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Impaired expression of key molecules of ammoniogenesis underlies renal acidosis in a rat model of chronic kidney disease

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ABSTRACT

Background. Advanced chronic kidney disease (CKD) is associated with the development of renal metabolic acidosis. Metabolic acidosis *per se* may represent a trigger for progression of CKD. Renal acidosis of CKD is characterized by low urinary ammonium excretion with preserved urinary acidification indicating a defect in renal ammoniogenesis, ammonia excretion or both. The underlying molecular mechanisms, however, have not been addressed to date.

Methods. We examined the Han:SPRD rat model and used a combination of metabolic studies, mRNA and protein analysis of renal molecules involved in acid–base handling.

Results. We demonstrate that rats with reduced kidney function as evident from lower creatinine clearance, lower haematocrit, higher plasma blood urea nitrogen, creatinine, phosphate and potassium had metabolic acidosis that could be aggravated by HCl acid loading. Urinary ammonium excretion was highly reduced whereas urinary pH was more acidic in CKD compared with control animals. The abundance of key enzymes and transporters of proximal tubular ammoniogenesis (phosphate-dependent glutaminase, PEPCK and SNAT3) and bicarbonate transport (NBCe1) was reduced in CKD compared with control animals. In the collecting duct, normal expression of the B1 H^+ -ATPase subunit is in agreement with

low urinary pH. In contrast, the RhCG ammonia transporter, critical for the final secretion of ammonia into urine was strongly down-regulated in CKD animals.

Conclusion. In the Han:SPRD rat model for CKD, key molecules required for renal ammoniogenesis and ammonia excretion are highly down-regulated providing a possible molecular explanation for the development and maintenance of renal acidosis in CKD patients.

Keywords: acidosis, ammoniogenesis, CKD

INTRODUCTION

Metabolic acidosis is common in chronic kidney disease (CKD) and is associated with several complications such as muscle wasting, impaired growth in children, bone disease, hypoalbuminaemia, inflammation and insulin resistance. Moreover, it has been associated with increased mortality in dialysis and non-dialysis-dependent CKD patients [1–5]. Numerous recent studies particularly highlighted the role of metabolic acidosis in the progression of CKD and the increased risk to develop end-stage renal disease [6–9]. The mechanisms of metabolic acidosis in CKD were first functionally investigated >50 years ago and it was shown that patients with metabolic acidosis and CKD demonstrate reduced excretion of ammonium, the most important

buffer for renal acid excretion and bicarbonate synthesis, with preserved ability to acidify urine [10–13]. However, the affected molecular pathways in the development of metabolic acidosis in CKD have not been addressed to date.

Hannover rat (Han:SPRD) is a commonly used model for autosomal dominant polycystic kidney disease, although the disease is caused by a mutation in the *Pkdr1* gene and no mutations are found in humans with polycystic kidney disease. Heterozygous male (cy/+) animals are characterized by a slow progression of kidney disease and proteinuria [14, 15]. Consequently, these rats have also been used as a model for CKD [16, 17].

Renal ammoniogenesis and ammonium excretion depend on a complex interplay between different nephron segments [18–21]. Ammoniogenesis in the proximal tubule serves mostly the *de novo* synthesis of bicarbonate to replenish bicarbonate buffers used by metabolism. Glutamine is taken up by proximal tubule cells, most likely by the SNAT3 amino acid transporter and metabolized by a series of enzymes located in mitochondria and cytosol. Phosphate-dependent glutaminase (PDG) and glutamate dehydrogenase (GDH) release two ammonia (NH₃) molecules and one bicarbonate (HCO₃⁻) ion whereas further metabolism of the resulting α -ketoglutarate by the phosphoenol pyruvate carboxy kinase (PEPCK) generates an additional HCO₃⁻ ion. The process of proximal tubular ammoniogenesis is highly stimulated during acidosis or by acid load [21, 22]. Ammonium (NH₄⁺) produced by the proximal tubule is mostly released into urine and is partly reabsorbed at the level of the thick ascending limb of the loop of Henle, concentrated in the medullary interstitium and secreted into urine [18–20]. The final secretory step depends on the capacity of H⁺-ATPases to acidify urine and the RhCG ammonia transporter [18, 19, 23].

Here, we used the Han:SPRD rats as a CKD model and investigated the regulation of key molecules in renal acid–base handling to identify the molecular mechanisms that contribute to the development of metabolic acidosis in CKD. Our data demonstrate a strongly reduced expression of enzymes and transporters involved in proximal ammoniogenesis and ammonia secretion whereas the capacity to acidify urine was preserved. Thus, our data suggest that metabolic acidosis is caused by the kidneys' impaired ability to produce and excrete adequate ammonium amounts thereby impairing bicarbonate synthesis and metabolic acid buffering.

MATERIALS AND METHODS

Animal model

The study was conducted in 4-month-old male heterozygous (cy/+) and wild-type (+/+) littermate Han:SPRD rats. Rats received standard diet (Provimi Kliba S.A., Switzerland) at Days 1 and 2, and consecutively HCl diet (500 g standard diet mixed with 750 mL of 333 mM HCl) to induce an additional acid-loader wet standard diet (analogously mixed with distilled water) for 2 days (Days 3 and 4). Rats were placed into metabolic cages on Day 1 for adaptation. Water and food intake as well as body weight were measured daily, and 24-h urine (under mineral oil) and stool were collected. All animal

experiments were performed according to national and international guidelines and laws of animal welfare and protocols approved by the local Veterinary Authority (Veterinäramt, Zürich, Switzerland). At the end of the experiments, rats were anaesthetized with isoflurane, heparinized blood was collected from the tail artery, and animals were perfused with PBS (136.9 mM NaCl, 2.68 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4 with HCl) to remove blood. Kidneys were immediately removed and subsequently snap-frozen in liquid nitrogen and stored at –80°C until further analysis.

Blood and urine analysis

Rats were anaesthetized with isoflurane (IsoFlo®, Abbott AG, Baar, Switzerland), 1.5–2.0%, and heparinized blood was collected from the tail artery and analysed immediately for pH, blood gases and electrolytes on a Radiometer ABL 800 Flex blood gas analyser (Radiometer, Copenhagen, Denmark) on Days 2, 3 and 5. In addition, at the end of the experiment (Day 5), blood was taken from the left ventricle and the inferior caval vein to measure plasma creatinine, phosphate, blood urea nitrogen (BUN), glucose and albumin using the clinical chemistry analyser Piccolo® Xpress (Abaxis, Union City, CA, USA). Ion chromatography (Metrohm ion chromatograph, Herisau, Switzerland) was performed to determine urinary Na⁺, K⁺ and Cl⁻ concentrations. Urine pH, pCO₂ and calculated HCO₃⁻ were determined by aspirating urine from the collectors into gas-tight syringes and injecting it into the blood gas analyser. Urinary creatinine was analysed using the Jaffé method [24, 25]. Urinary ammonium concentration was determined using the Berthelot protocol [26]. Urinary phosphate was measured by standards of Urine Precision Controls (Randox Lab, UK). Creatinine clearance was calculated from the measured values.

RNA extraction

Approximately 25 mg of snap-frozen kidney tissue was homogenized in RLT-Buffer (Qiagen, Hilden, Germany) supplemented with 2-mercaptoethanol to a final concentration of 1%. Total RNA was extracted from 600 μ L of each homogenized sample using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. DNase digestion was performed using the RNase-Free DNase Set (Qiagen). Quality, purity and concentration of the isolated RNA preparations were analysed spectroscopically using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA samples were stored at –80°C.

