t* test. P values <0.05 were considered statistically significant.

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DISCLOSURES

None

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FIGURE LEGENDS

Figure 1: Western blot of microdissected CNT and CCD from CD-specific KO mice and their corresponding controls (panel A, lane 1-4) and from the CNT/CD-specific KO mice and littermate controls (Panel B, lane 1-8). Anti-αENaC antibody recognizes a band at ~95 kDa corresponding to full length αENaC and a band just above 26 kDa that may correspond to a cleaved fragment of αENaC (indicated by ◄*). Some unspecific bands were also observed (indicated by ◄). The blots were reprobed with anti-actin antibody to examine the amount of protein loaded on the gels.

Figure 2: Western blots of microdissected proximal tubules (Prox), CNT and CCD from CNT/CD-specific KO mice (KO, n=4, panel A-D) and corresponding controls (Ctrl, n=4, panel A-D) subjected to sodium-deficient diet. Anti-αENaC antibody recognizes a band at ~95 kDa corresponding to full length αENaC (blots on top of panel A-D). The blots were reprobed with anti-actin antibody (blots on bottom of panel A-D).

Figure 3: Immunohistochemistry using whole kidney sections from sodium-restricted control (D-F) and knockout mice (A-C and G-I). Panel A-B: Double labeling of whole kidney sections from a knockout mouse was performed with polyclonal primary antibodies that recognize Cre recombinase (brown) and AQP2 (gray-blue). Along the CD nuclear Cre recombinase staining was observed in AQP2 expressing cells (A, arrows). Within the CNT some AQP2 expressing cells were negative for Cre recombinase (arrowhead, B). Panel C: Double-labeling with primary antibodies recognizing Cre recombinase (brown) and H^+-ATPase (gray-blue) showed no co-localization of the two proteins (arrows, C). Panel D-I: Serial kidney sections from a control mouse (D-F) and knockout mouse (G-I) incubated with anti-TSC antibody (D and G), anti-αENaC antibody (E and H) and anti-AQP2 antibody (F

and I). In control mice, αENaC labeling was observed in the CNT (AQP2 positive and TSC negative, asterisks, D-F) including the early CNT, which was identified adjacent to the transition from the TSC-positive DCT to the TSC-negative CNT (filled squares, D-F). In the knockout mice, αENaC was absent from part of the CNT (triangles, G-I), but present in other parts (asterisks, G-I) including the early CNT (filled squares, inset, G-I).

Figure 4: Knockout (n=18, filled triangles) and control mice (n=23, open squares) were subjected to a sodium-deficient diet for 4 days and the body weight was measured daily (A). The body weight is presented as percentage of the initial weight. Cumulative sodium (B) and potassium (C) balance in knockout mice (n=18, black column) and control mice (n=23, white column) subjected to a sodium-deficient diet for up to 4 days. *, $p<0.05$. **, $p<0.01$. ***, $p<0.001$.

Figure 5: Knockout (n=7, filled triangles) and control mice (n=7, open squares) were subjected to a sodium-deficient diet for 15 days in standard cages. Body weight was measured daily, and is presented as percentage of the initial body weight (at day 0. **, $p<0.01$. ***, $p<0.001$).

Figure 6: Urine excretion (A), urine osmolality (B) and water intake (C) were measured daily in knockout mice (n=18, black column) and control mice (n=23, white column) subjected to a sodium-deficient diet for up to 4 days. *, $p<0.05$. **, $p<0.01$. ***, $p<0.001$.

Figure 7: Knockout mice (n=11, filled triangles) and control mice (n=11, open squares) were subjected to a high-potassium diet for 2 days. Urinary potassium excretion (A) and urinary sodium excretion (B) were measured daily. *, $p<0.05$. **, $p<0.01$.

