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Pendrin in mouse kidney

is primarily regulated by chloride excretion

but also by systemic metabolic acidosis

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ABSTRACT

The chloride/anion exchanger pendrin (SLC26A4) is expressed on the apical side of renal non-type A intercalated. Pendrin abundance is reduced during metabolic acidosis induced by oral NH_4Cl-loading. More recently, it has been shown that pendrin expression is increased during conditions associated with decreased urinary chloride excretion and decreased upon chloride loading. Hence, it is unclear if pendrin regulation during NH_4Cl induced acidosis is primarily due the chloride load or acidosis. Therefore, we treated mice to increase urinary acidification, induce metabolic acidosis, or provide an oral chloride load and examined systemic acid-base status, urinary acidification, urinary chloride excretion, and pendrin abundance in kidney. NaCl or NH_4Cl increased urinary Cl^- excretion whereas $(NH_4)_2SO_4$, Na_2SO_4 and acetazolamide treatments decreased urinary Cl^- excretion. NH_4Cl, $(NH_4)_2SO_4$, and acetazolamide caused metabolic acidosis and stimulated urinary net acid excretion. Pendrin expression was reduced under NaCl, NH_4Cl, and $(NH_4)_2SO_4$ loading and increased with the other treatments. $(NH_4)_2SO_4$ and acetazolamide treatments reduced the relative number of Pendrin expressing cells in the collecting duct. In a second series, animals were kept for 1 and 2 weeks on a low (20 %) and high protein (50 %) diet. High protein diet slightly increased urinary Cl^- excretion, strongly stimulated net acid excretion but did not alter pendrin expression. Thus, pendrin expression is primarily correlated with urinary chloride excretion but not blood chloride. However, metabolic acidosis caused by acetazolamide or $(NH_4)_2SO_4$-loading prevented the increase or even reduced pendrin expression despite low urinary chloride excretion suggesting independent regulation by acid-base status.

INTRODUCTION

The kidney plays a central role in regulating the systemic homeostasis for electrolytes and acid-base homeostasis which are intricately linked together. The bulk reabsorption of electrolytes and bicarbonate is mediated by several transport proteins in the proximal tubule and thick ascending limb of the loop of Henle. The fine control, however, takes place along the connecting segment and collecting duct. There, the reabsorption of water and sodium by principal or segment-specific cells is regulated, as well as the secretion of protons or bicarbonate, by different subtypes of intercalated cells. The exact mechanisms underlying trans- and paracellular chloride absorption in these segments have not been fully clarified yet (10, 11).

The chloride/ anion exchanger pendrin (SLC26A4) belongs to a large superfamily of anion transporters and is predominantly expressed in the inner ear, thyroid gland, and kidney (8, 16). Mutations in pendrin cause Pendred syndrome associated with sensorineural deafness and goiter (OMIM #274600)(7). In kidney, pendrin expression is restricted to the apical side of non-type A intercalated cells (i.e. type B and non-type A/B intercalated cells) in the connecting segment and cortical collecting duct (13, 20, 29). There, pendrin has been implicated in bicarbonate secretion into urine stimulated during metabolic alkalosis (20). Indeed, pendrin can function as Cl^-/HCO_3^- -exchanger as evident from experiments in cells with exogenous expression of pendrin (21). Moreover, pendrin deficient mice secrete less bicarbonate in isolated perfused cortical collecting ducts during metabolic alkalosis (20). Also consistent with a role in regulated secretion of bicarbonate during metabolic alkalosis we and several other groups observed that pendrin abundance was reduced during metabolic acidosis induced by oral NH_4Cl-loading (9, 18, 28).

These findings were interpreted as the down-regulation of pendrin due to metabolic acidosis.

More recently, Eladari et al. and Wall et al. described that pendrin expression was regulated by urinary chloride excretion and suggested a role in transcellular chloride absorption (19, 24, 26, 30). In particular, they noted that manoeuvres increasing urinary Cl^- -excretion decreased pendrin expression. Treatments inducing chloride depletion and stimulating renal chloride reabsorption were associated with higher pendrin protein levels (19, 24, 26, 30).

These data raised the question if the down-regulation in pendrin abundance observed in NH_4Cl-loaded animals was solely due to chloride-loading or was also, at least in part, due to the metabolic acidosis induced by ammonium-loading. To this end, we examined here the correlation between pendrin expression and urinary acid and chloride excretion by Western blotting in several animal models. Systemic acid-base homeostasis and urinary acid excretion were manipulated by providing mice with different diets including NH_4Cl, $(NH_4)_2SO_4$, the carbonic anhydrase inhibitor acetazolamide and high protein (50 %). All these treatments have been shown to induce metabolic acidosis or to provide an acid challenge leading to increased urinary acid excretion (i.e. high urinary ammonium and net acid excretion (NEA)) (6, 12, 22, 31). In addition, other groups of animals received either only sucrose which was added to all diets, a diet containing a normal protein content (20 %), or NaCl and Na_2SO_4 to provide a chloride load or a control for $(NH_4)_2SO_4$-loading, respectively. The results indicate pendrin expression is regulated by urinary chloride excretion but the abundance may also be reduced during metabolic acidosis despite low urinary chloride excretion.