Semi-quantitative real-time RT-PCR

The RNA samples were diluted to 100 ng/ μ L. First, reverse transcription was performed using the TaqMan Reverse Transcription Reagents (Roche Molecular Systems, Branchburg, NJ, USA/Applied Biosystems, Foster City, CA, USA). Briefly, 12.4 μ L RNase-free water, 4 μ L 10 \times RT buffer, 8.8 μ L MgCl₂ solution, 2 μ L random hexamers, 8 μ L dNTP mixture, 0.8 μ L RNase inhibitor and 1 μ L MultiScribe Reverse Transcriptase (respectively, 1 μ L of additional RNase-free water for negative controls) were mixed with 3 μ L of RNA to perform reverse transcription by using the *Tpersonal* Combi thermocycler (Biometra, Göttingen, Germany) with thermo-cycling

conditions set at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. Next, relative mRNA expression was determined using quantitative real-time PCR with the 7500 Fast Real-Time PCR System (Applied Biosystems) on the basis of the standard 7500 run mode. Primers for all genes of interest were designed using Primer3-web software (frodo.wi.mit.edu/primer3) (see Supplementary Table 1). Probes of the tested genes were labelled with the reporter dye FAM at the 5' end and the quencher dye TAMRA at the 3' end (Microsynth, Balgach, Switzerland). The probe of the housekeeping gene rat 18S rRNA (Applied Biosystems) was labelled with the reporter dye VIC at the 5' end and the quencher dye TAMRA at the 3' end. The specificity of all primers was first tested in a standard PCR and always resulted in a single product of the expected size on 1.5% agarose gels (data not shown). Briefly, 2 µL cDNA, 0.8 µL of each primer (25 µM), 0.4 µL labelled probe (5 µM), 6 µL RNase-free water and 10 µL TaqMan Universal PCR Master Mix (Applied Biosystems) reached 20 µL of final reaction volume for the tested gene. The protocol for the housekeeping gene was slightly different: 2 µL cDNA, 1 µL of the human 18S rRNA (20×) Control primers and probe reagent, 7 µL RNase-free water and 10 µL TaqMan Universal PCR Master Mix. Reaction conditions were denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 60 s with auto-ramp time. All reactions were run in triplicates, including a negative control. To analyse the data, we set the threshold to 0.06 as this value had been determined to be in the linear range of the amplification curves for all mRNAs in all experimental runs. The expression of gene of interest was calculated in relation to Human 18S rRNA. Relative expression ratios were calculated as $R = 2^{[C_t(18S) - C_t(\text{gene of interest})]}$, where C_t represents the cycle number at the threshold 0.06.

Western immunoblotting

For immunoblotting brush border membrane (BBM) proteins were prepared using the Mg^{2+} precipitation technique [27]. Additionally, protein extraction of total membrane fractions (MF) of kidney tissue was performed. Briefly, kidney tissue was homogenized in ice-cold HEPES buffer (200 mM mannitol, 80 mM HEPES, 41 mM KOH, titrated with KOH to pH 7.5) with Protease Inhibitors Cocktail Tablets complete, Mini (Roche Diagnostics GmbH, Mannheim, Germany). Both BBM and MF protein samples were used for western blotting. After measurement of the protein concentration (Bio-Rad Dc Protein Assay, Bio-Rad, Hercules, CA, USA), 40 µg of MF protein or 20 µg of BBM protein was solubilized in Laemmli sample buffer, and SDS-PAGE was performed on 8–10% polyacrylamide gels. Proteins were transferred electrophoretically from gels to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). After blocking with 5% milk powder in Tris-buffered saline containing 0.1% (v/v) Tween-20 for 60 min at room temperature, the blots were incubated with the respective primary antibodies overnight at 4°C or 2 h at room temperature (see Supplementary Table 2). Antibodies against SNAT3 produced two bands, a specific band of ~55–60 kDa and an unspecific band of lower molecular weight of ~40 kDa. PDG appears as two bands of 66 and

68 kDa and both bands were quantified together [28]. Similarly, pendrin antibodies recognized two specific bands of ~100 and 150 kDa [29].

After washing and subsequent blocking, blots were incubated with the secondary antibody [donkey anti-rabbit or sheep anti-mouse antibodies linked to horseradish peroxidase 1 : 10 000 (GE Healthcare, Little Chalfont, Buckinghamshire, UK) or goat anti-rabbit and goat anti-mouse antibody 1 : 5000 linked to alkaline phosphatase (Promega, Madison, WI, USA)] for 1 h at room temperature. Antibody binding was detected with appropriate Immobilon Western chemiluminescence substrates (Millipore, Billerica, MA, USA), using the LAS-4000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan). All images were analysed using appropriate software (Advanced Image Data Analyzer version 3.44, Raytest) to calculate the protein of interest/actin ratio.

Statistical analysis

All data are presented as means ± SE. Unpaired two-tailed *t*-test or ANOVA with Bonferroni correction were applied for inference statistics and result with *P*-value <0.05 were considered statistically significant.

RESULTS

Han:SPRD *cy/+* rats recapitulate hallmarks of CKD and develop severe hyperchloraemic metabolic acidosis

Cy/+ compared with *+/+* rats receiving standard diet had lower blood bicarbonate, higher chloride and reduced pCO_2 levels indicating a partly compensated hyperchloraemic metabolic acidosis. Potassium and phosphate plasma levels were elevated and creatinine clearance and haematocrit were lower demonstrating advanced CKD (Tables 1 and 2) in *cy/+* rats. Urine analysis showed more acidic urine, lower urinary bicarbonate and higher phosphate excretion in *cy/+* compared with *+/+* rats whereas ammonium excretion was similar between groups (Table 2). Thus, *cy/+* rats display the major hallmarks of advanced CKD confirming and expanding previous reports [30, 31].

Next, we tested the rats' ability to respond to an additional acid load by adding HCl to food for 48 h. Arterial blood gas analysis confirmed induction of metabolic acidosis in HCl-treated animals (Table 1). However, *cy/+* rats developed a more severe metabolic acidosis compared with *+/+* littermates (blood pH 7.13 ± 0.00 versus 7.34 ± 0.01). Accordingly, arterial bicarbonate levels were lower in *cy/+* animals compared with *+/+* rats (8.3 ± 0.4 versus 15.6 ± 0.3 mmol/L). These results indicate that *cy/+* animals' ability to buffer an acid load is impaired. Interestingly, induction of acidosis was associated with a fall in creatinine clearance in both wild-type and *cy/+* animals (Table 2).

The dietary acid load caused a markedly acidic urinary pH in both *cy/+* and *+/+* rats (Table 2). Thus, *cy/+* rats with markedly reduced renal function were also able to acidify urine to the same extent as wild-type animals with normal renal function. Similarly, patients with advanced CKD maintain their ability to acidify urine [10, 12] suggesting that the Han:SPRD

Table 1. Summary of arterial blood parameters from wild-type and cy/+ CKD rats on standard diet or after 2 days of HCl diet