Sup figure: Immunohistochemistry using serial kidney sections from control (A-C) and knockout mice (D-F). Sections were incubated with anti-αENaC antibody (A and D), anti-AQP2 antibody (B and E) and anti-calbindin antibody (C and F). In the early part of the CCD of control mice (AQP2 positive and with very weak calbindin staining, B-C), apical αENaC staining was observed (C, asterisks). αENaC staining was not observed in the early part of the CCD of knockout mice (D, triangles).

Table 1. *Urinary and blood parameters from mice kept on a standard salt diet*

	Knockout	Control
Body weight, g, females	21.3 ± 0.66 (n=13)	22.1 ± 0.65 (n=24)
Body weight, g, males	27.7 ± 0.89 (n=13)	28.2 ± 1.08 (n=15)
Food intake, g/g BW/24 h	0.13 ± 0.01 (n=8)	0.12 ± 0.01 (n=10)
Na^+ intake, mmol/24 h	0.27 ± 0.03 (n=8)	0.26 ± 0.03 (n=10)
U-Na^+, mmol/24 h	0.23 ± 0.01* (n=31)	0.19 ± 0.01 (n=46)
U- Na^+, mM	136 ± 11 (n=31)	155 ± 7 (n=46)
U-K^+, mmol/24 h	0.33 ± 0.04 (n=31)	0.28 ± 0.03 (n=46)
U-K^+, mM	213 ± 38 (n=31)	242 ± 23 (n=46)
U-osm, mosm/kgH_2O	1809 ± 119** (n=26)	2442 ± 133 (n=39)
Urine output, ml/g BW/24 h	0.085 ± 0.008** (n=31)	0.055 ± 0.004 (n=46)
Water intake, ml/g BW/24 h	0.25 ± 0.03* (n=31)	0.16 ± 0.01 (n=46)
Serum-osm, mosm/kgH_2O	309 ± 4 (n=5)	313 ±34 (n=6)
Serum-Na^+, mM	145.8 ± 1.0* (n=5)	149.8 ± 1.1 (n=6)
Plasma/serum-K^+, mM	6.3 ± 0.2* (n=10)	5.6 ± 0.1 (n=16)
Mean BP, mmHg	127 ± 2 (n=13)	135 ± 3 (n=19)

Mean HR, beats/min	632 ± 18 (n=13)	630 ± 13 (n=19)

Values are means ± SEM. n, number of mice. *, $p<0.05$. **, $p<0.01$. BW, body weight.

BP, blood pressure. HR, heart rate.

Table 2. *Plasma aldosterone in mice kept on a standard salt diet or sodium-deficient diet*

	Knockout	Control
Standard diet (*Ren-2*$^{-/-}$ gene mice)	1915 ± 396* (n=5)	381 ± 55 (n=8)
Sodium-deficient diet, 4 d (*Ren-2*$^{-/-}$ gene mice)	3085 ± 400** (n=4)	1064 ± 213 (n=6)
Sodium-deficient diet, 15 d (*Ren-2*$^{+/-}$ gene mice)	20984 ± 2775*** (n=7)	1218 ± 304 (n=7)

Values are means ± SEM. n, number of mice. *, $p<0.05$. **, $p<0.01$. ***, $p<0.001$.

Table 3. *Functional data from mice subjected to a high potassium diet for 48 hrs*

	Knockout	Control
Serum-Na^+, mM	147.6 ± 1.4* (n=8)	151.5 ± 0.7 (n=11)
Serum-K^+, mM	7.7 ± 0.8* (n=9)	5.2 ± 0.2 (n=11)
Serum-HCO_3^-, mM	12.8 ± 1.1*** (n=7)	18.3 ± 0.5 (n=10)
Serum-osm, mosm/kgH_2O	329 ± 4.7 (n=8)	325 ± 1.5 (n=9)
Serum-Creatinine, mM	19.1 ± 2.3* (n=7)	12.1 ± 1.5 (n=10)
Serum-Cl^-, mM	114.4 ± 1.4 (n=7)	114.8 ± 0.7 (n=10)
FE(Na^+), %	0.27 ± 0.05 (n=7)	0.25 ± 0.02 (n=10)

Values are means ± SEM. n, number of mice. *, $p<0.05$. **, $p<0.01$. ***, $p<0.001$.