MATERIAL AND METHODS

Animal studies

All animal studies were according to Swiss Animal Welfare Laws and approved by the local Veterinary Authority (Kantonales Veterinäramt, Zurich). NMRI mice (Charles River Laboratories, Germany) (male, 12 weeks, 35 - 45 g) were maintained on standard chow and had access to drinking water ad libitum. Animals were divided into two main groups. Group one was subdivided into 6 subgroups receiving different additives in their drinking water: control (2 % sucrose), NH_4Cl (280 mM NH_4Cl, 2 % sucrose), NaCl (0.28 M NaCl, 2 % sucrose), $(NH_4)_2SO_4$ (0.28 M $(NH_4)_2SO_4$, 2 % sucrose), Na_2SO_4 (0.28 M Na_2SO_4, 2 % sucrose), Azetazolamide (acetazolamide 80 mg/100 ml water, 2 % sucrose) for 7 days each. The second group of animals received normal drinking water and an isocaloric diet containing either normal protein content (20 %) or a high protein (50 %) content for 7 or 14 days. Care was taken that all other constituents, particularly electrolytes, of the diet were kept constant (Kliba AG, Switzerland). Each group consisted of 5 animals for each time point and treatment. Animals were kept in normal cages for the first 5 days of the diet and were slowly adapted to metabolic cages for several hours every day. The last two days animals were kept constantly in metabolic cages (Tecniplast, Buguggiate, Va, Italy) and food and water intake, faeces and urine output monitored and 24 hrs urine collected under mineral oil. At the end of the collection period, mice were anesthetized with ketamine/xylazine i.p. and heparinized venous blood samples collected and analyzed immediately for blood gases and electrolytes on a Radiometer ABL 505, (Radiometer, Copenhagen, Denmark) blood gas analyzer. Urine was collected and pH measured immediately using a pH microelectrode connected to a Thermo Orion 290 pH meter. Serum and urine were frozen until

further analysis. Creatinine in serum and urine were measured using an enzymatic reaction (WAKO Chemicals, Germany) and the Jaffe reaction, respectively. Electrolytes in diluted urine samples were determined using ion chromatography (Metrohm ion chromatograph, Switzerland). Phosphate in urine was measured using a commercial kit (Sigma) and ammonium using the Berthelot protocol (19). Titratable acids were measured in 200 µl urine samples diluted with an equal volume of 0.1 M HCl after boiling for 2 min at at 37 °C by titrating with 0.1 M NaOH to pH 7.4 as described previously (5). Net acid excretion was calculated as the sum of the measured titratable acidity and ammonium.

Antibodies against pendrin

The antibodies against mouse pendrin were raised in rabbit and guinea-pig against the very C-terminal sequence CKDPLDLMEAEMNAEELDVQDEAMRRLAS coupled to KLH (Pineda Antibody Service, Berlin, Germany). This sequence has been used previously to raise antibodies against pendrin (14). The guinea-pig and rabbit antisera recognized only one major band of approximately 120 kDa and the band was abolished by preincubation of serum with the immunizing peptide and absent in pre-immune serum (supplementary fig. 1). Both sera were also tested by immunohistochemistry and stained the luminal side of intercalated cells only in the late distal tubule, connecting segment and cortical collecting duct as described previously for other anti-pendrin antibodies (14, 20, 28, 29). No staining was observed when pre-immune serum was used or the immune-serum was preincubated with the immunizing peptide (supplementary fig. 2). We used the guinea-pig serum for immunoblotting and the rabbit serum for immunohistochemistry.

Western blotting

Mice were sacrificed, kidneys rapidly harvested and transferred into ice-cold K-HEPES buffer (200 mM mannitol, 80 mM K-HEPES, 41 mM KOH, pH 7.5) with pepstatin, leupeptin, K-EDTA, and phenylmethylsulfonyl fluoride (PMSF) added as protease inhibitors. The samples were homogenized with a tip sonicator, centrifuged at 1,000 g for 10 min at 4 °C and the supernatant saved. Subsequently, the supernatant was centrifuged at 100,000 g for 1 h at 4°C and the resultant pellet resuspended in K-HEPES buffer containing protease inhibitors. After measurement of the total protein concentration (Biorad Protein kit), 75 µg of membrane protein were solubilized in Laemmli sample buffer, and SDS-Page was performed on 10 % polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically from unstained gels to PVDF-membranes (Immobilon-P, Millipore, Bedford, MA, USA). After blocking with 5 % milk powder in Tris-buffered saline/0.1% Tween-20 for 60 min., the blots were incubated with guinea-pig anti-pendrin immune serum and mouse monoclonal anti-actin (42 kDa, Sigma) 1: 500 either for 2 h at room temperature or overnight at 4 °C. After washing and subsequent blocking, blots were incubated with secondary antibodies conjugated with alkaline phosphatase or horse radish peroxidase (goat anti-rabbit 1: 5'000, goat anti-guinea pig 1:5'000, and donkey anti-mouse 1: 10'000 (Promega)), for 1 h at room temperature. Antibody binding was detected with the enhanced chemiluminescence kit (Pierce) in the case of HRP-linked antibodies and with the CDP Star kit (Roche, USA) for AP linked antibodies before detection of chemiluminescence with the Diana III Chemiluminescence detection system. Bands were quantified with the Aida Image Analyzer software (Raytest, Germany).

