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MOLECULAR AND CELLULAR MECHANISMS OF DISEASE

ENaC activity in collecting ducts modulates NCC in cirrhotic mice

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Abstract Cirrhosis is a frequent and severe disease, complicated by renal sodium retention leading to ascites and oedema. A better understanding of the complex mechanisms responsible for renal sodium handling could improve clinical management of sodium retention. Our aim was to determine the importance of the amiloride-sensitive epithelial sodium channel (ENaC) in collecting ducts in compensate and decompensate cirrhosis. Bile duct ligation was performed in control mice (CTL) and collecting duct-specific αENaC knockout (KO) mice, and ascites development, aldosterone plasma concentration, urinary sodium/potassium ratio and sodium transporter expression were compared. Disruption of ENaC in collecting ducts (CDs) did not alter ascites development, urinary sodium/potassium ratio, plasma aldosterone concentrations or Na,K-ATPase abundance in CCDs. Total αENaC abundance in whole kidney increased in cirrhotic mice of both genotypes and cleaved forms of α and γ ENaC increased only in ascitic mice of both genotypes. The sodium chloride cotransporter (NCC) abundance was lower in non-ascitic KO, compared to non-ascitic CTL, and increased when ascites appeared. In ascitic mice, the lack of αENaC in CDs induced an upregulation of total ENaC and NCC and correlated with the cleavage of ENaC subunits. This revealed compensatory mechanisms which could also take place when treating the patients with diuretics. These compensatory mechanisms should be considered for future development of therapeutic strategies.

Keywords Ascites · Aldosterone · Cirrhosis · Cortical collecting ducts · ENaC · NCC

Introduction

Cirrhosis is a frequent and severe disease, complicated by renal sodium retention leading to ascites and oedema. The development of the disease starts with damages to the liver architecture causing an increase of intrahepatic resistance and leading to portal hypertension. The latter is known to stimulate the production of nitric oxide, which, in turn, induces a peripheral arterial vasodilatation and causes intravascular volume insufficiency. This triggers mechanisms of sodium and water conservation through the renin-angiotensin system, the sympathetic nervous system and the vasopressin pathway. It is hypothesized that an inadequate stimulation of these pathways leads to renal sodium retention which will favour ascites accumulation [13, 25]. However, cellular and molecular mechanisms responsible for unbalanced renal sodium transport are incompletely understood.

We showed previously that bile duct-ligated mice developed ascites concomitantly to Na,K-ATPase stimulation in cortical collecting ducts exclusively [1]. Underlining the role

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of the aldosterone-sensitive distal nephron in ascites development, studies performed with rats showed an increased apical targeting of ENaC in ascitic animals [20, 21]. In order to investigate the role of collecting ducts (CDs) in cirrhosis-induced sodium retention, we used a transgenic mouse model with a CD-specific inactivation of the amiloride-sensitive sodium channel (ENaC) [30], which is crucial for regulated renal sodium reabsorption. Rubera et al. showed that mice with disruption of αENaC in CDs were still able to maintain sodium and potassium balance, even when challenged by salt restriction, water deprivation or potassium loading [30]. Our hypothesis was that in pathological conditions, such as cirrhosis, CD function may become of importance.

The aim of the present study was to determine the importance of ENaC in CDs using the bile duct ligation-induced cirrhosis mouse model. We investigated ascites development, plasma aldosterone concentrations, urinary sodium and potassium excretion, as well as the abundance of ENaC subunits, Na,K-ATPase and sodium chloride cotransporter (NCC) in control (CTL) and αENaC KO (KO) mice.

Subjects and methods

Animals

Animal studies were approved by the Veterinary Service of the Canton de Vaud, Switzerland. Experiments were performed on adult CTL (*Scnn1a*$^{lox/lox}$) and αENaC KO (*Hoxb7*::*cre*/*scnn1a*$^{lox/lox}$) mice.

Bile duct ligation

The bile duct ligation was performed under anaesthesia mediated by isoflurane inhalation (57 CTL and 53 KO). A ventral incision was made; ligatures were tightened around the bile duct and the segment in between excised. The same procedure was performed in SHAM-operated mice (12 CTL and 10 KO) except that no ligatures were tightened. After ligation, abdominal muscles were sutured and skin closed with *Michel's* suture clips. Mice were observed daily and received paracetamol (200 mg/kg/day) in drinking water for 2 days following surgery.

Ascites quantitation

Ascites development was monitored by body weight measurement. Groups were determined *a posteriori* at the time of sacrifice. Mice with ascites at the time of sacrifice were included in BDL+ group and the other in BDL−. The rate of ascites accumulation was estimated by the calculation of bodyweight difference between a measurement and the previous one.

Plasma aldosterone concentrations

Plasma aldosterone concentrations were measured by RIA (Coat-a-Count; Diagnostics Products Inc.).

Immunostaining

Kidneys of anesthetized mice were fixed for 5 min with 3 % PFA in phosphate buffer by retrograde perfusion via the abdominal aorta [22]. Kidneys were cut in thin sections, frozen in liquid propane and stored at −80 °C until further analysis. Immunohistochemistry was performed on 4 μm cryosections. Sections were blocked with 10 % normal goat serum and subsequently incubated over night at 4 °C with the primary antibodies (N-ter αENaC 1/5000 [33]); followed by a Cy3-conjugated donkey anti-rabbit IgG (Jackson Immuno Research Laboratories) diluted 1/1000. The sections were analysed by a fluorescence microscope (Leica). Pictures were taken with a CCD camera and processed with Adobe Photoshop and Microsoft PowerPoint softwares.

Urine collection

Mice were installed into restraining tube, every 3 to 4 days from 8:00 am to 11:00 am, for urine collection. Urinary sodium and potassium were measured by flame photometry (IL943, Instrumentation Laboratory).

Microdissection of renal tubules

The left kidney was perfused with a liberase-containing solution (liberase TM 33 μg/ml from Roche in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12), 21041-025; Gibco). Pyramids cut along the corticomedullary axis were incubated at 30 °C for 40 min in perfusion medium. Tubules were isolated in ice-cold DMEM/F-12 supplemented with 0.05 % BSA without liberase. Tubules were transferred into 96-well plates and photographed. The total length of tubules was measured using Image J [32].

Na,K-ATPase assay

Na,K-ATPase activity was determined as previously described [5]. Total ATPase activity was determined in a solution containing 50 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 100 mM Tris-HCl, 10 mM Na_2ATP and 8 nCi/μl of ATP [γ-^{32}P] (10 Ci/mmol, 2 mCi/ml, Perkin Elmer: BLU002250UC) at pH 7.4. For Na^+, K^+-independent ATPase activity measurements, NaCl and KCl were omitted, Tris-HCl was 150 mM, and 2 mM ouabain was added. Na,K-ATPase activity was taken as the difference between total and Na^+, K^+-independent ATPase activities and expressed as the mean in pmole/mm/h of *n* measurements.