Figure 1

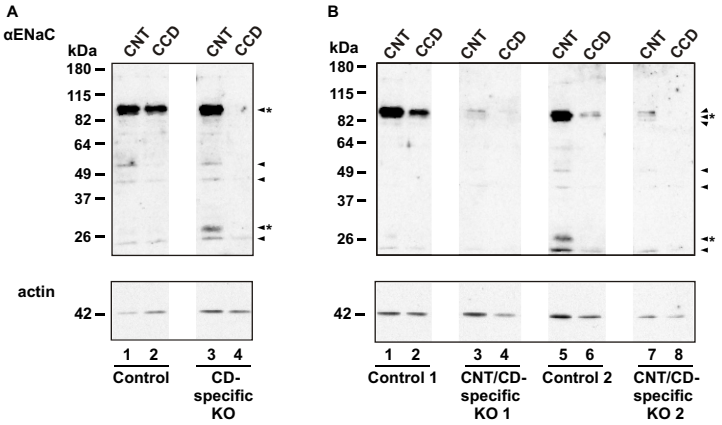

A
αENaC
kDa
CNT
CCD
CNT
CCD
180
115
82
64
49
37
26
actin
42
1 2
3 4
Control
CD-specific KO
B
kDa
CNT
CCD
CNT
CCD
CNT
CCD
CNT
CCD
180
115
82
64
49
37
26
42
1 2
3 4
5 6
7 8
Control 1
CNT/CD-specific KO 1
Control 2
CNT/CD-specific KO 2