Immunohistochemistry

Mice were anesthetized with ketamine/xylazine and perfused through the left ventricle with PBS followed by paraformaldehyde-lysine-periodate fixative (PLP) (15). Kidneys were removed and fixed overnight at 4°C by immersion in PLP. Kidneys were washed three times with PBS, rapidly frozen with liquid propane, and 5 µm cryosections were cut after cryoprotection with 2.3 M sucrose in PBS.

Indirect immunohistochemistry was carried out as described previously (4, 27). Sections were incubated with 1% SDS for 5 min, washed three times with PBS, and incubated with PBS containing 1% BSA for 15 min before the primary antibodies. The primary antibodies rabbit and guinea-pig anti-pendrin, mouse monoclonal anti-calbindin D28k (SWANT, Bellinzona, Switzerland) and goat-anti-AQP2 (Chemicon (now Millipore)) were diluted in PBS 1:1000, 1: 20'000, and 1:400, respectively, and applied either for 75 min at room temperature or overnight at 4°C. Sections were then washed twice for 5 min with high NaCl PBS (PBS + 2.7% NaCl), once with PBS, and incubated with dilutions of the secondary antibodies (donkey anti-rabbit Alexa 594, donkey-anti guinea-pig Cy3, donkey anti-mouse Alexa 488, donkey anti-goat 488, Molecular Probes, OR, USA) at 1:1000; 1:400, 1:400, and 1:400, respectively, combined with DAPI to counterstain cell nuclei (1:200) for 1 h at room temperature. Sections were again washed twice with high-NaCl PBS and once with PBS before mounting with VectaMount (Vector Laboratories, Burlingame, CA). Sections were viewed with a confocal microscope (Leica CLSM). Images were processed (overlays) using Adobe Photoshop. Cells were counted as described previously in at least 3 sections from 4 animals each for each treatment group (28). Omission of primary antibodies or preincubation of anti-pendrin sera with the immunizing peptide abolished all staining (see supplementary figure).

Statistics

All data were tested for significance using the student's t-test and ANOVA followed by Bonferroni's multiple comparison test. Only data with $p < 0.05$ were considered statistically significant. Data are reported as mean ± SEM.

RESULTS

Treatment of animals with NH_4Cl for 7 days induced metabolic acidosis as evident from blood gas analysis and urinary net acid excretion (table 1, fig. 1). Urinary total and fractional chloride excretion were strongly increased. Immunoblotting showed a reduction in relative pendrin protein expression levels to 62 ± 13 % as compared to control animals as described previously in mice and rats (9, 18, 19, 28) (fig. 1). In contrast, addition of NaCl to the drinking water provided a chloride load as evident from the urinary fractional chloride excretion but did not affect systemic acid-base status or urinary acid excretion (table 1, fig. 1). As expected, pendrin protein abundance was reduced to 80 ± 16 % (fig. 1).

In a second pair of animals, mice received $(NH_4)_2SO_4$ to induce metabolic acidosis without providing a direct chloride load. $(NH_4)_2SO_4$ induced a severe hyperchloremic metabolic acidosis with high net acid excretion, low urinary fractional Cl^- excretion but high blood chloride concentrations possibly due to dehydration (as also evident from the increased Na^+ concentration and weight loss)(table 1, fig. 2). Relative pendrin expression levels were decreased to 49 ± 5 % (fig. 2). Na_2SO_4 was given to control for effects of sulfate which increases the lumen-negative potential in the collecting duct as an impermeable anion and facilitates H^+-secretion. Na_2SO_4 did not affect NAE, but caused urinary Cl^- retention most likely due to dehydration. Pendrin expression was highly stimulated to 252 ± 12 % (fig. 2). Thus, low urinary chloride excretion stimulates pendrin expression levels as described previously (19, 30). However, concomitant metabolic acidosis as in the case of $(NH_4)_2SO_4$-loading seems to reverse this effect and reduces pendrin expression.

In an alternative model of metabolic acidosis, acidosis was induced in mice treated with the carbonic anhydrase inhibitor acetazolamide. Acetazolamide causes metabolic acidosis by inducing urinary bicarbonate wasting due to inhibition of proximal tubular bicarbonate reabsorption (3, 6). This type of metabolic acidosis is thus associated with high urinary pH (table 1) and high NAE and allows distinguishing between effects of urinary and systemic pH. Acetazolamide caused metabolic acidosis with high NAE as expected. Urinary chloride excretion was low possibly due to dehydration (high blood Na^+ and Cl^- concentrations, weight loss) and relative pendrin expression levels were not changed (106 ± 12 %)(fig.3, table 1). Hence, metabolic acidosis with high urinary pH prevents the increase in pendrin protein abundance despite low urinary chloride excretion.