Table 1 Primers and probe number used for qPCR

Gene	Forward primer	Reverse primer	Probe
scnn1a	CCAAGGGTGTAGAGTTCTGTGA	AGAAGGCAGCCTGCAGTTTA	45
scnn1b	TTCAACTGGGGCATGACAG	CCGATGTCCAGGATCAACTT	29
scnn1g	AACAGAGAAAACGCCACCAT	TTATGTATAAGATGACTTGCAGACCA	16
slc12a3	CCTCCATCACCAACTCACCT	CCGCCCACTTGCTGTAGTA	12

SDS-PAGE and immunoblotting

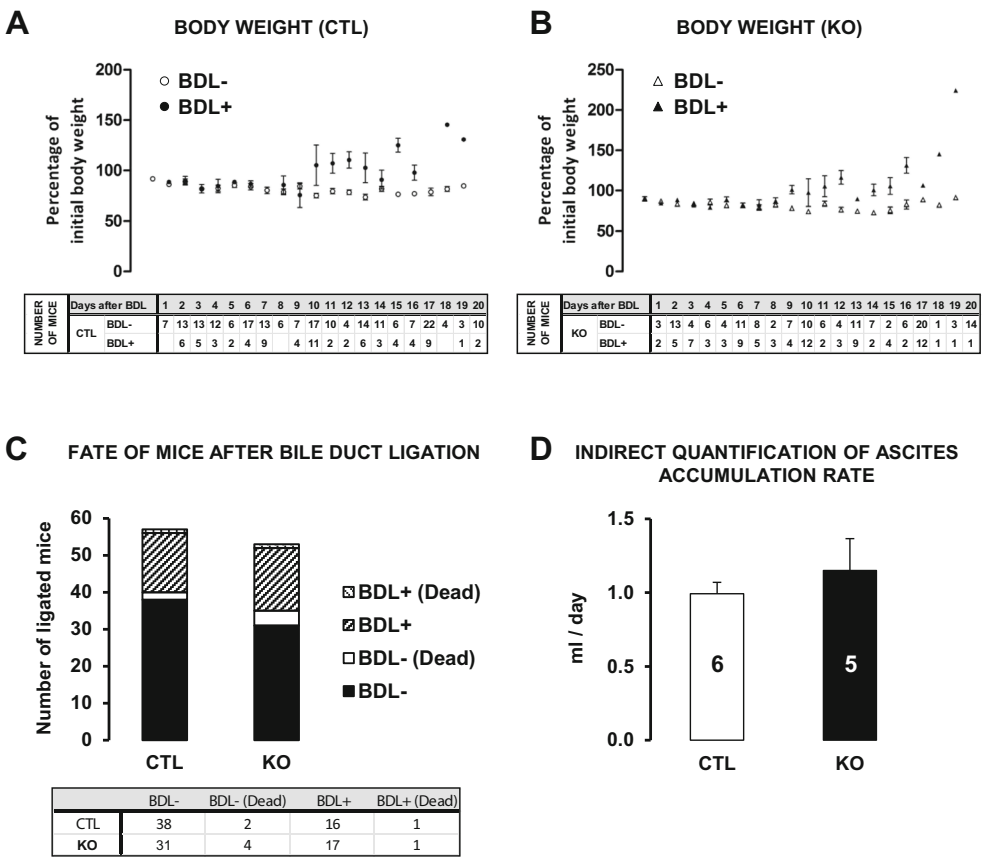
Kidneys were homogenized using a Dounce tissue grinder and membrane proteins extracted in the presence of protease and phosphatase inhibitors (No. 78440, Pierce) according to the manufacturer's protocol (MEM Per Plus Kit No. 89842, Pierce). Protein concentration was quantified using the BCA protein assay kit (No. 23225, Pierce). Ten micrograms was separated by SDS-PAGE, then transferred on nitrocellulose membrane and stained with Ponceau red before immunodetection. Nitrocellulose membranes were incubated with primary antibodies detecting αENaC (1/10,000) [33], βENaC (1/10,000) [34] and γENaC (1/10,000) [34] and NCC (1/10,000) [33]. Immunoblots were scanned using the Molecular Imager Chemidoc XRS+ (Bio-Rad). Relative quantification was obtained by dividing the densitometric values of the proteins of interest by the densitometric values obtained with Ponceau red staining for the corresponding lane.

Total RNA extraction and qPCR

Total RNA was extracted from kidneys with Trizol according manufacturer's protocol (Life Technologies). Reverse transcription was performed on 1 μg of total RNA with the ImProm-II™ Reverse Transcription System (Promega). Relative abundances of transcripts were calculated after qPCR amplification. Primers and probe number (corresponding to the Universal ProbeLibrary (Roche)), targeting transcripts of interest and described in Table 1, were designed using ProbeFinder software (Roche).

Statistics

Results are expressed as means±SEM from several animals. Two-way ANOVA, one-way ANOVA followed by Bonferroni's post-test or Student's *t* test were used



NUMBER OF MICE	Days after BDL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
CTL	BDL-	7	13	13	12	6	17	13	6	7	17	10	4	14	11	6	7	22	4	3	10
	BDL+		6	5	3	2	4	9		4	11	2	2	6	3	4	4	9		1	2

NUMBER OF MICE	Days after BDL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
KO	BDL-	3	13	4	6	4	11	8	2	7	10	6	4	11	7	2	6	20	1	3	14
	BDL+	2	5	7	3	3	9	5	3	4	12	2	3	9	2	4	2	12	1	1	1

	BDL-	BDL- (Dead)	BDL+	BDL+ (Dead)
CTL	38	2	16	1
KO	31	4	17	1

Fig. 1 Evolution of body weight after bile duct ligation of CTL (**a**) or αENaC KO (**b**) mice without (BDL−) and with (BDL+) ascites. The number of animals weighed is indicated in tables underneath (*BDL* bile duct ligation). Fate of mice after bile duct ligation (**c**). Indirect quantification of ascites accumulation rate six CTL and five KO mice (**d**). Values are expressed as mean±SEM

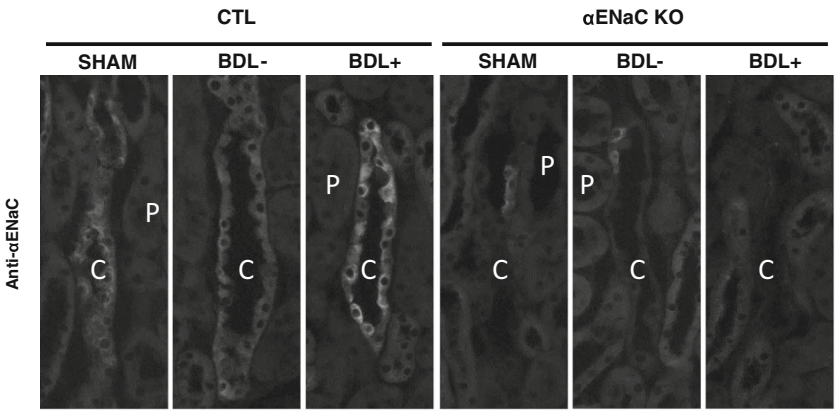


Fig. 2 Renal immunolocalization of αENaC for SHAM, BDL− and BDL+ mice. Collecting ducts (*C*), proximal tubules (*P*)

to determine statistical differences. A two-sample test for the equality of proportions was used to analyse the fate of mice after bile duct ligation.