Figure 2

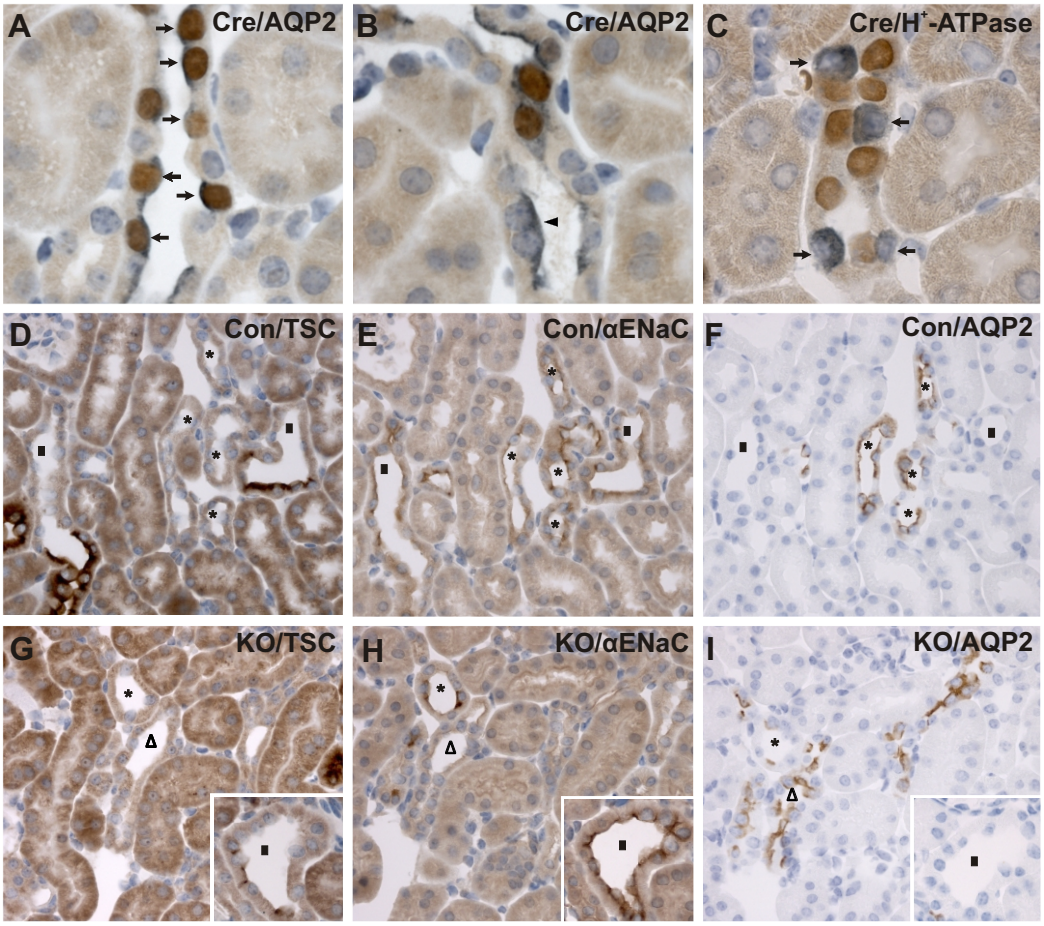

A
Cre/AQP2
B
Cre/AQP2
C
Cre/H+-ATPase
D
Con/TSC
E
Con/αENaC
F
Con/AQP2
G
KO/TSC
H
KO/αENaC
I
KO/AQP2

Figure 3

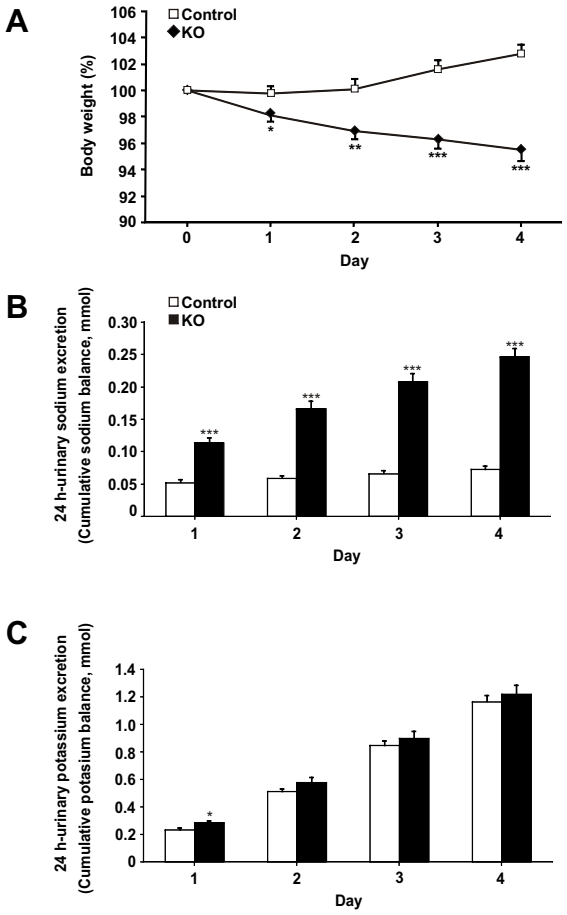

A
Control
KO
Body weight (%)
106
104
102
100
98
96
94
92
90
0
1
2
3
4
Day
*
**
***
***
B
Control
KO
24 h-urinary sodium excretion
(Cumulative sodium balance, mmol)
0.30
0.25
0.20
0.15
0.10
0.05
0
***
***
***
***
1
2
3
4
Day
C
24 h-urinary potassium excretion
(Cumulative potasium balance, mmol)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
1
2
3
4
Day