Immunohistochemistry was performed on kidneys from control mice or mice treated with $NH_4(SO_4)_2$, Na_2SO_4, or acetazolamide. Kidneys were stained for pendrin and the principal cell markers calbindin D28k and AQP2. Cells were counted for the absence or presence of these cell-specific markers separately along the connecting tubule (CNT) and cortical collecting duct (CCD). In kidneys from control mice the relative abundance of Pendrin positive or negative cells and AQP2/calbindin D28k expressing cells was similar to results described previously in mouse kidney (22, 23, 28). Treatment with $NH_4(SO_4)_2$ or acetazolamide significantly reduced the relative abundance of pendrin positive cells and increased the abundance of cells lacking both pendrin and AQP2/ calbindin D28k both in the connecting tubule and cortical collecting duct (figs. 4 and 5, table 3). Application of Na_2SO_4 had no effect on the relative abundance of the different cell types.

A second group of animals was given low (20 % protein, LP) or high (50 % protein, HP) protein containing diets which provide a mild and more physiological acid-load without altering chloride balance. Control mice received the low protein diet for 7 days, two groups were given the high protein diet for 7 or 14 days, respectively. High protein diet increased urinary NAE strongly without altering otherwise systemic acid-base status, urinary Cl^- excretion, and blood chloride concentrations (table 2). The relative pendrin abundance levels were not affected by the high protein diet (HP 7 days: 105 ± 4 %, HP 14 days: 107 ± 10 %)(table 2, fig. 6). Thus, increasing urinary acid excretion without inducing metabolic acidosis does not alter pendrin protein abundance.

DISCUSSION

The connecting segment and collecting duct are the main sites where the fine regulation of excretion or reabsorption of several ions including Na^+, K^+, Cl^- and acid-base equivalents such as H^+ and HCO_3^- take place. These highly regulated transport processes are mediated by a set of specific ion channels and transport proteins expressed in principal cells and different subtypes of intercalated cells. The transport of particular ions is often linked directly or indirectly to the transport of other ions as exemplified by the reabsorption of Na^+ and the secretion of K^+, or the direct coupling of H^+ and K^+ in the H^+/K^+-ATPase. Another example of mutual transport coupling is provided by the chloride/ anion exchanger pendrin that is thought to couple the uptake of chloride from urine to the secretion of bicarbonate into urine at the apical side of non-type A intercalated cells along the connecting tubule and cortical collecting duct (20, 21). Thus, pendrin could provide for bicarbonate secretion as well as for chloride absorption and thereby contribute to acid-base status and/or systemic chloride balance. Obviously, both processes need to be regulated even in opposite directions. Thus, the abundance of pendrin may be influenced by more than one factor and regulation by different factors may require coordination.

Previous experiments have provided evidence for regulation of pendrin during NH_4Cl-loading at the level of protein abundance and subcellular localization (9, 18, 19, 28). These results implied that pendrin is regulated by acid-base status focussing on its function as bicarbonate-secretory protein. More recently, it was shown that pendrin regulation directly correlates with urinary chloride absorption suggesting that pendrin was an important determinant for chloride conservation and thus blood pressure regulation (19, 30). However, these findings raised the question if pendrin is

only regulated by urinary chloride excretion and load or if metabolic acidosis is a second determinant of pendrin abundance.

Our data demonstrate: i) pendrin expression is reduced during chloride-loading with NH_4Cl or NaCl as demonstrated previously (9, 18, 28, 30); ii) pendrin expression is increased when urinary chloride excretion is reduced as in the case of Na_2SO_4-loading similar to what has been demonstrated for furosemide-treatment and NaCl restriction (19, 30), iii) severe metabolic acidosis with low urinary chloride excretion as induced by $(NH_4)_2SO_4$-loading is associated with reduced pendrin expression and relative abundance of Pendrin expressing cells, iv) mild metabolic acidosis with low urinary chloride excretion as induced by acetazolamide treatment prevents the expected stimulation of pendrin expression and even reduced the relative abundance of Pendrin expressing cells, v) increased urinary acidification and stimulated NAE does not influence pendrin expression as evident from high protein diet experiments, and vi) systemic acid-base status but not urinary pH or acid excretion are associated with down-regulation of pendrin abundance as demonstrated by the fact that $(NH_4)_2SO_4$ caused an acidic urinary pH whereas acetazolamide an alkaline urine and both prevented the expected increase in pendrin expression. The reduction in pendrin expression during $(NH_4)_2SO_4$-loading may be most likely caused by the more severe metabolic acidosis as compared to the acetazolamide treatment. Interestingly, this and previous reports suggest that pendrin abundance in kidney may be regulated either on the cellular levels (as seen in cases where chloride was primarily manipulated) (19, 24) or on the level of the whole collecting duct system altering the number of pendrin expressing cells (as in cases where primarily acid-base status is altered). Underlying mechanisms of this potentially differential regulation should be addressed in future studies.

Distal chloride delivery has been suggested to be a major regulator of pendrin expression (24). In our set of data, no clear correlation between total chloride excretion, FE_{Cl} and pendrin expression could be established if acid-base status is not taken into consideration. However, the actual distal chloride delivery rates are difficult to estimate in our mouse models since we did not investigate other transport proteins affecting transcellular or paracellular chloride transport such as NCC, ENaC, claudin 2, the WNK kinases, or ClC-k channels.