Results

Control and collecting duct alpha ENaC KO mice develop ascites

The presence of ascites was a prerequisite to identify cirrhotic mice with sodium retention. Ascites development was indirectly estimated by body weight measurement over 20 days following surgery (Fig. 1a, b). Around 10 days after BDL, 30 % of CTL (17 out of 57) and 34 % of KO (18 out of 53) of bile duct-ligated mice rapidly gained weight due to ascites accumulation (BDL+). Mice were sacrificed when ascites was observed for several consecutive days. The proportion of mice developing ascites and their survival rate after bile duct ligation were not affected by the genotype ($p=0.79$) (Fig. 1c). Ascites accumulated at 1 ml per day, and its volume reached about 10 ml at the time of sacrifice (Fig. 1d). Mice which did not gain weight over a period of 20 to 30 days after BDL were sacrificed and considered to be mice with compensated cirrhosis (BDL−).

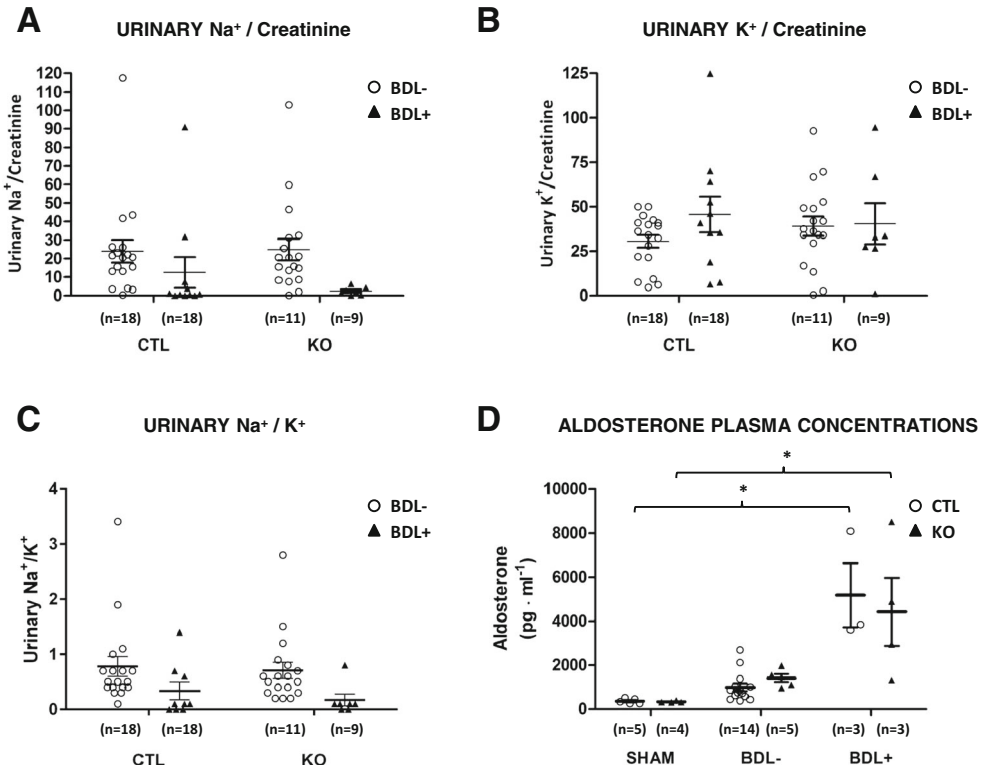


Fig. 3 Urinary Na^+, K^+/creatinine and Na^+/K^+ ratio and plasma aldosterone concentrations. Urinary Na^+/creatinine (**a**). Urinary K^+/creatinine (**b**). Urinary Na^+/K^+ ratio of CTL and KO mice: without ascites, more than 2 weeks after bile duct ligation (BDL−) and with ascites, more than 2 weeks after bile duct ligation (BDL+) (**c**). Aldosterone plasma concentrations of CTL and KO mice: sham-operated (SHAM), without (BDL−) and with (BDL+) ascites (**d**). Values are expressed as mean±SEM. *Significant differences were revealed by two-way ANOVA followed by Bonferroni's post-test

Immunolocalization confirms αENaC disruption in CDs

Deletion of αENaC along CDs of αENaC KO mice was assessed by immunostaining (Fig. 2). In CTL mice, αENaC was seen in all principal cells of CDs, independent from the group (SHAM, BDL− and BDL+). In KO mice, αENaC was absent from CD cells. A very few principal cells with remaining αENaC expression were seen in the initial cortical collecting duct.

Urinary sodium and potassium excretion are similar in CTL and αENaC KO mice

As expected in mice retaining sodium, we observed a significant reduction of the urinary Na^+/creatinine (two-way ANOVA with genotype and presence of ascites as factors: interaction, p=not significant (ns); genotype, p=ns; presence of ascites, p=0.0217) (Fig. 3a) and of the urinary Na^+/K^+ ratio (two-way ANOVA: interaction, p=ns; genotype, p=ns;