Figure 4

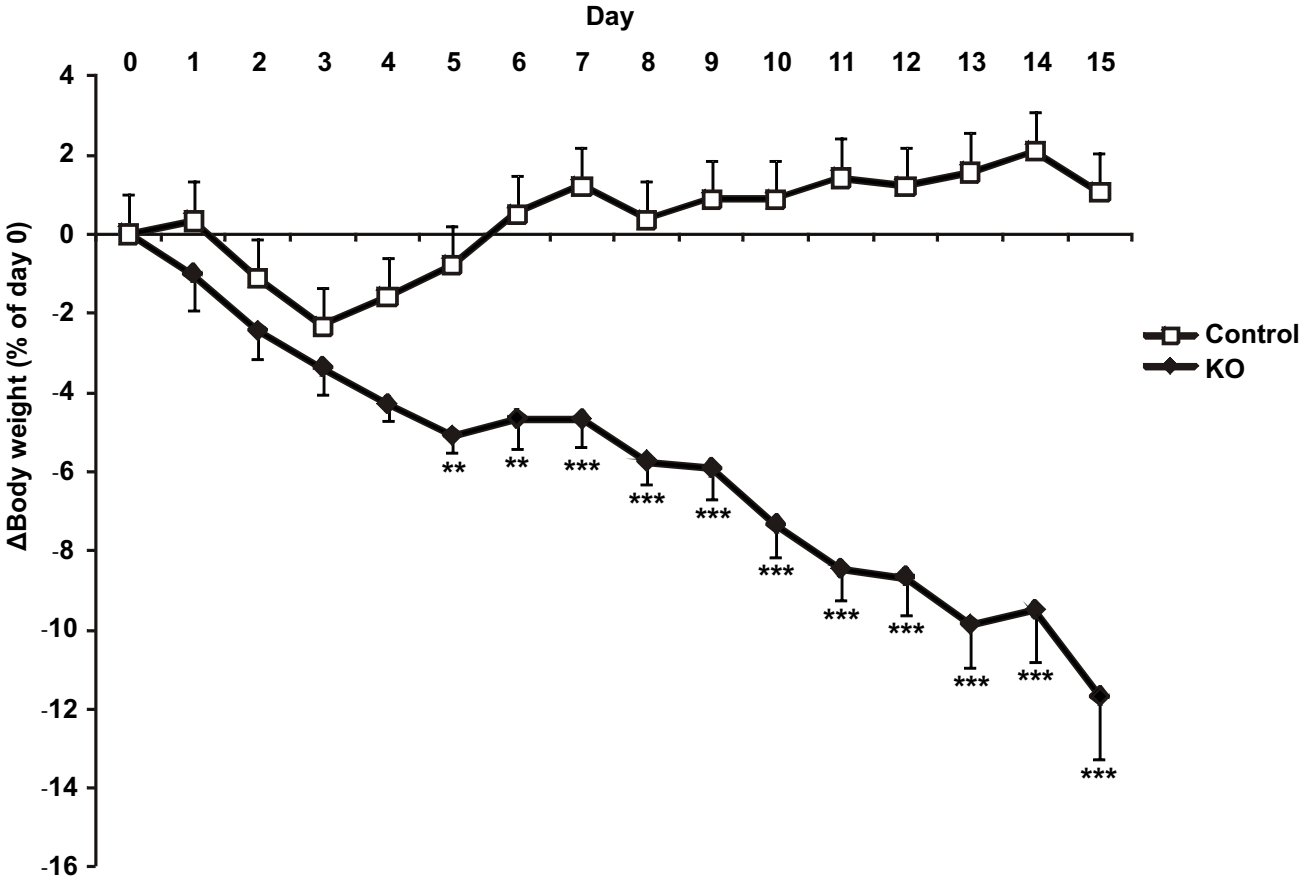

Day
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
ΔBody weight (% of day 0)
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
**
**
***
***
***
***
***
***
***
***
Control
KO