Aldosterone, angiotensin II, and vasopressin have been implicated in the regulation of pendrin expression and activity (2, 17, 25). Verlander et al. suggested that NaCl restriction and aldosterone increase pendrin expression and translocation into the luminal membrane (25, 26, 30). In contrast, Adler et al. recently demonstrated that aldosterone decreased endogenous pendrin mRNA abundance in HEK293 cells. Moreover, aldosterone reduced the pendrin-promotor driven luciferase activity in HEK293 cells (1). Thus, the role of aldosterone in pendrin regulation in kidney remains controversial. Even though we did not measure aldosterone levels in our experiments, we hypothesize that aldosterone is not a major regulator of pendrin expression since we imposed various conditions where aldosterone levels are either increased (NH_4Cl, acetazolamide) or decreased (NaCl-loading) but pendrin abundance was found to be decreased in all conditions. The vasopressin analogue dDAVP increased in Brattleboro rats pendrin mRNA, an effect possibly mediated by V2-receptors (2). We did not measure vasopressin levels in our mouse models, but serum osmolarity was increased in two groups, $(NH_4)_2SO_4$ and acetazolamide, and associated with lower pendrin expression levels. Thus, vasopressin may be an additional and independent regulator of pendrin expression.

Our data are consistent with the recent report from Adler et al. where a pH dependent regulation of pendrin promoter activity in HEK293 cells was demonstrated. There, extracellular acidification reduced and alkalinization increased promoter activity (1).

Taken together, the data suggest that pendrin expression is primarily correlated to urinary chloride excretion and may function there in the regulation of transcellular chloride absorption. However, pendrin abundance may also be regulated by systemic acid-base status despite low urinary chloride excretion. Metabolic acidosis associated with low urinary chloride excretion down-regulates pendrin expression or at least prevents its stimulation. The sensing mechanism for metabolic acidosis must be located either intracellularly or at the basolateral side of the cells as only metabolic acidosis but not urinary acidification per se influence pendrin expression as evident from the $(NH_4)_2SO_4$, acetazolamide and protein diet experiments. Alternatively, the signal(s) for pendrin expression could be generated in other cells or organs but must involve sensing of urinary chloride excretion and systemic acid-base status.

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FIGURE LEGENDS

TABLE 1

Blood and urine analysis. 6 groups of mice (each 5 animals) were subjected to different diets by adding to their drinking water 2 % sucrose (control), 0.28 M NH_4Cl/ 2 % sucrose, 0.28 M NaCl/ 2 % sucrose, 0.28 M $(NH_4)_2SO_4$/ 2 % sucrose, 0.28 M Na_2SO_4/ 2 % sucrose, or the carbonic anhydrase inhibitor acetazolamide (80 mg/ 100 ml water)/ 2 % sucrose, respectively, for 7 days. Animals were kept for the last two days in metabolic cages and urine was collected over 24 hrs under mineral oil. Venous blood was taken under anesthesia for blood analysis. Fe_{Cl} % fractional urinary chloride excretion, TA titratable acidity, NAE net acid excretion. Levels of statistical significance were: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

TABLE 2

Blood and urine analysis. 3 groups of mice (each 5 animals) were kept on low (20 %) or high (50 %) protein containing diets for 7 or 14 days, respectively. Animals were kept for the last two days in metabolic cages and urine was collected over 24 hrs under mineral oil. Venous blood was taken under anesthesia for blood analysis. Fe_{Cl} % fractional urinary chloride excretion, TA titratable acidity, NAE net acid excretion. Levels of statistical significance were: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

TABLE 3

Relative cell abundance of pendrin expressing cells in the connecting tubule and cortical collecting duct. Kidney sections were stained with antibodies against pendrin and calbindin D28k/AQP2 to identify principal cells (calbindin D28k/AQP2

positive), non-type A intercalated cells (Pendrin positive, calbindinD28k/AQP2 negative), and type A intercalated cells (negative for all markers). Pictures were taken from two kidney sections from 4 mice in each group and cells counted separately in the connecting tubule (CNT) and cortical collecting duct (CCD). Data are presented as percentage (Mean ± SEM) of total cells counted per segment where n represents the total number of cells counted. Levels of statistical significance were: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGURE 1

NH_4Cl and NaCl decrease pendrin abundance. Mice were given 0.28 M NH_4Cl/ 2 % sucrose or 0.28 M NaCl/ 2 % sucrose in the drinking water, respectively, for 7 days. Control mice received only 2 % sucrose. NH_4Cl-loading decreased relative pendrin abundance and increased blood and urinary Cl^- and net acid excretion (NAE). Addition of NaCl also reduced the relative pendrin abundance without altering NAE and blood chloride concentration but increased urinary Cl^- excretion. n = 5 animals for each group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

FIGURE 2

$(NH_4)_2SO_4$ decreases and Na_2SO_4 increases pendrin abundance. Mice were given 0.28 M $(NH_4)_2SO_4$/ 2 % sucrose or 0.28 M Na_2SO_4/ 2 % sucrose in the drinking water, respectively, for 7 days. Control mice received only 2 % sucrose for 7 days. $(NH_4)_2SO_4$ loading reduced the relative pendrin abundance, decreased urinary Cl^- excretion while increasing blood Cl^- concentration and urinary NAE. In contrast, Na_2SO_4 stimulated pendrin expression while reducing urinary Cl^- and net acid excretion and increased blood Cl^- concentration due to dehydration. n = 5 animals for each group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

FIGURE 3

Inhibition of carbonic anhydrase with acetazolamide does not alter pendrin abundance. Mice were given the carbonic anhydrase inhibitor acetazolamide in drinking water for 7 days which caused metabolic acidosis, increased urinary NAE and blood Cl^- concentration while decreasing urinary Cl^- excretion. The relative pendrin abundance was not affected by acetazolamide treatment. n = 5 animals for each group, *p < 0.05, **p < 0.01, ***p < 0.001..