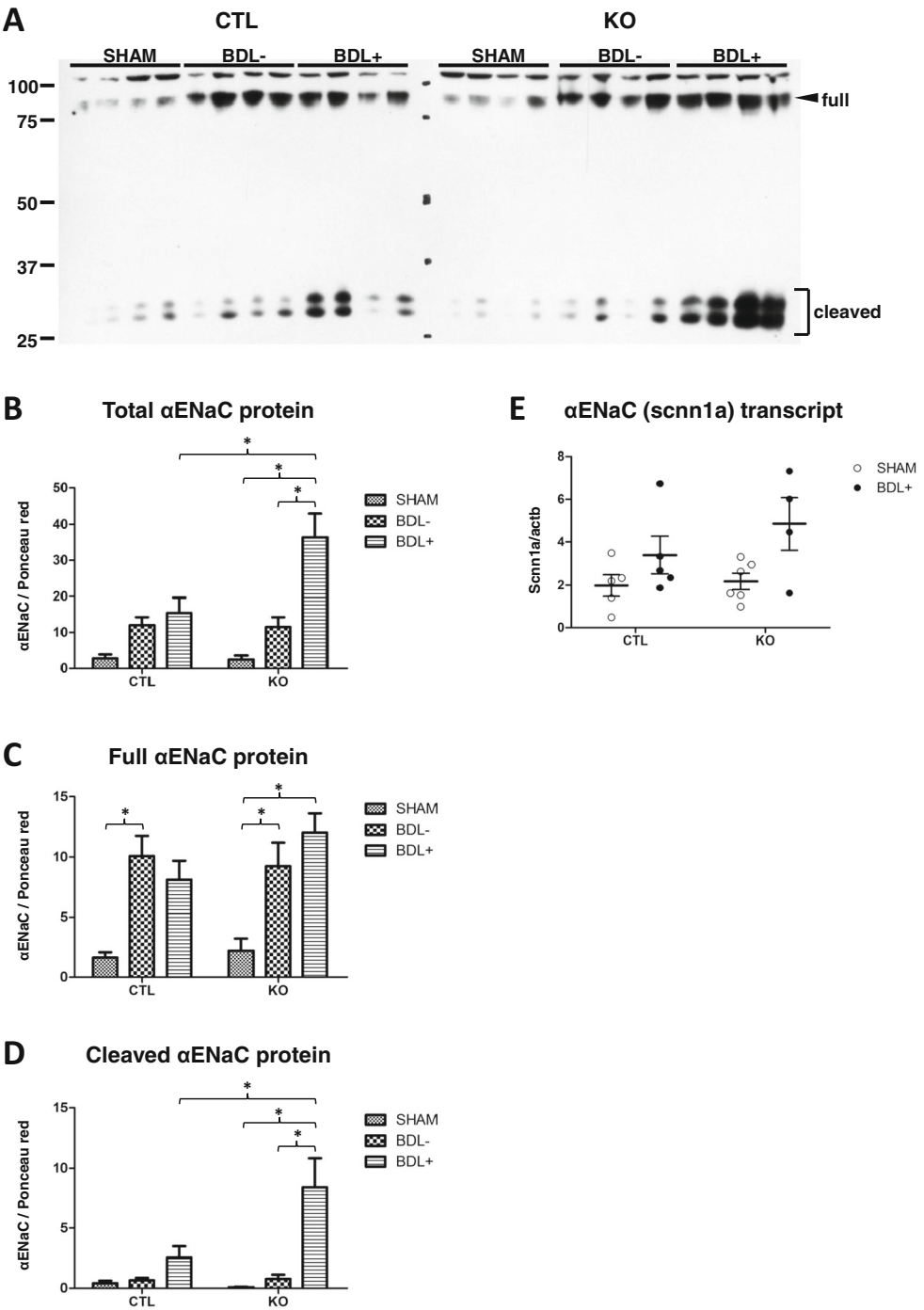


Fig. 4 αENaC abundance. Membrane protein extracts from whole kidneys from CTL (n=4) and KO (n=4) mice were loaded on a single 10 % polyacrylamide gel (**a**) and quantified. Abundance of the total αENaC protein (**b**). Abundance of the full αENaC protein (**c**). Abundance of the αENaC cleaved protein (**d**). Abundance of αENaC transcript (scnn1a) (**e**). Values are expressed as mean±SEM. *Significant differences were revealed by two-way ANOVA followed by Bonferroni's post-test

presence of ascites, $p=0.0133$) (Fig. 3c). The urinary K^+/creatinine (Fig. 3b) remained unchanged.

Plasma aldosterone concentrations increase in CTL and αENaC KO mice with ascites

Plasma aldosterone concentrations increased independently of genotypes following bile duct ligation (two-way ANOVA with health status: SHAM, BDL−, BDL+ and genotype as factors: interaction, p=ns; genotype, p=ns; presence of ascites, $p<0.0001$) (Fig. 3d); the difference between SHAM and BDL− group was not significant.

ENaC is upregulated in cirrhotic CTL and KO mice

Immunoblots on membrane proteins extracted from kidney homogenates showed increased αENaC abundance in cirrhotic mice (BDL− or BDL+) (Fig. 4a). The cleaved form of αENaC appeared as doublets, as previously observed by others [6, 10, 11]. Statistical analysis of band density quantifications revealed (Fig. 4b) an upregulation of total αENaC (full and cleaved forms) (two-way ANOVA with health status: SHAM, BDL− and BDL+ and genotype as factors: interaction, $p=0.0107$; genotype, $p=0.0348$; bile duct ligation, $p<0.0001$—Bonferroni's post-test: KO SHAM vs KO BDL+, $p<0.0001$; KO BDL− vs KO BDL+, $p=0.0017$; CTL BDL+ vs KO BDL+ $p=0.0096$).

Analysis of the αENaC full form showed an increase in BDL− mice (two-way ANOVA: interaction=ns; genotype, p=ns; bile duct ligation, $p<0.0001$—Bonferroni's post-test: CTL SHAM vs CTL BDL−, $p=0.0107$, KO SHAM vs KO BDL−, $p=0.0498$ and KO SHAM vs KO BDL+, $p=0.0025$) (Fig. 4c).

An increase of the αENaC cleaved form was observed in ascitic mice; however, it was significant only in the KO BDL+ likely due to the variability observed in CTL BDL+ mice (two-way ANOVA: interaction, $p=0.0175$; genotype, $p=0.0462$; bile duct ligation, $p=0.0002$—Bonferroni's post-test: KO SHAM vs KO BDL−, $p=0.0005$; KO SHAM vs KO BDL+, $p=0.0014$; CTL BDL+ vs KO BDL+, $p=0.018$) (Fig. 4d). The abundance of αENaC transcript (scnn1a) was affected in BDL+ mice independently of genotypes (two-way ANOVA: interaction, p=ns; genotype, p=ns, bile duct ligation, $p=0.0133$) (Fig. 4e). The expression of βENaC subunit is illustrated in Fig. 5a (two-way ANOVA: interaction, $p=0.0169$; genotype, p=ns; bile duct ligation $p<0.0001$—Bonferroni's post-test: KO SHAM vs KO BDL−, $p<0.0003$; KO BDL− vs KO BDL+, $p=0.0001$; CTL BDL− vs KO BDL−, $p=0.0375$) (Fig. 5b). The abundance of βENaC transcript (scnn1b) was affected by the bile duct ligation (two-way