Figure 5

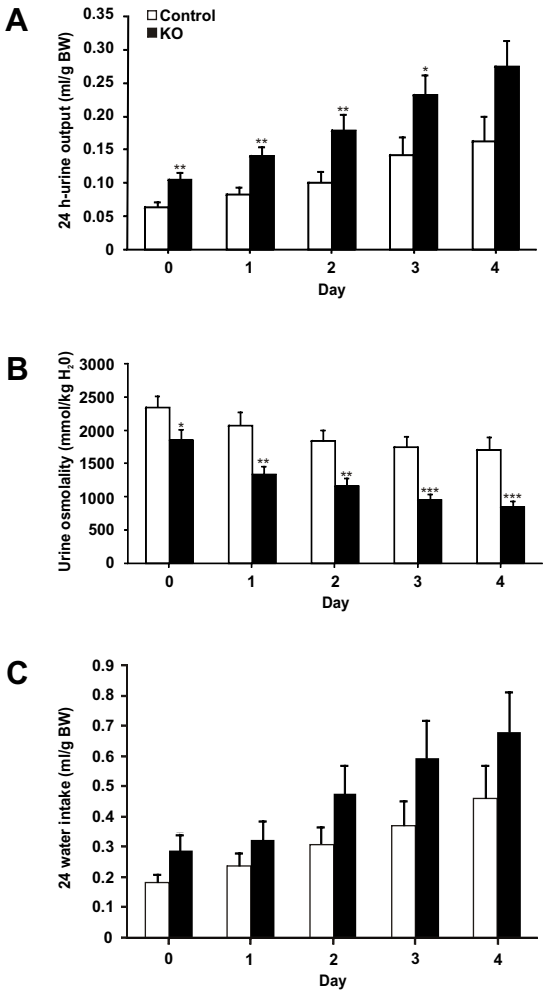
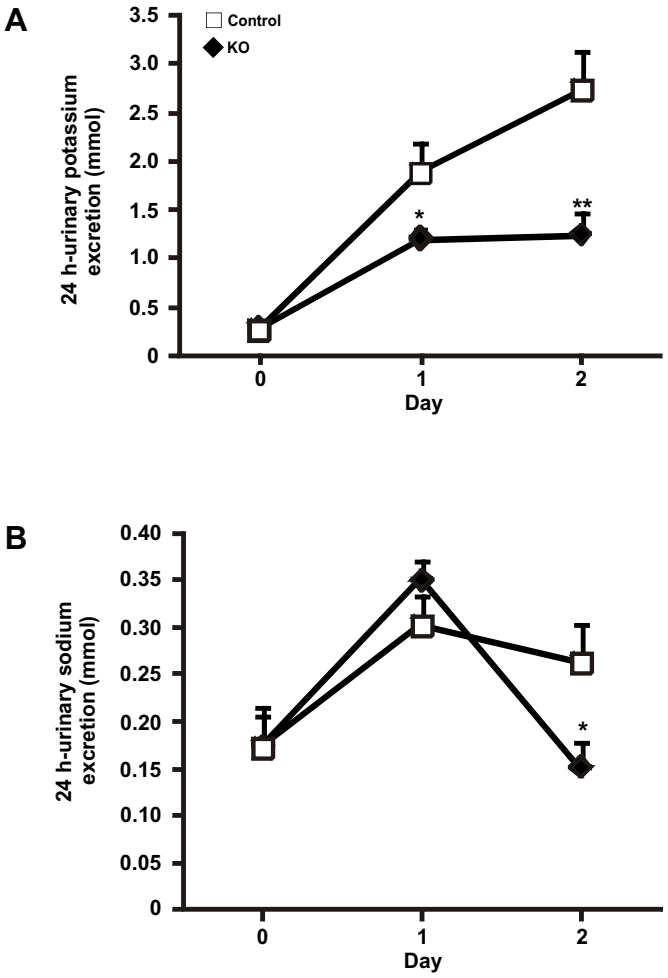

A
Control
KO
24 h-urine output (ml/g BW)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
**
**
**
*
0
1
2
3
4
Day
B
Urine osmolality (mmol/kg H2O)
3000
2500
2000
1500
1000
500
0
*
**
**
***
***
0
1
2
3
4
Day
C
24 water intake (ml/g BW)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
Day

Figure 6


A
Control
KO
24 h-urinary potassium excretion (mmol)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
**
0
1
2
Day
B
24 h-urinary sodium excretion (mmol)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
*
0
1
2
Day