FIGURE 4

Abundance of pendrin expressing cells in kidneys from mice with different treatments. Kidney sections were stained with antibodies against pendrin (red) and calbindinD28k/ AQP2 (green) and cells counted in the connecting tubule and cortical collecting duct. **(A-D)** Pendrin positive cells in the connecting tubule: A: control, B: $(NH_4)_2SO_4$ treated, C: Na_2SO_4 treated, D: acetazolamide treated. **(E-H)** Pendrin positive cells along the cortical collecting duct: E: control, F: $(NH_4)_2SO_4$ treated, G: Na_2SO_4 treated, H: acetazolamide treated. Arrow head: pendrin positive cells, asterisks: pendrin negative, calbindinD28k/AQP2 negative cells. Original magnification: 630 x

FIGURE 5

Ammoniumsulfate and acetazolamide reduce the number of pendrin expressing intercalated cells. Kidney sections from 5 mice each group treated with sucrose (control), $NH_4(SO_4)_2$, Na_2SO_4, or acetazolamide were stained for Pendrin, calbindin D28k, and AQP2, and cells along the connecting tubule (CNT) and cortical collecting duct (CCD) were counted for their expression of these cell markers. Data

are presented as percent of total cells counted, s.e.m ± mean. The number of total cells counted is given. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 6

Dietary protein content and acid-load does not affect pendrin expression. Mice received a dietary acid load by varying dietary protein content from low protein (LP, 20 %) to high protein (HP, 50 %) for 7 or 14 days, respectively. Diets were otherwise identical in their fat and electrolyte composition. High protein caused strongly increased NAE without altering pendrin expression, blood, and urine Cl^- concentration. n = 5 animals for each group, *p < 0.05, **p < 0.01, ***p < 0.001.

SUPPLEMENTARY FIGURE 1

Specificity of anti-pendrin antibody in immunoblotting. Total kidney membranes (75 μg) were loaded and membranes probed with guinea-pig anti pendrin or rabbit anti-pendrin antibodies. Anti-pendrin antibodies detected one major band around 120-130 kDa. No bands were detected with the pre-immune sera or when the immune sera were preincubated with the immunizing peptide.

SUPPLEMENTARY FIGURE 2

Specificity of anti-pendrin antibodies in immunohistochemistry. Kidney sections were stained with **(A)** rabbit anti-pendrin or **(C)** guinea-pig anti-pendrin antibodies (red). Sections were also stained with antibodies against the principal cell specific AQP2 water channel (green). **(B, D)** Anti-pendrin antibodies were preincubated with the immunizing peptide which completely abolished the signal. Cell nuclei are stained with DAPI (blue). Original magnification 400 x.