Blood	Baseline		48-h HCl diet	
	WT	Cy/+	WT	Cy/+
pH	7.45 ± 0.01	7.44 ± 0.01	7.34 ± 0.01 ⁺⁺⁺	7.13 ± 0.00 ^{*****}
pCO ₂ , mmHg	36.9 ± 1.1	31.3 ± 0.3 ^{**}	30.1 ± 0.6 ⁺⁺	26.3 ± 1.2 ⁺⁺⁺
pO ₂ , mmHg	90.9 ± 1.4	93.4 ± 1.4	96.7 ± 1.1 ⁺⁺	95.3 ± 4.3
HCO ₃ ⁻ , mM	25.2 ± 0.7	20.6 ± 0.2 ^{***}	15.6 ± 0.3 ⁺⁺⁺	8.3 ± 0.4 ^{*****}
SO ₂ , %	95.4 ± 0.3	95.6 ± 0.2	95.2 ± 0.1	93.3 ± 0.8 [*]
Haematocrit	43.9 ± 2.9	40.6 ± 2.1 ^{**}	46.0 ± 0.6	37.3 ± 0.7 ^{***}
Sodium, mM	139.0 ± 1.0	137.0 ± 0.4	145 ± 2 ⁺	145 ± 4 ⁺
Potassium, mM	3.8 ± 0.1	4.7 ± 0.1 ^{**}	5.4 ± 0.2 ⁺⁺	5.4 ± 0.3
Chloride, mM	100.0 ± 1.0	103 ± 1.0 [*]	106 ± 1 ^{*****}	115 ± 2 ^{*****}
Phosphate, mg/dL	6.8 ± 0.2	7.9 ± 0.3 ^{**}	6.9 ± 0.1	8.3 ± 0.4 ^{**}
Creatinine, mg/dL	0.3 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	1.3 ± 0.1 ^{*****}
BUN, mg/dL	15 ± 1	43 ± 2 ^{***}	14 ± 1	48 ± 3 ^{***}
Albumin, g/dL	1.8 ± 0.1	1.8 ± 0.0	1.9 ± 0.0	1.7 ± 0.1 ^{**}
Glucose, mg/dL	247 ± 18	197 ± 3 [*]	220 ± 7	173 ± 5 ^{*****}

Values are means ± SE; n = 5–6 animals/group.

^{**}P < 0.05, ^{***}P < 0.01, ^{****}P < 0.001, ^{*****}P < 0.0001, ^{*}significantly different between wild-type and CKD rats (cy/+) at the same time point, ⁺significantly different between baseline and 48-h HCl diet for the same genotype.

Table 2. Summary of urine parameters from wild-type and cy/+ CKD rats on standard diet or after 2 days of HCl diet

Urine	Baseline		24-h HCl diet		48-h HCl diet	
	WT	Cy/+	WT	Cy/+	WT	Cy/+
pH	6.61 ± 0.03	5.74 ± 0.06 ^{***}	5.73 ± 0.03 ⁺⁺⁺	5.48 ± 0.02 ^{*****}	5.37 ± 0.03 ⁺⁺⁺	5.29 ± 0.02 ^{*****}
24-h urine/body weight, mL/g	0.09 ± 0.00	0.09 ± 0.01	0.11 ± 0.02 ⁺⁺	0.10 ± 0.01	0.10 ± 0.01	0.12 ± 0.00
Creatinine clearance, mL/min	8.3 ± 0.9	2.5 ± 0.2	n/a	n/a	3.68 ± 0.22 ⁺⁺⁺	0.82 ± 0.08 ^{*****}
Na ⁺ , mmol/24 h	1.06 ± 0.12	1.33 ± 0.05	2.64 ± 0.17 ⁺⁺⁺	1.22 ± 0.10 ^{***}	2.18 ± 0.06 ⁺⁺⁺	1.33 ± 0.14 ^{***}
K ⁺ , mmol/24 h	4.30 ± 0.36	6.77 ± 0.15 ^{***}	6.57 ± 0.31 ⁺⁺⁺	5.99 ± 0.27 ⁺	6.92 ± 0.16 ⁺⁺⁺	4.50 ± 0.29 ^{*****}
Phosphate, mmol/24 h	0.63 ± 0.03	0.96 ± 0.04 ^{***}	1.05 ± 0.08 ⁺⁺⁺	1.08 ± 0.07	1.18 ± 0.05 ⁺⁺⁺	1.27 ± 0.07 ⁺⁺
NH ₄ ⁺ , mmol/24 h	0.11 ± 0.01	0.07 ± 0.00	3.18 ± 0.22 ⁺⁺⁺	1.10 ± 0.24 ^{*****}	8.32 ± 0.25 ⁺⁺⁺	1.71 ± 0.09 ^{*****}
HCO ₃ ⁻ , mmol/24 h	5.78 ± 0.44	1.17 ± 0.13 ^{***}	0.29 ± 0.04 ⁺	0.39 ± 0.03 ⁺⁺	0.18 ± 0.02 ⁺⁺⁺	0.22 ± 0.01 ⁺⁺⁺

Values are means ± SE; n = 3–6 animals/group.

^{**}P < 0.05, ^{***}P < 0.01, ^{****}P < 0.001, ^{*****}P < 0.0001, ⁺significantly different between wild-type and CKD rats (cy/+) for the same time point, ⁺significantly different between baseline and/or 24-h HCl and/or 48-h HCl diet for the same genotype.

rat model serves as a useful CKD model for studying metabolic acidosis.

After acid load urinary ammonium excretion, a major component of overall net acid excretion, was significantly lower in cy/+ compared with +/+ rats (Table 2) whereas, urinary phosphate excretion, a major component of titratable acidity, increased modestly in both animal groups. Our data suggest that urinary acidification is preserved in CKD animals whereas urinary ammonium excretion is decreased by ~60–80% which is paralleled by more severe metabolic acidosis.

Expression of ammoniogenic key molecules is reduced in CKD rats

We performed real-time RT-qPCR on kidney tissue for SNAT3, PDG, PEPCK and the sodium/proton exchanger Type 3 (NHE3), key molecules of the renal ammoniogenesis pathway. The mRNA abundance of PEPCK, SNAT3 and NHE3 was reduced in cy/+ CKD rats (Figure 1) compared with wild-type animals under standard diet and after 48-h acid load. In contrast, PDG mRNA expression was increased after the acid load in cy/+ CKD animals (Figure 1).

Similarly, protein abundance of SNAT3 and PEPCK in cy/+ animals was markedly decreased compared with wild-type littermates (Figure 2). PDG protein levels were decreased after the acid load, opposite to mRNA levels (Figure 2). Protein levels of NHE3 were not different compared with wild-type animals (Figure 2).

The electrogenic sodium/bicarbonate co-transporter Type 1 (NBCe1) is located on the basolateral membrane of the proximal tubule cell and releases bicarbonate into blood. mRNA expression of NBCe1 was decreased in cy/+ CKD animals under standard diet and increased after acid load (Figure 3). Protein levels were also decreased under both standard diet and after the acid load (Figure 3).

Taken together, these data demonstrate a strong reduction in the expression of key molecules of the ammoniogenesis pathway in kidneys from cy/+ CKD animals under standard diet. CKD animals were able to modestly increase urinary ammonium excretion after acid loading; however, to a much lesser extent than wild-type animals. Thus, reduced expression of key molecules of the ammoniogenesis pathway parallels lower urinary ammonium excretion in CKD rats.