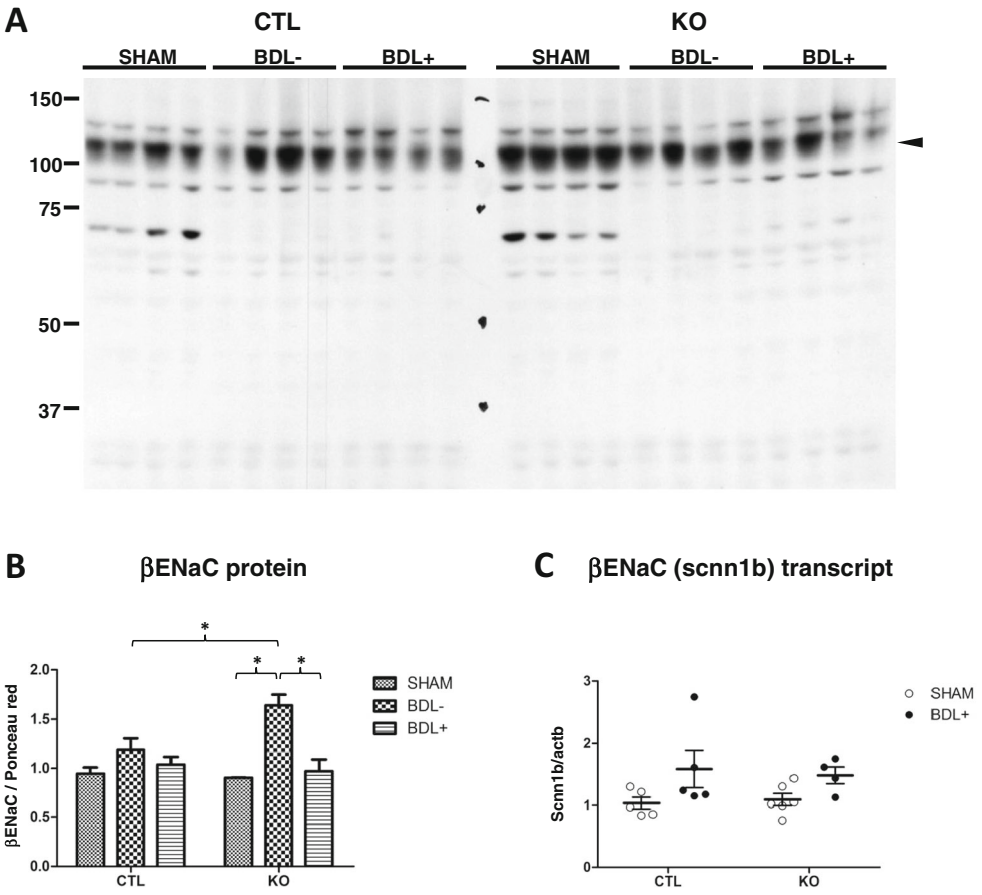


Fig. 5 βENaC abundance. Membrane protein extracts from whole kidneys from CTL (n=4) and KO (n=4) mice were loaded on a single 10 % polyacrylamide gel (**a**) and quantified. Abundance of βENaC protein (**b**). Abundance of βENaC transcript (scnn1b) abundance (**c**). Values are expressed as mean±SEM. *Significant differences were revealed by two-way ANOVA followed by Bonferroni's post-test

ANOVA: interaction, p=ns; genotype, p=ns, bile duct ligation, p=0.0185) (Fig. 5c).

Immunoblots showed a higher abundance of the γENaC cleaved form compared to its full form for two CTL BDL+ and KO BDL+ mice and the quasi absence of the γENaC cleaved form in BDL− (Fig. 6a). No differences were seen in the total γENaC protein (full and cleaved). The full γENaC was less abundant in BDL+ than in BDL− in both genotypes

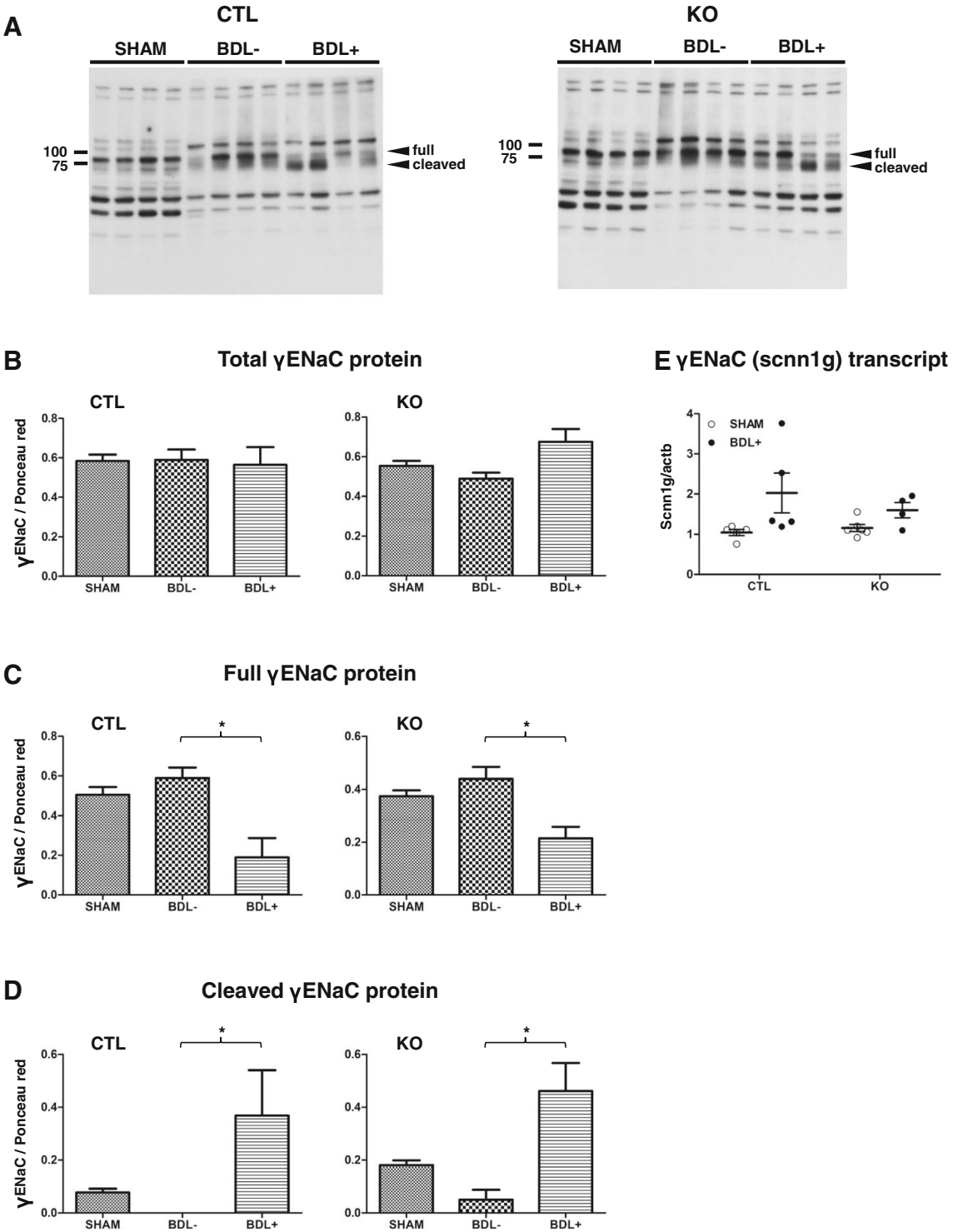


Fig. 6 γENaC abundance. Membrane protein extracts from whole kidneys CTL (n=4) of and KO (n=4) of mice were loaded on two different 4–20 % polyacrylamide gels (**a**). Abundance of the total γENaC protein (full and cleaved) (**b**); abundance of the full γENaC protein (**c**); abundance of the cleaved γENaC protein (**d**); abundance of γENaC transcript (scnn1g) (**e**). Values are expressed as mean±SEM. *Significant differences were revealed by one-way ANOVA followed by Bonferroni's post-test

(one-way ANOVA, Bonferroni's post-test: CTL BDL− vs CTL BDL+, adj. p=0.0134; KO BDL− vs KO BDL+, adj. p=0.0243) (Fig. 6c) while γENaC cleaved form was more abundant in BDL+ than in BDL− mice (one-way ANOVA, Bonferroni's post-test: CTL BDL− vs CTL BDL+, adj. p=0.044; KO BDL− vs KO BDL+, adj. p=0.0098) (Fig. 6d). The abundance of γENaC transcript (scnn1g) was affected by the bile duct ligation (two-way ANOVA: interaction, p=ns; genotype, p=ns, bile duct ligation, p=0.0185) (Fig. 6e).