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Table 1

	Control	NH_4Cl	NaCl	$(NH_4)_2SO4$	Na_2SO_4	Acetazolamide
Blood						
pH	7.35 ± 0.02	7.23 ± 0.03 **	7.30 ± 0.03	7.14 ± 0.02 ***	7.32 ± 0.01	7.18 ± 0.00 ***
pCO_2 mmHg	44.6 ± 3.1	47.1 ± 4.5	47.9 ± 4.5	53.5 ± 1.7	53.7 ± 2.2	51.5 ± 1.8
$[HCO_3]$ mM	23.8 ± 0.7	16.1 ± 1.3 **	22.5 ± 0.9	17.4 ± 0.9 ***	26.8 ± 1.2 *	18.6 ± 0.8 *
$[Na^+]$ mM	144.8 ± 0.8	146.2 ± 0.7	146.6 ± 1.2	151.0 ± 1.8 **	156.4 ± 2.3 **	151.6 ± 1.7 **
$[K^+]$ mM	4.3 ± 0.1	4.4 ± 0.3	4.6 ± 0.1	4.1 ± 0.2	4.1 ± 0.1	4.1 ± 0.1
$[Cl^-]$ mM	109.2 ± 1.2	119.2 ± 1.5 *	111.0 ± 1.2	123.4 ± 0.9 ***	115.0 ± 2.5	124.4 ± 1.4 ***
Creatinine (mg/dl)	0.06 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.02	0.10 ± 0.03	0.08 ± 0.01
Hematocrit	46.8 ± 0.7	43.4 ± 0.6	44.6 ± 0.7	44.6 ± 1.0	48.8 ± 1.5	44.2 ± 0.6
Osmolarity (mOsm)	303.5 ± 1.1	296.4 ± 1.1	296.8 ± 0.7	372.34 ± 13.5***	298.5 ± 1.5	339.6 ± 4.0***
Urine						
pH	6.70 ± 0.14	5.73 ± 0.08 **	6.77 ± 0.15	5.85 ± 0.24 **	6.60 ± 0.13	8.08 ± 0.05 ***
Urine volume/ 24 hrs	1.82 ± 0.59	1.24 ± 0.27	2.34 ± 0.91	0.57 ± 0.16	0.92 ± 0.28	1.21 ± 0.25
Creatinine mM	59.4 ± 8.0	101.4 ± 10.6 *	133.1 ± 36.4 *	114.1 ± 5.2 *	141.9 ± 4.6 **	80.0 ± 5.8 *
NH_4/Creatinine	0.51 ± 0.13	1.4 ± 0.15 ***	0.23 ± 0.07	1.25 ± 0.06 ***	0.23 ± 0.05	1.35 ± 0.13 ***
Na/Creatinine	3.09 ± 0.33	1.52 ± .010	7.61 ± 3.68 *	1.70 ± 0.20	5.60 ± 0.39 *	1.59 ± 0.14
K/Creatinine	4.93 ± 0.26	2.65 ± 0.34 ***	2.11 ± 0.48 ***	3.12 ± 0.34 ***	2.52 ± 0.17 ***	2.83 ± 0.08 ***
Cl/Creatinine	4.03 ± 0.44	6.31 ± 0.66*	6.75 ± 2.3*	1.78 ± 0.37*	1.43 ± 0.10*	2.28 ± 0.21*
FE_Cl %	0.32 ± 0.08	0.47 ± 0.09 *	0.51 ± 0.21 *	0.13 ± 0.04 *	0.13 ± 0.04 *	0.15 ± 0.04 *
TA	13.4 ± 2.5	25.7 ± 5.2 *	12.2 ± 2.7	32.9 ± 8.5 *	16.0 ± 3.5	-21.5 ± 2.2 ***
NAE/Creatinine	0.58 ± 0.07	1.66 ± 0.17 ***	0.36 ± 0.10	1.53 ± 0.05 ***	0.34 ± 0.04	1.13 ± 0.15**
Food intake (g/24 hrs)	4.81 ± 0.48	5.20 ± 0.62	4.70 ± 0.27	5.26 ± 0.47	5.90 ± 0.28	4.88 ± 0.26
Water intake (ml/24 hrs)	8.87 ± 1.49	6.50 ± 0.51	7.56 ± 1.67	4.24 ± 0.16**	5.78 ± 0.34	5.44 ± 0.37
Feces (g/24 hrs)	1.65 ± 0.18	1.64 ± 0.29	1.60 ± 0.05	1.76 ± 0.11	2.76 ± 0.25	1.56 ± 0.18
Weight change %	-1.8 ± 1.3	-2.2 ± 0.7 *	-3.6 ± 0.9 *	-12.8 ± 7.2 ***	-5.7 ± 1.6 *	-9.52 ± 1.6 ***

Table 2

	Low protein 7 days	High protein 7 days	High protein 14 days
Blood			
pH	7.35 ± 0.03	7.33 ± 0.00	7.30 ± 0.02
pCO_2 mmHg	45.8 ± 4.2	49.6 ± 2.9	57.3 ± 3.2
$[HCO_3]$ mM	24.7 ± 0.6	25.3 ± 1.2	27.1 ± 1.6
$[Na^+]$ mM	146.4 ± 0.4	146.2 ± 0.7	146.8 ± 0.6
$[K^+]$ mM	4.4 ± 0.2	4.5 ± 0.2	4.4 ± 0.2
$[Cl^-]$ mM	111.8 ± 0.2	113.2 ± 0.5	112.2 ± 1.6
Creatinine (mg/dl)	0.13 ± 0.04	0.10 ± 0.02	0.08 ± 0.02
Hematocrit	36.5 ± 3.6	41.2 ± 1.8	n.t.
Osmolarity (mOsm)	309.8 ± 2.3	310.6 ± 2.1	n.t.
Urine			
pH	7.35 ± 0.08	6.81 ± 0.24 ***	6.55 ± 0.04 ***
Urine volume/ 24 hrs	1.38 ± 0.26	3.83 ± 0.59***	3.66 ± 0.79***
Creatinine mM	37.8 ± 2.4	20.5 ± 1.7 ****	20.7 ± 1.1 ****
NH_4/Creatinine	0.32 ± 0.01	2.14 ± 0.34 ***	1.97 ± 0.18 ***
Na/Creatinine	7.36 ± 0.80	9.38 ± 0.65	9.08 ± 0.39
K/Creatinine	5.49 ± 0.27	5.86 ± 0.41	5.49 ± 0.27
Cl/Creatinine	1.32 ± 0.16	1.76 ± 0.14	1.59 ± 0.15
FE_{Cl} %	0.14 ± 0.03	0.16 ± 0.06	0.11 ± 0.02
TA	9.0 ± 2.1	54.5 ± 14.7 ***	52.0 ± 10.3 ***
NAE/Creatinine	0.57 ± 0.05	4.9 ± 0.88 ***	4.47 ± 0.63 ***
Food intake (g/24 hrs)	4.68 ± 0.47	3.76 ± 0.37	4.02 ± 0.22
Water intake (ml/24 hrs)	5.92 ± 0.82	7.66 ± 0.65***	9.00 ± 0.46***
Feces (g/24 hrs)	0.52 ± 0.06	0.54 ± 0.07	0.58 ± 0.11
Weight change %	2.0 ± 1.6	-6.2 ±1.4 **	-3.8 ± 1.6 **