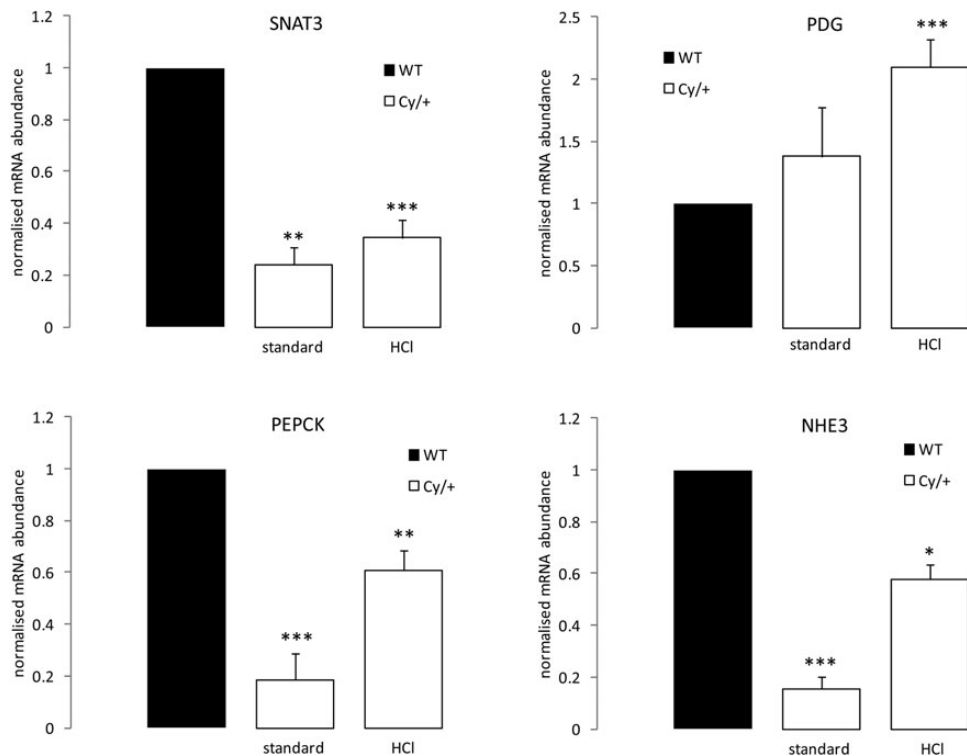


FIGURE 1: Reduced mRNA expression of key ammoniagenic molecules in kidneys from CKD rats. Normalized mRNA abundance of PEPCK, SNAT3, PDG and NHE3 in kidneys from controls and CKD rats (*cy/+*) under standard diet and 2 days of acid load with HCl. $n = 5-6$ per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different between wild-type and CKD rats (*cy/+*).

Sodium-dependent phosphate transporters are altered in CKD rats

The major renal phosphate transporters NaPi-IIa and NaPi-IIc and the third sodium-dependent phosphate transporter Pit-2 are expressed on the apical membrane of the proximal tubule cells and mediate phosphate reabsorption [32]. mRNA abundance of NaPi-IIa and NaPi-IIc was decreased during standard diet and after acid loading in *cy/+* rats compared with control rats (Figure 4) while Pit-2 mRNA expression was not altered. BBM protein levels of the three phosphate transporters were regulated differently. NaPi-IIc protein abundance was decreased while NaPi-IIa and Pit-2 remained unchanged. However, after an acid load, Pit-2 protein levels were increased (Figure 5).

Ammonium excretion is reduced in CKD rats

The last set of experiments examined the abundance of proteins contributing to final urinary acidification and ammonium excretion along the collecting duct system: the Type A intercalated cell specific AE1 anion exchanger, the non-Type A intercalated cell marker pendrin, the ammonia transporter RhCG, and the B1 subunit of the H^+ -ATPase enriched in intercalated cells [23]. The mRNA abundance of AE1 was decreased in CKD animals while B1 mRNA abundance was markedly increased after acid loading compared with wild-type animals (Figure 6). RhCG and pendrin mRNA abundance was not different. AE1 protein levels were reduced in CKD rats while B1 protein levels were unchanged in comparison to wild-type rats (Figure 7). RhCG protein abundance was strongly decreased

under standard diet and after acid loading compared with wild-type animals. Thus, normal H^+ -ATPase expression and reduced RhCG abundance are consistent with the preserved urinary acidification and reduced ammonium excretion in CKD rats.

DISCUSSION

Metabolic acidosis is a common complication of CKD and is associated with increased mortality in dialysis and non-dialysis-dependent CKD patients. Particularly, the impact of metabolic acidosis on the progression of CKD and the increased risk to develop end-stage renal disease have been recently highlighted by studies investigating the effect of alkali supplementation as a potential nephroprotective treatment in patients with CKD [3, 4, 6–9].

The major findings in our study are (i) *cy/+* Han:SPRD rats mimic major features of CKD, (ii) metabolic acidosis in this CKD rat model is paralleled by the reduced expression of key molecules of proximal tubular ammoniogenesis, (iii) urinary acidification and expression of H^+ -ATPases in the collecting duct are preserved, (iv) the RhCG ammonia transporter required for final urinary excretion of ammonium is down-regulated and (v) renal phosphate reabsorption was reduced in CKD rats.

Han:SPRD rats mimic features of CKD

Humans with CKD have reduced glomerular filtration rate (GFR) and develop several complications such as anaemia,

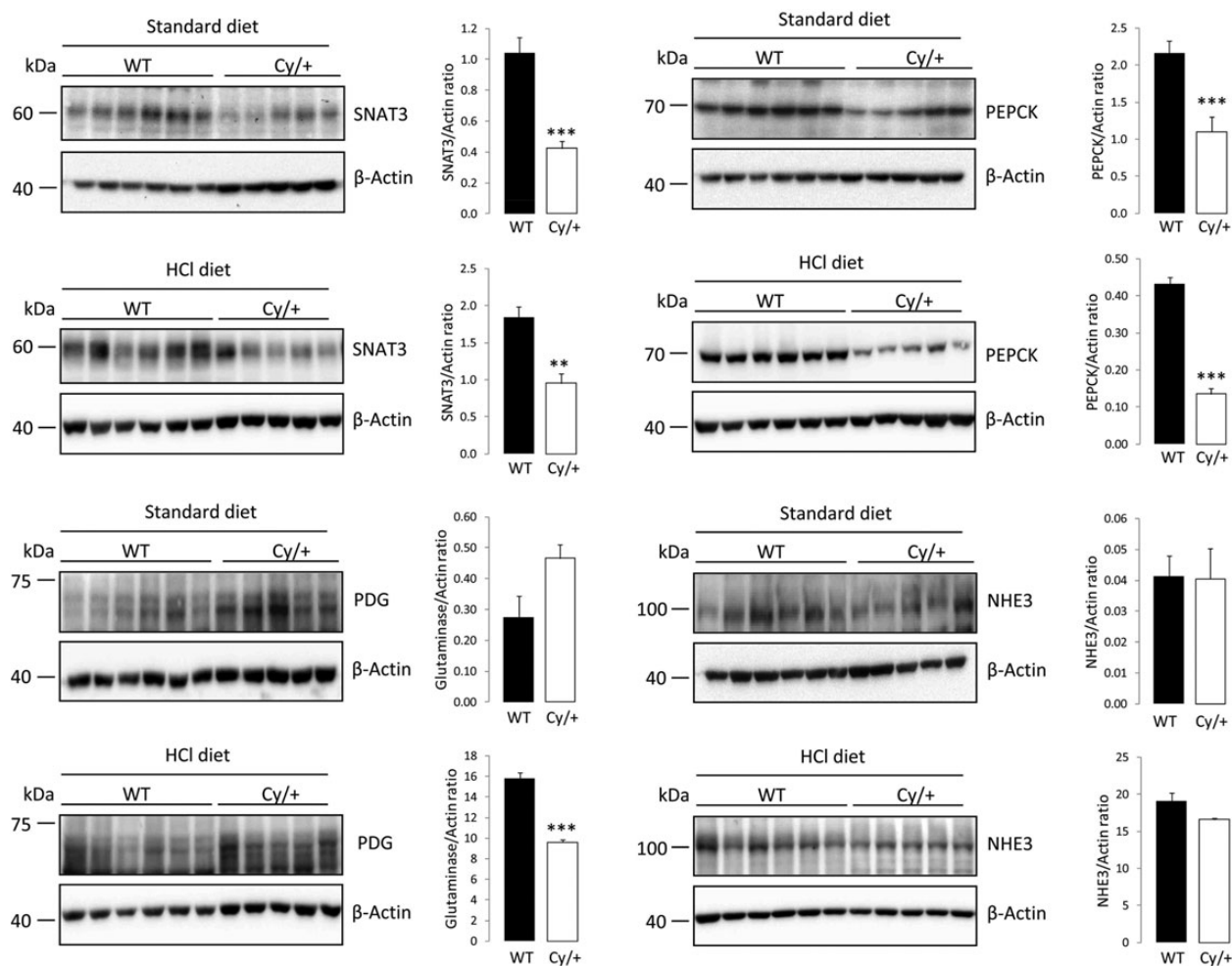


FIGURE 2: Altered expression of key molecules of ammoniogenesis in kidneys from CKD rats. Protein abundance of PEPCK, SNAT3, PDG and NHE3 are shown in controls and CKD rats (*cy/+*) on standard diet and after 2 days of acid load with HCl. The immunoblots were stripped and re-probed for all proteins and β-actin. All data were normalized against β-actin. *n* = 5–6 per group, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 significantly different between wild-type and CKD rats (*cy/+*).