The absence of ENaC activity in collecting ducts does not influence Na,K-ATPase abundance

Previously used as a marker for sodium reabsorption along renal tubules [1], the Na,K-ATPase activity measurements performed in microdissected tubules at V_{max} reflects the abundance of Na,K-ATPase holoenzyme. In this study, the measurements did not reveal differences between groups (Fig. 7).

Sodium chloride cotransporter abundance differs between CTL and KO cirrhotic mice

Immunoblots on membrane proteins extracted from whole kidney showed the variation of NCC abundance in cirrhotic mice (Fig. 8a). Quantification of band density showed a lower expression of NCC in cirrhotic KO BDL− (one-way ANOVA, Bonferroni's post-test: SHAM vs BDL−, p=0.0287 and BDL − vs BDL+, p=0.0075) (Fig. 8b). Since samples from CTL and KO were loaded on different gels and thus could not be compared, a second electrophoresis was performed to investigate differences between CTL and KO samples in SHAM, BDL− and BDL+ (Fig. 9a, b). The abundance of NCC protein was lower in KO BDL− compared to CTL BDL− (Student's t test: p<0.01) but higher in KO BDL+ compared to CTL BDL+ (Student's t test: p<0.01).

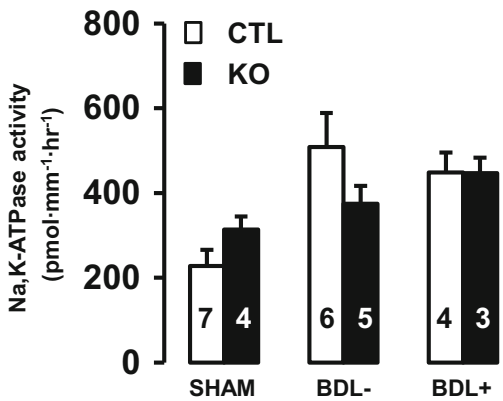


Fig. 7 Na,K-ATPase abundance in cortical collecting ducts. Na,K-ATPase abundance was quantified by measuring the Na,K-ATPase activity at V_{max} in microdissected CCDs from CTL and collecting ducts specific αENaC KO mice for the three groups: SHAM-operated, without ascites (BDL−) and with (BDL+). Values are expressed as mean±SEM. The *number into the columns* represents the *n*

Discussion

To our knowledge, this is the first study investigating the mechanisms of sodium retention by bile duct ligation in genetically modified animals. It demonstrated in ascitic mice that the disruption of ENaC and thus a lack of its activity in CDs induced ENaC and NCC in upstream segments. These results on ENaC expression are in line with previous studies demonstrating the importance of ENaC in CNTs for the regulation of sodium reabsorption [9, 12, 27].

The absence of ENaC in CDs did not affect ascites formation. Ascites developed in 30 % of CTL and 34 % of KO after bile duct ligation.

The immunohistochemical studies confirmed the disruption of ENaC in CDs of KO mice. Although a very few single cells in cortical collecting ducts escape the cre recombinase mediated inactivation of αENaC, as previously reported [30], we consider it highly unlikely that these few single CD cells with persistent αENaC expression can account for the absence of differences between CTL and KO mice. It is likely that upstream segments contribute to sodium retention.

The urinary Na^+/creatinine as well as Na^+/K^+ ratios were reduced and plasma aldosterone concentrations increased in BDL+, as observed in humans [4].

Western blot analysis not only showed for both genotypes an increase of αENaC abundance in BDL− and BDL+ but it also revealed that the cleavage of α and γ subunits was significant only in BDL+ mice, which were retaining sodium. In kidneys, αENaC protein abundance has been shown to be regulated by aldosterone, while βENaC and γENaC not or only weakly [6, 26]. Moreover, aldosterone stimulation or low salt diet was shown to induce an apical redistribution of αENaC, βENaC and γENaC [8, 10, 23, 24, 26] and cleavage of αENaC and γENaC [6, 26]. Cleavage of ENaC by exogenous trypsin has been linked to an increase of channel activity [8, 28]. Kim et al. observed, in ascitic rats, an increased apical targeting of αβγENaC in DCTs, CNTs and CDs and cleavage of γENaC, without changes of αβγENaC protein abundance [21, 20]. Altogether, these results showed that in ascitic animals, α and γ ENaC are cleaved, suggesting an increase in ENaC activity and thus demonstrate its importance for sodium retention. Interestingly, in our study, the αENaC cleaved form was more abundant in KO BDL+ than in CTL BDL+ mice, suggesting insufficient sodium reabsorption and a need to compensate.

In contrast to Ackermann et al., we did not observe any significant upregulation of the Na,K-ATPase activity in isolated CCDs between SHAM-operated and cirrhotic mice [1]. This difference could be due to the mouse strains used. In

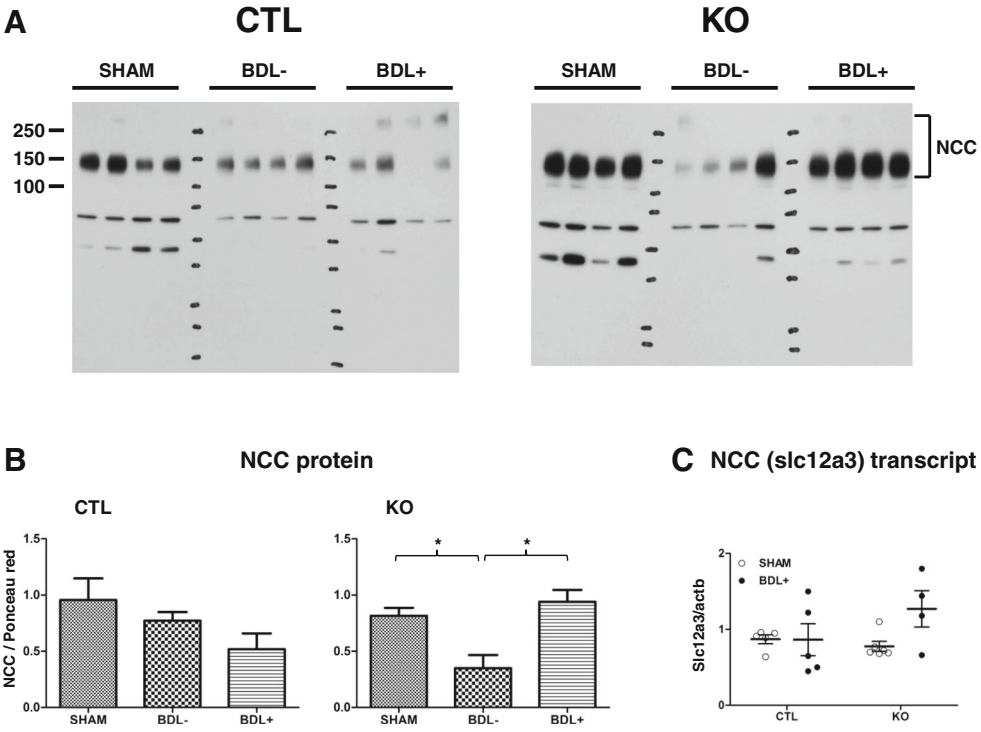


Fig. 8 NCC abundance: comparison between SHAM, BDL− and BDL+. Membrane protein extracts from whole kidneys of CTL (n=4) and KO (n=4) were loaded on a 10 % polyacrylamide gel (**a**). NCC protein abundance (**b**). NCC transcript (sl12a3) abundance transcript (**c**). Values are expressed as mean±SEM. *Significant differences were revealed by one-way ANOVA followed by Bonferroni's post-test