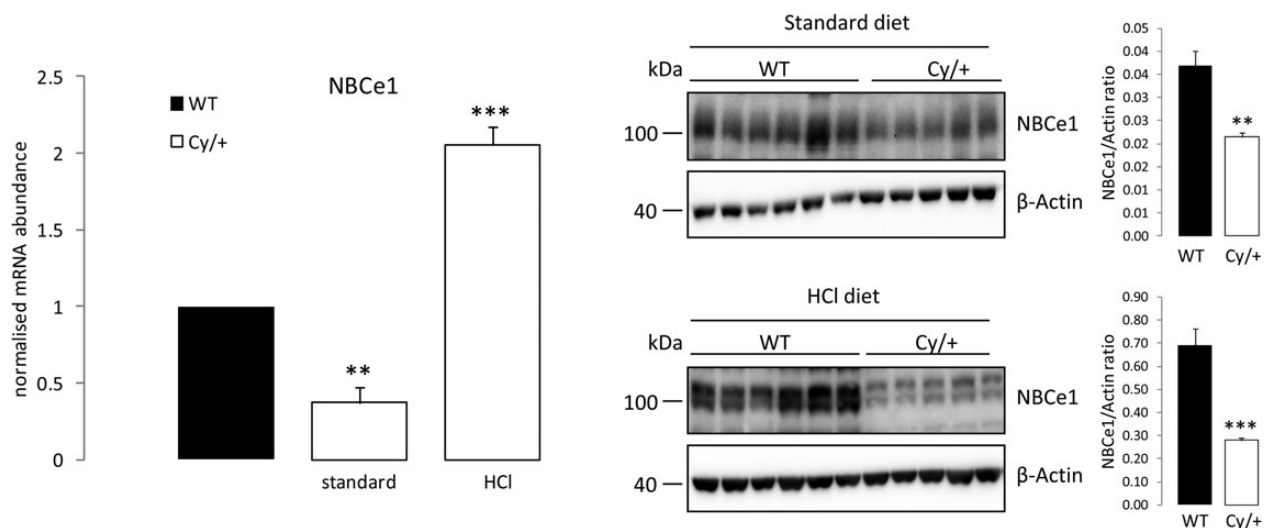


FIGURE 3: Altered expression of the proximal tubular bicarbonate transporter NBCe1. Normalized mRNA and protein abundance of NBCe1 are shown in controls and CKD rats (*cy/+*) on standard diet and after 2 days of acid load with HCl. Immunoblots were normalized against β-actin. *n* = 5–6 per group, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 significantly different between wild-type and CKD rats (*cy/+*).

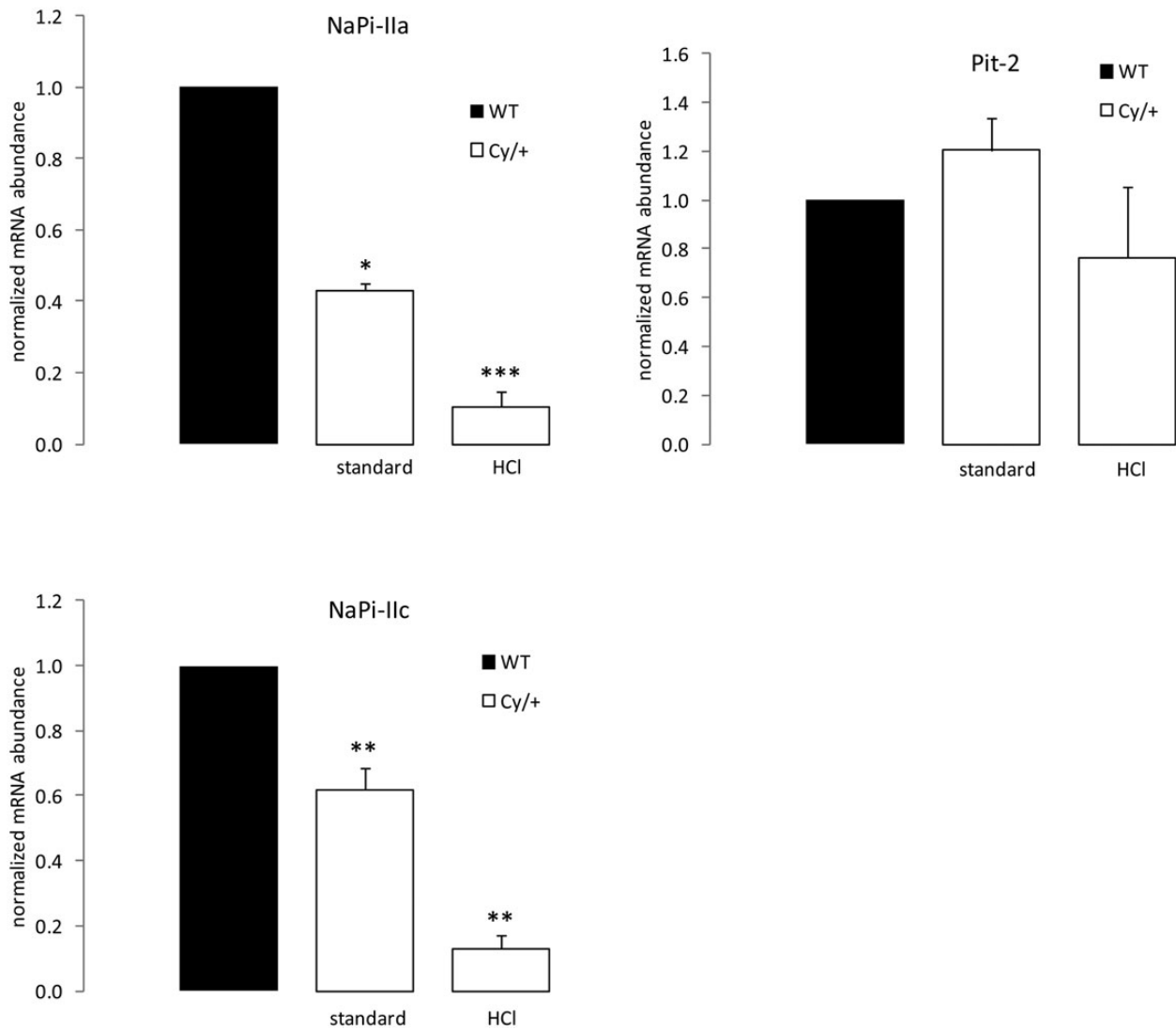


FIGURE 4: mRNA expression of sodium-dependent phosphate transporters is altered in CKD rats. Normalized mRNA abundance of NaPi-IIa, NaPi-IIc and Pit-2 are shown in controls and CKD rats (*cy/+*) on standard diet and after 2 days of acid load with HCl. $n = 5-6$ per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different between wild-type and *cy/+* rats.

metabolic acidosis, mineral bone disease, neuropathy etc. with progressive disease [33]. Similarly, *cy/+* rats presented with CKD confirmed by elevated creatinine and BUN values in blood and reduced creatinine clearance. *Cy/+* rats also had metabolic acidosis, lower haematocrit, hyperkalaemia and hyperphosphataemia. These findings are consistent with and expand previous reports using this rat strain as model for CKD or polycystic kidney disease [30, 31]. Acid loading wild-type and *cy/+* rats, caused a fall in creatinine clearance in both groups. The mechanism(s) by which acidosis may reduce GFR is currently not exactly known.

Metabolic acidosis in CKD is due to reduced expression of key molecules of ammoniogenesis and decreased ammonium excretion by the kidney

Patients with CKD and metabolic acidosis maintain their ability to excrete acidic urine but have a reduced capacity to increase their ammonium excretion [11, 12]. Ammoniogenesis is markedly reduced in patients with CKD which correlates with decreased renal metabolism of glutamine [34]. However,

whether reduced total ammoniogenesis reflects only a lower number of nephrons or a decreased capacity of surviving nephrons has been intensively studied in rat models of partial nephrectomy or infarction [13, 35]. These experiments indicated that the kidneys capacity to produce ammonium was proportional to nephron number, ammoniogenesis could be stimulated by diet and that a compensatory increase in ammonium producing cells could be found. In our experiments, urinary ammonium excretion was inappropriately low in CKD rats and responded only with a small increase to the acid load. This may result from impaired ammoniogenesis, defective ammonium excretion or urinary acidification, or a combination of these factors. mRNA abundance and protein expression of PEPCK and SNAT3 were highly reduced in CKD rats. Additionally, PDG and NBCe1 protein abundance were also decreased in acid-loaded CKD rats. Of note, the direction of changes in mRNA expression and protein abundance differed for NHE3, PDG, NBCe1, NaPi-IIa and Pit-2 for some conditions. The causes for these differences are currently not known

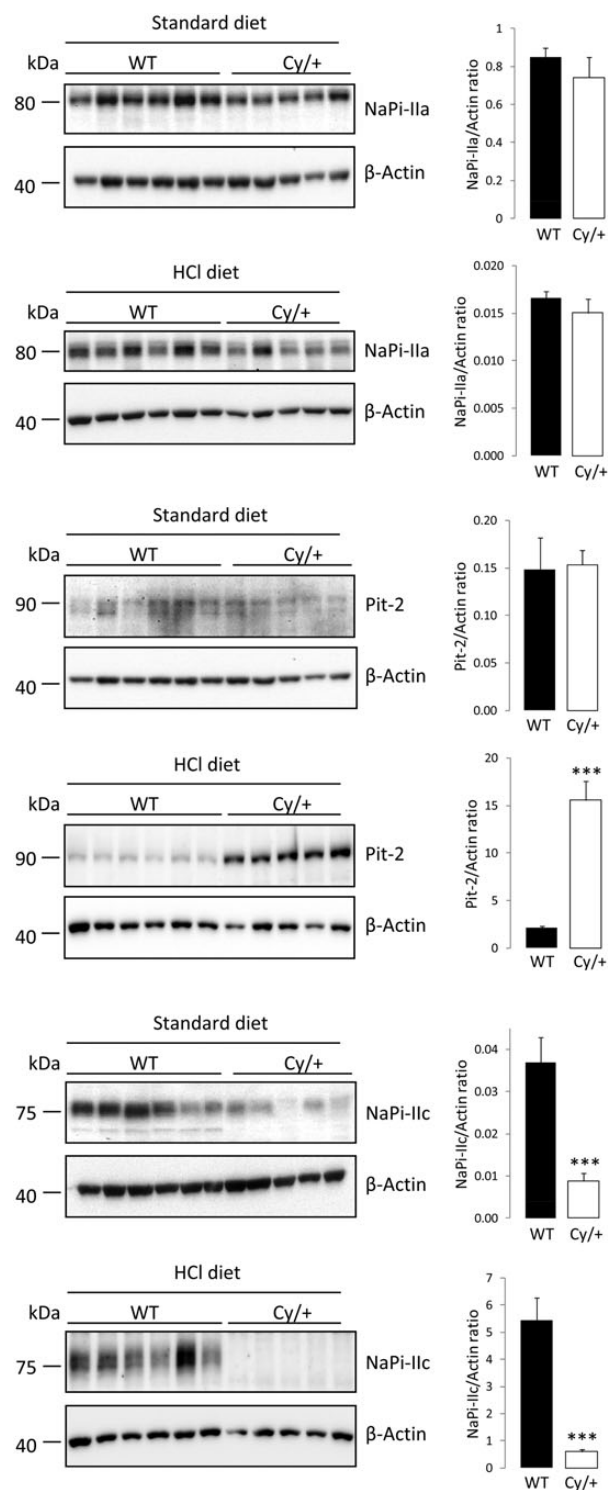


FIGURE 5: Expression of renal sodium-dependent phosphate transporters in CKD rats. Protein abundance of NaPi-IIa, NaPi-IIc and Pit-2 are shown in controls and CKD (cy/+) rats on standard diet and after 2 days of acid load with HCl. The immunoblots were stripped and re-probed for all proteins and β -actin. All data were normalized against β -actin. $n = 5-6$ per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different between wild-type and CKD rats (cy/+).

but these differences indicate that the interpretation of changes in mRNA expression without analysis of protein abundance can be misleading.

SNAT3 is expressed on the basolateral membrane of proximal tubule cells mediating glutamine uptake for ammoniogenesis [36–38]. Glutamine is further metabolized by a series of enzymes including PDG, GDH and finally PEPCK resulting in the cumulative synthesis of two NH_3 and two HCO_3^- ions [21]. Bicarbonate generated during these metabolic steps is released into blood by the basolaterally located NBCe1 sodium/bicarbonate co-transporter. Reduced expression of all these key molecules in the cy/+ CKD rats may severely impair ammoniogenesis and bicarbonate transport.

We and others have previously shown that many of these transporters and enzymes investigated here respond with changes in their expression to endogenous or exogenous acid loads. The expression of NHE3, NaPi-IIa, PDG, PEPCK, SNAT3, NBCe1, RhCG and AE1 is increased whereas pendrin expression decreased in kidneys from acid-loaded kidneys [39–42]. Here, we compared only control animals versus cy/+ CKD rats as we wanted to assess how renal impairment impacted on the ability of the kidney to excrete acid but were not interested to study the renal response *per se* to an acid load. However, the regulation can be inferred from the ratios between control and Cy/+ CKD animals at baseline and after the acid load showing that most proteins were up-regulated in the Cy/+ CKD rats with the acid load as the ratios remain constant, e.g. for SNAT3, NHE3, NBCe1, NaPi-IIa, NaPi-IIc, AE1, B1 and RhCG. In the case of pendrin, acid loading reduced pendrin expression in WT to the same level as in Cy/+ under baseline conditions most likely reflecting the already existing acidosis in CKD rats. In contrast, acid loading revealed a strong increase in Pit-2 expression in CKD rats and reversed a trend to higher expression of PDG in CKD rats to a lower expression in acid-loaded CKD rats.