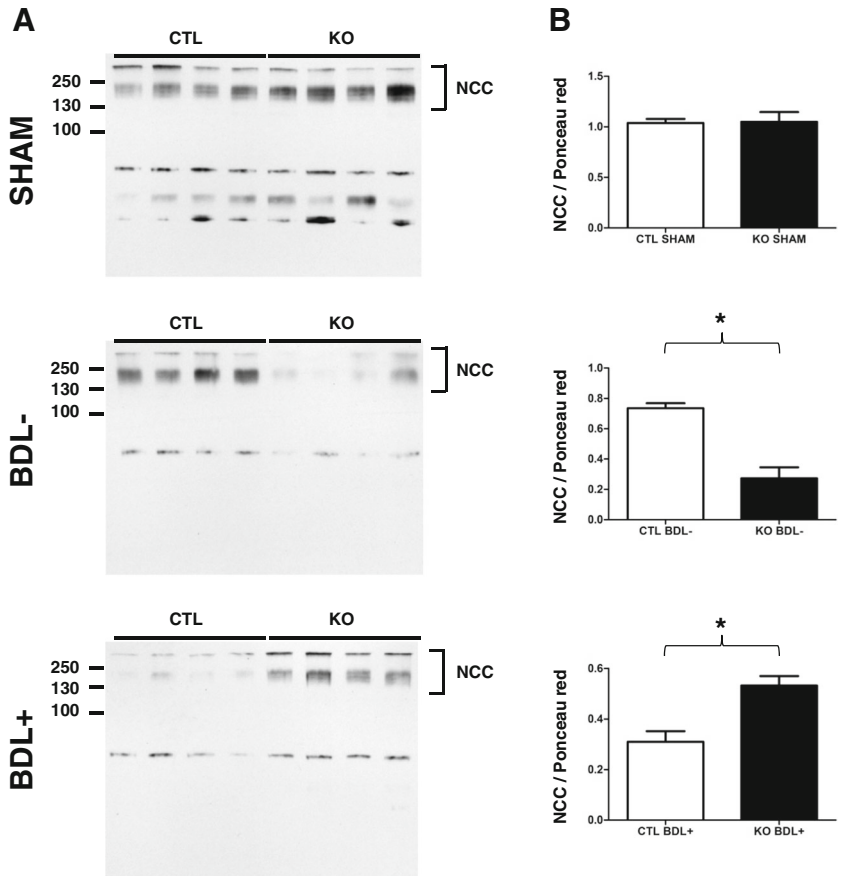


Fig. 9 NCC abundance: comparison between CTL and KO. Membrane protein extracts from whole kidneys SHAM, BDL − and BDL+ were loaded on a 10 % polyacrylamide gel (**a**) and quantified (**b**). Values are expressed as mean±SEM. *Significant differences were revealed by Student's t test

the previous study, CD1 mice were used whereas in the actual one, we used 129/Sv x C57BL/6, since they are more prone to develop ascites [2]. Additionally, mice were analysed at different time point.

We did not observe an increase in NCC abundance, although NCC is described as an aldosterone-induced protein [19]. The slight decrease in NCC abundance observed in CTL cirrhotic mice was not significant in contrast to what was previously reported [7, 14, 35]. In BDL− mice, NCC protein expression was lower than in SHAM, whereas in BDL+ mice, in which sodium retention occurs, it was higher in KO than CTL mice and probably reached the same level as in the SHAM-operated mice as shown by the results in Fig. 8. These results suggested compensation by NCC of an insufficient ENaC activity in CDs. The reduced abundance of NCC in non-ascitic mice may reflect the aldosterone escape phenomenon, characterized by a downregulation of NCC in the presence of high plasma aldosterone concentrations and high salt diet [31]. Conditions which are similar to those encountered by the CTL cirrhotic mice, their plasma aldosterone concentrations are high and their salt intake sufficient. The downregulation of NCC could also be due to an increase in plasma potassium concentration [3]. However, this hypothesis is unlikely since several studies performed in rats reported normal plasma potassium values in cirrhotic animals [14, 15, 17, 18].

In summary, our study showed that in pathological conditions such as cirrhosis, the amount of sodium reabsorbed through ENaC in CDs is likely not negligible and if not sufficient, ENaC and NCC upregulation in upstream segments may happen. Similar ENaC upregulation was suggested by results obtained by Ronzaud et al. in $MR^{AQP2Cre}$ mice [29]. The upregulation of NCC could explain the blunted natriuretic effect of the amiloride observed in cirrhotic rats treated with amiloride [16].

The control of renal sodium retention and ascites development is not trivial in cirrhotic patients. A better understanding of the mechanisms responsible for renal sodium handling would improve its clinical management. This study illustrates the usability of gene-modified mouse models to dissect the complex mechanisms of sodium retention. It revealed compensation mechanisms which could take place when we downregulate artificially ion transporters, similarly as what is done with diuretics. In conclusion, to develop efficient therapeutic strategies, we have to understand pathways leading to sodium/potassium imbalance and to further consider compensatory mechanisms.

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References

1. Ackermann D, Mordasini D, Cheval L, Imbert-Teboul M, Vogt B, Doucet A (2007) Sodium retention and ascites formation in a cholestatic mice model: role of aldosterone and mineralocorticoid receptor? Hepatology 46:173–179. doi:10.1002/hep.21699
2. Alaish SM, Torres M, Ferlito M, Sun CC, De Maio A (2005) The severity of cholestatic injury is modulated by the genetic background. Shock 24:412–416
3. Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ (2001) Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. J Biol Chem 276:28857–28865. doi:10.1074/jbc.M011610200
4. Arroyo V, Bernardi M, Epstein M, Henriksen JH, Schrier RW, Rodes J (1988) Pathophysiology of ascites and functional renal failure in cirrhosis. J Hepatol 6:239–257
5. Doucet A, Katz AI, Morel F (1979) Determination of Na-K-ATPase activity in single segments of the mammalian nephron. Am J Physiol 237:F105–113
6. Ergonul Z, Frindt G, Palmer LG (2006) Regulation of maturation and processing of ENaC subunits in the rat kidney. Am J Physiol Renal Physiol 291:F683–693. doi:10.1152/ajprenal.00422.2005
7. Fernandez-Llama P, Jimenez W, Bosch-Marce M, Arroyo V, Nielsen S, Knepper MA (2000) Dysregulation of renal aquaporins and Na-Cl cotransporter in CCl4-induced cirrhosis. Kidney Int 58:216–228. doi:10.1046/j.1523-1755.2000.00156.x
8. Frindt G, Ergonul Z, Palmer LG (2008) Surface expression of epithelial Na channel protein in rat kidney. J Gen Physiol 131:617–627. doi:10.1085/jgp.200809989
9. Frindt G, Palmer LG (2004) Na channels in the rat connecting tubule. Am J Physiol Renal Physiol 286:F669–674. doi:10.1152/ajprenal.00381.2003
10. Frindt G, Palmer LG (2009) Surface expression of sodium channels and transporters in rat kidney: effects of dietary sodium. Am J Physiol Renal Physiol 297:F1249–1255. doi:10.1152/ajprenal.00401.2009
11. Frindt G, Palmer LG (2012) Regulation of epithelial Na+ channels by adrenal steroids: mineralocorticoid and glucocorticoid effects. Am J Physiol Renal Physiol 302:F20–26. doi:10.1152/ajprenal.00480.2011
12. Fu Y, Gerasimova M, Batz F, Kuczkowski A, Alam Y, Sanders PW, Ronzaud C, Hummler E, Vallon V (2015) PPARgamma agonist-induced fluid retention depends on alphaENaC expression in connecting tubules. Nephron 129:68–74. doi:10.1159/000370254
13. Gines P, Cardenas A, Arroyo V, Rodes J (2004) Management of cirrhosis and ascites. N Engl J Med 350:1646–1654. doi:10.1056/NEJMra035021
14. Graebe M, Brond L, Christensen S, Nielsen S, Olsen NV, Jonassen TE (2004) Chronic nitric oxide synthase inhibition exacerbates renal dysfunction in cirrhotic rats. Am J Physiol Renal Physiol 286:F288–297. doi:10.1152/ajprenal.00089.2003
15. Jonassen TE, Brond L, Torp M, Graebe M, Nielsen S, Skott O, Marcussen N, Christensen S (2003) Effects of renal denervation