We assessed the abundance of these molecules in tissue homogenates from whole kidneys. Thus, our results do not directly indicate whether reduced abundance is due to reduced nephron number or rather due to decreased expression per nephron. Data from animal models with reduced kidney mass suggested that the reduced number of functional nephrons would decrease the total capacity to produce ammonium and that the rate of ammoniogenesis was even stimulated at the level of the single nephron [13, 35]. However, our results may also be interpreted as a down-regulation of key molecules of ammoniogenesis. Reduced expression was found only for few molecules such as SNAT3, PEPCK, NBCe1 and NaPi-IIc whereas other proximal tubule proteins such as NaPi-IIa, Pit2, NHE3 or PDG remained unaltered or were even up-regulated. Therefore, the results may suggest that reduced expression of SNAT3, PEPCK and NBCe1 may be caused by a down-regulation in surviving nephrons. In fact, the degree of metabolic acidosis found in CKD rats at baseline would cause a strong up-regulation of these enzymes and transporters in the healthy kidney [22]. The mechanisms that may induce a specific down-regulation of some proximal tubular proteins involved in ammoniogenesis and bicarbonate transport remain to be identified. Clearly, this important question warrants further experiments in this CKD animal model as well as in additional models to clarify the underlying mechanisms and whether these mechanisms are model specific or occur in different models of CKD and also in humans.

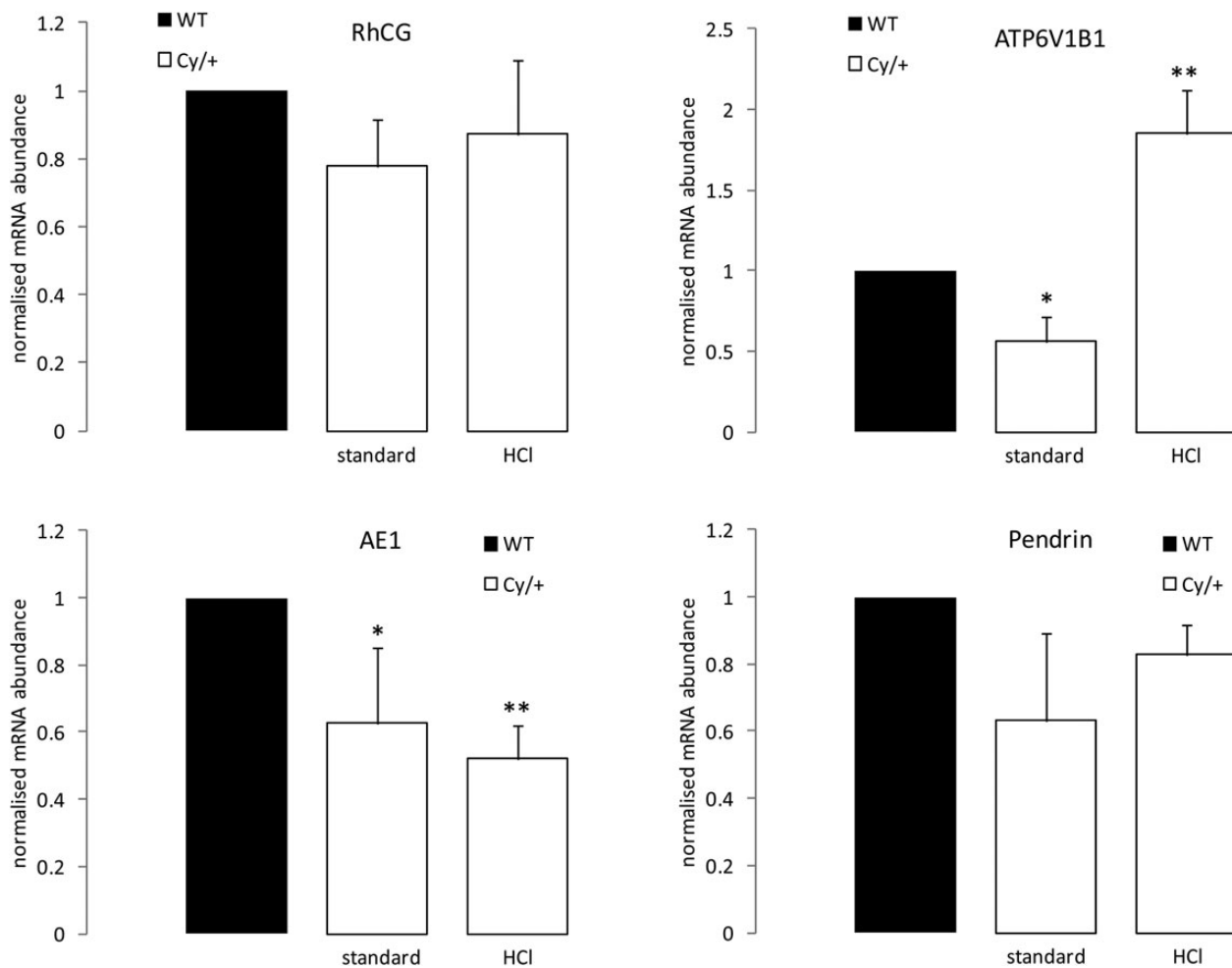


FIGURE 6: Regulation of mRNA abundance of collecting duct acid–base transporters. Normalized mRNA abundance of AE1, pendrin, RhCG and B1 subunit of the H^+ -ATPase are shown in controls and CKD rats (*cy/+*) on standard diet and after 2 days of acid load with HCl. $n = 5-6$ per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different between wild-type and CKD rats (*cy/+*).

Accumulation of ammonium in the medullary interstitium is necessary to facilitate excretion into the collecting duct. The transport of ammonium into the interstitium is mediated by the $Na^+/K^+/2Cl^-$, co-transporter NKCC2 and the Na^+/H^+ exchanger NHE4 and may involve interactions with sulphatides [18, 43, 44]. We tested the expression of NKCC2 and found no differences between control and *Cy/+* CKD rats (data not shown). However, we cannot rule out that expression of NHE4 (for which no specific antibodies exist) or the interstitial concentration of ammonium were not altered.

Ammonium excretion and final urinary acidification are mediated by the late distal convoluted tubule, connecting tubule and collecting duct [23]. Type A intercalated cells secrete protons and ammonia whereas neighbouring segment-specific cells (principal cells) also contribute to ammonia secretion [19, 23, 45]. The final step of ammonia excretion depends on the generation of a cortico-papillary gradient of ammonia from interstitium into the lumen of the collecting duct [20, 43]. Ammonia secretion is mediated by the two rhesus proteins RhBG and RhCG where RhCG appears to play a major role [45, 46]. The driving force for ammonia secretion, however, depends on the degree of urinary acidification by Type A intercalated cells. The importance of acid–base transport proteins in these

cells is highlighted by inherited disorders in humans with mutations in the AE1/SLC4A1 chloride/bicarbonate exchanger, and the B1/ATP6V1B1 and a4/ATP6V0A4 H^+ -ATPase subunits [47–50]. Urinary pH was more acidic in *cy/+* CKD rats under baseline conditions and was further acidified after the HCl diet demonstrating an intact capacity to generate and maintain a steep proton gradient from interstitium to the collecting duct lumen. Surprisingly, AE1 mRNA and protein abundance was decreased in CKD animals whereas the abundance of the B1 H^+ -ATPase subunit remained unaltered. Thus, the maximal capacity of the collecting duct to maximally acidify urine may be decreased; however, in the presence of greatly reduced ammonia concentrations urinary pH remains very acidic. The reduced expression of pendrin, a marker of non-Type A intercalated cells, is most likely caused by metabolic acidosis [29, 51, 52]. Impaired ammonia secretion by the collecting duct may not only be the result of decreased proximal tubular ammoniogenesis but also due to lower expression of the ammonia transporter RhCG. Thus, reduced secretion of ammonia by the collecting duct is likely by a combination of decreased ammoniogenesis and reduced RhCG expression. The reason for lower RhCG expression is not known and may be either the direct consequence of renal disease or a response to reduced

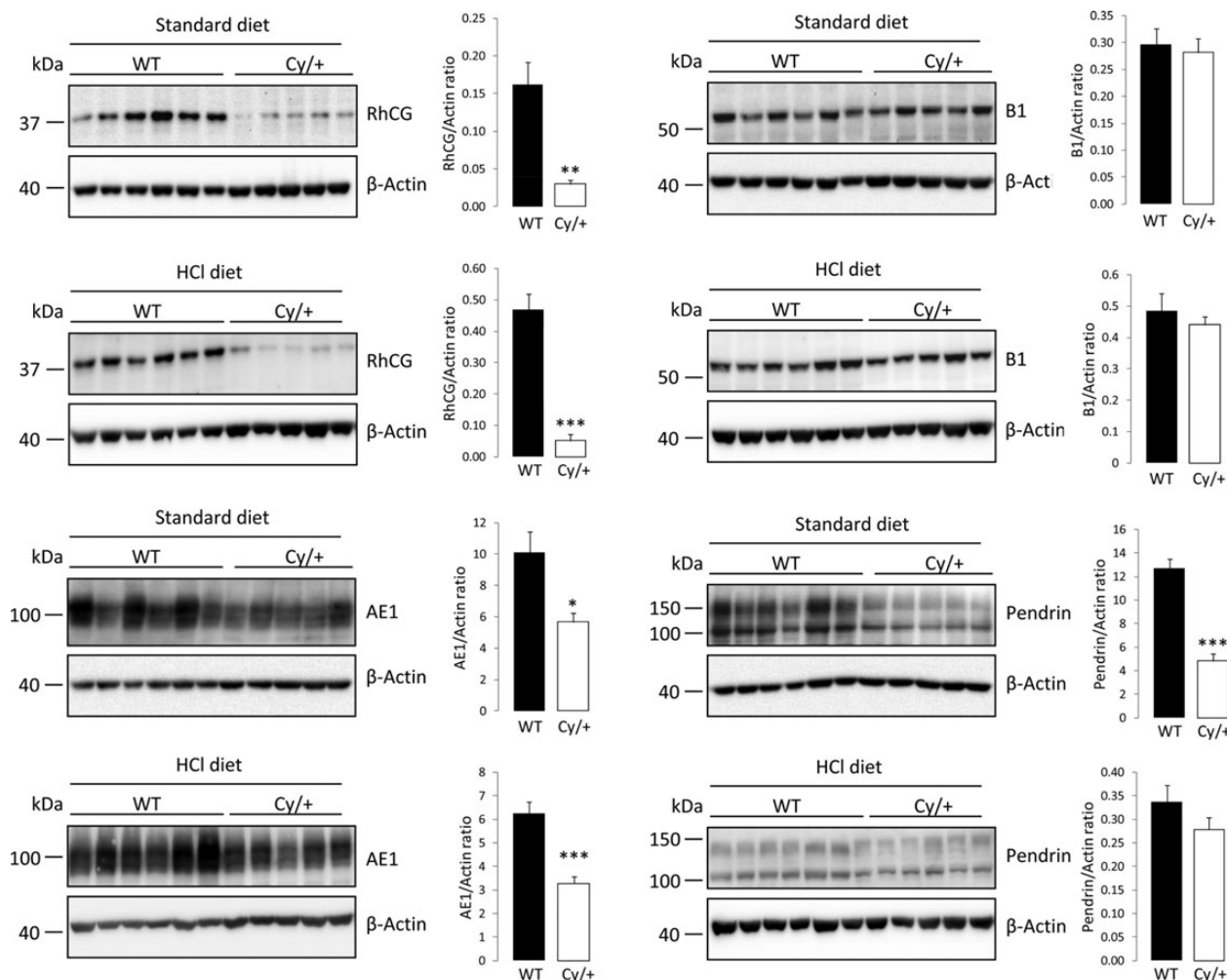


FIGURE 7: Reduced expression of the ammonia transporters RhCG. Protein abundance of AE1, pendrin, RhCG and the B1 subunit of the H^+ -ATPase are shown in controls and CKD rats (cy/+) on standard diet and after 2 days of acid load with HCl. The immunoblots were stripped and re-probed for all proteins and β -actin. All data were normalized against β -actin. $n = 5-6$ per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different between wild-type and CKD rats (cy/+).

ammoniogenesis. Similarly, in a mouse model with haploinsufficiency of Rhcg, reduced urinary acid excretion was observed indicating that low levels of RhCG expression *per se* may reduce renal acid excretion [45].

Renal reabsorption of phosphate is reduced in Han:SPRD rats

Efficient proton secretion depends in part on the availability of titratable acids, mostly phosphate, that buffer protons and thereby prevent an excessive urinary acidification that would impair further acid excretion [39, 49]. In metabolic acidosis, phosphate excretion is increased to increase buffer capacity and to reduce extracellular phosphate derived from bone [53]. Phosphate excretion in the kidney is mainly controlled by reabsorption of phosphate in the proximal tubule mediated by a set of sodium-dependent transporter, namely NaPi-IIa and NaPi-IIc [32]. Protein abundance of NaPi-IIc but not of NaPi-IIa was down-regulated in CKD rats after HCl load. Urinary phosphate excretion was higher after acid loading. Moreover, CKD rats had increased phosphaturia under normal conditions that was paralleled by reduced expression of NaPi-IIc but not

NaPi-IIa. Thus, phosphaturia under these conditions may result from a combination of direct inhibition of NaPi-IIa and NaPi-IIc transport activity by lower urinary pH in the proximal tubule due to lower plasma bicarbonate levels and the down-regulation of NaPi-IIc [39]. The up-regulation of Pit-2 abundance in acid-loaded cy/+ rats may reflect a compensatory mechanism to limit urinary phosphate losses.

In conclusion, we demonstrated that renal ammonium excretion is severely impaired in a CKD rat model while urinary acidification is mostly preserved. The expression of key molecules of the ammoniogenesis pathway in the proximal tubule and the excretory ammonium transporter RhCG is reduced in kidney. Whether the reduced number of functional nephrons and/or the down-regulation of these molecules cause the renal acidosis in CKD remains to be further explored.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://ndt.oxfordjournals.org>.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they are not aware of any conflicts of interest and that the results presented in this paper have not been published previously in whole or part, except in abstract format.

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Diabetic nephropathy is associated with increased urine excretion of proteases plasmin, prostatic and urokinase and activation of amiloride-sensitive current in collecting duct cells

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ABSTRACT

Background. Diabetic nephropathy (DN) is associated with hypertension, expanded extracellular volume and impaired renal Na⁺ excretion. It was hypothesized that aberrant glomerular filtration of serine proteases in DN causes proteolytic activation of the epithelial sodium channel (ENaC) in the kidney by excision of an inhibitory peptide tract from the γ subunit.

Methods. In a cross-sectional design, urine, plasma and clinical data were collected from type 1 diabetic patients with DN ($n = 19$) and matched normoalbuminuric type 1 diabetics (controls, $n = 20$). Urine was examined for proteases by western immunoblotting, patch clamp and ELISA. Urine exosomes were isolated to elucidate potential cleavage of γ ENaC

by a monoclonal antibody directed against the ‘inhibitory’ peptide tract.

Results. Compared with control, DN patients displayed significantly higher blood pressure and urinary excretion of plasmin(ogen), prostatic and urokinase that correlated directly with urine albumin. Western blotting confirmed plasmin, prostatic and urokinase in urine from the DN group predominantly. Urine from DN evoked a significantly larger amiloride-sensitive inward current in single collecting duct cells compared with controls. Immunoblotting of urine exosomes showed aquaporin 2 in all patient samples. Exosomes displayed a virtual absence of intact γ ENaC while moieties compatible with cleavage by furin only, were shown in both groups. Proteolytic cleavage by the extracellular serine proteases plasmin or prostatic was observed in DN samples predominantly.