on tubular sodium handling in rats with CBL-induced liver cirrhosis. Am J Physiol Renal Physiol 284:F555–563. doi:10.1152/ajprenal.00258.2002

16. Jonassen TE, Heide AM, Janjua NR, Christensen S (2002) Collecting duct function in liver cirrhotic rats with early sodium retention. Acta Physiol Scand 175:237–244
17. Jonassen TE, Marcussen N, Haugan K, Skyum H, Christensen S, Andreasen F, Petersen JS (1997) Functional and structural changes in the thick ascending limb of Henle's loop in rats with liver cirrhosis. Am J Physiol 273:R568–577
18. Jonassen TE, Nielsen S, Christensen S, Petersen JS (1998) Decreased vasopressin-mediated renal water reabsorption in rats with compensated liver cirrhosis. Am J Physiol 275:F216–225
19. Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB, Knepper MA (1998) The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. Proc Natl Acad Sci U S A 95:14552–14557
20. Kim SW, Wang W, Nielsen J, Praetorius J, Kwon TH, Knepper MA, Frokiaer J, Nielsen S (2004) Increased expression and apical targeting of renal ENaC subunits in puromycin aminonucleoside-induced nephrotic syndrome in rats. Am J Physiol Renal Physiol 286:F922–935. doi:10.1152/ajprenal.00277.2003
21. Kim SW, Wang W, Sassen MC, Choi KC, Han JS, Knepper MA, Jonassen TE, Frokiaer J, Nielsen S (2006) Biphasic changes of epithelial sodium channel abundance and trafficking in common bile duct ligation-induced liver cirrhosis. Kidney Int 69:89–98. doi:10.1038/sj.ki.5000018
22. Loffing J, Loffing-Cueni D, Valderrabano V, Klausli L, Hebert SC, Rossier BC, Hoenderop JG, Bindels RJ, Kaissling B (2001) Distribution of transcellular calcium and sodium transport pathways along mouse distal nephron. Am J Physiol Renal Physiol 281:F1021–1027
23. Loffing J, Pietri L, Aregger F, Bloch-Faure M, Ziegler U, Meneton P, Rossier BC, Kaissling B (2000) Differential subcellular localization of ENaC subunits in mouse kidney in response to high- and low-Na diets. Am J Physiol Renal Physiol 279:F252–258
24. Loffing J, Zecevic M, Feraille E, Kaissling B, Asher C, Rossier BC, Firestone GL, Pearce D, Verrey F (2001) Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. Am J Physiol Renal Physiol 280:F675–682
25. Martin PY, Gines P, Schrier RW (1998) Nitric oxide as a mediator of hemodynamic abnormalities and sodium and water retention in cirrhosis. N Engl J Med 339:533–541. doi:10.1056/NEJM199808203390807
26. Masilamani S, Kim GH, Mitchell C, Wade JB, Knepper MA (1999) Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. J Clin Invest 104:R19–23. doi:10.1172/JCI7840
27. Meneton P, Loffing J, Warnock DG (2004) Sodium and potassium handling by the aldosterone-sensitive distal nephron: the pivotal role of the distal and connecting tubule. Am J Physiol Renal Physiol 287:F593–601. doi:10.1152/ajprenal.00454.2003
28. Nesterov V, Dahlmann A, Bertog M, Korbmacher C (2008) Trypsin can activate the epithelial sodium channel (ENaC) in microdissected mouse distal nephron. Am J Physiol Renal Physiol 295:F1052–1062. doi:10.1152/ajprenal.00031.2008
29. Ronzaud C, Loffing J, Bleich M, Gretz N, Grone HJ, Schutz G, Berger S (2007) Impairment of sodium balance in mice deficient in renal principal cell mineralocorticoid receptor. J Am Soc Nephrol 18:1679–1687. doi:10.1681/ASN.2006090975
30. Rubera I, Loffing J, Palmer LG, Frindt G, Fowler-Jaeger N, Sauter D, Carroll T, McMahon A, Hummler E, Rossier BC (2003) Collecting duct-specific gene inactivation of alphaENaC in the mouse kidney does not impair sodium and potassium balance. J Clin Invest 112:554–565. doi:10.1172/JCI16956
31. Sandberg MB, Maunsbach AB, McDonough AA (2006) Redistribution of distal tubule Na+-Cl- cotransporter (NCC) in response to a high-salt diet. Am J Physiol Renal Physiol 291:F503–508. doi:10.1152/ajprenal.00482.2005
32. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675
33. Sorensen MV, Grossmann S, Roesinger M, Gresko N, Todkar AP, Barmettler G, Ziegler U, Odermatt A, Loffing-Cueni D, Loffing J (2013) Rapid dephosphorylation of the renal sodium chloride cotransporter in response to oral potassium intake in mice. Kidney Int 83:811–824. doi:10.1038/ki.2013.14
34. Wagner CA, Loffing-Cueni D, Yan Q, Schulz N, Fakitsas P, Carrel M, Wang T, Verrey F, Geibel JP, Giebisch G, Hebert SC, Loffing J (2008) Mouse model of type II Bartter's syndrome. II. Altered expression of renal sodium- and water-transporting proteins. Am J Physiol Renal Physiol 294:F1373–1380. doi:10.1152/ajprenal.00613.2007
35. Yu Z, Serra A, Sauter D, Loffing J, Ackermann D, Frey FJ, Frey BM, Vogt B (2005) Sodium retention in rats with liver cirrhosis is associated with increased renal abundance of NaCl cotransporter (NCC). Nephrol Dial Transplant 20:1833–1841. doi:10.1093/ndt/gfh916