Table 3

	Pendrin neg Calbindin D28k/ AQP2 neg	**Pendrin pos Calbindin D28k/ AQP2 neg**	**Pendrin neg Calbindin D28k/ AQP2 pos**	**Cells counted**
CNT				
Control	7.0 ± 0.6	29.1 ± 1.3	63.9 ± 1.1	715
$NH_4(SO_4)_2$	21.0 ± 1.2***	15.6 ± 1.6***	63.5 ± 1.7	624
Na_2SO_4	13.0 ± 1.0***	25.1 ± 1.3*	61.9 ± 1.1	873
Acetazolamide	22.3 ± 1.3***	16.7 ± 1.4***	61.0 ± 1.2	839
CCD				
Control	14.6 ± 0.8	21.0 ± 1.5	64.4 ± 1.5	855
$NH_4(SO_4)_2$	22.3 ± 1.5***	12.4 ± 1.9***	65.3 ± 1.0	644
Na_2SO_4	16.7 ± 0.8	17.9 ± 0.9	65.4 ± 0.8	1087
Acetazolamide	23.1 ± 1.1***	11.9 ± 1.2***	65.0 ± 0.9	978

Fig. 1

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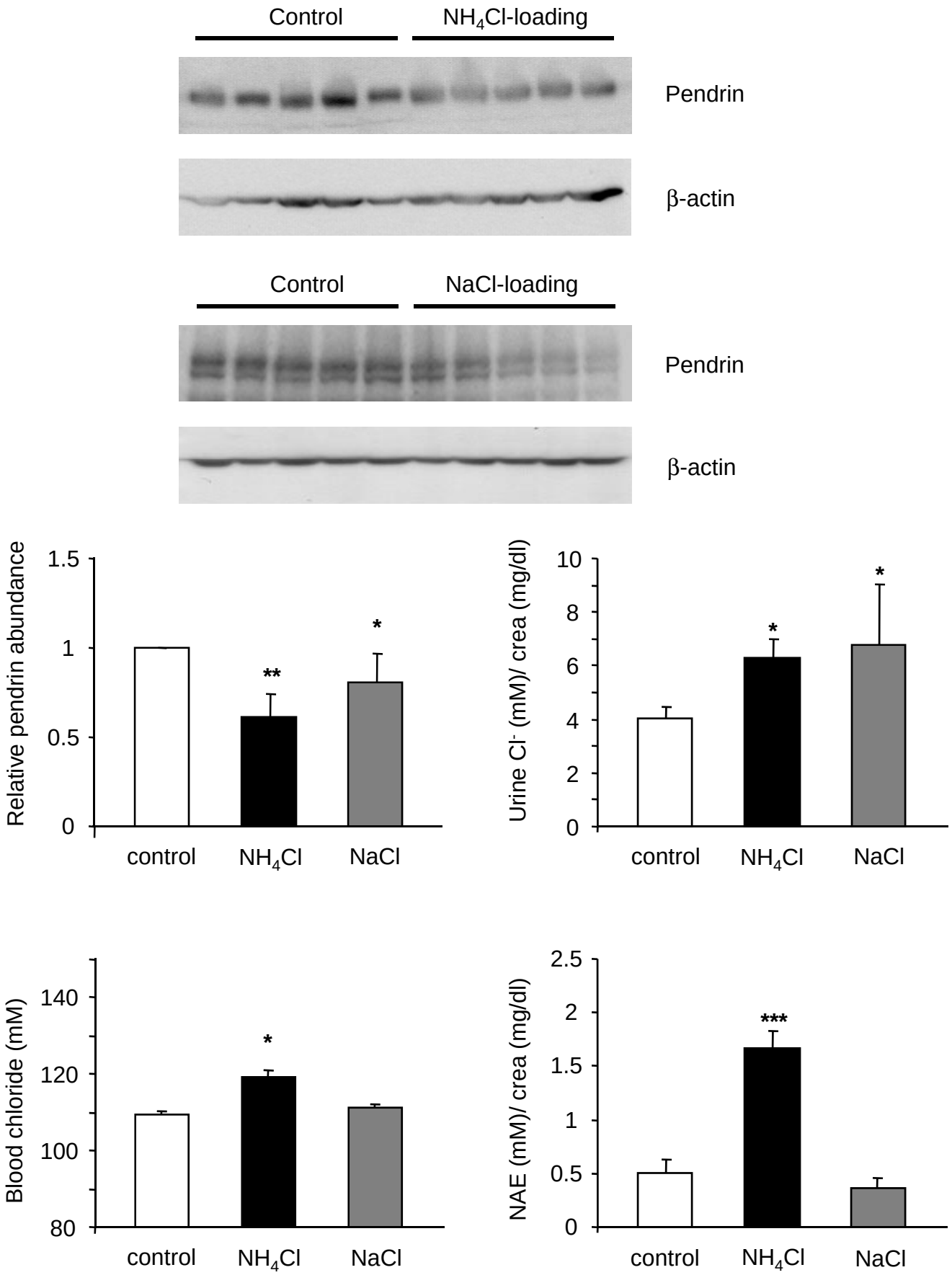

Fig. 2

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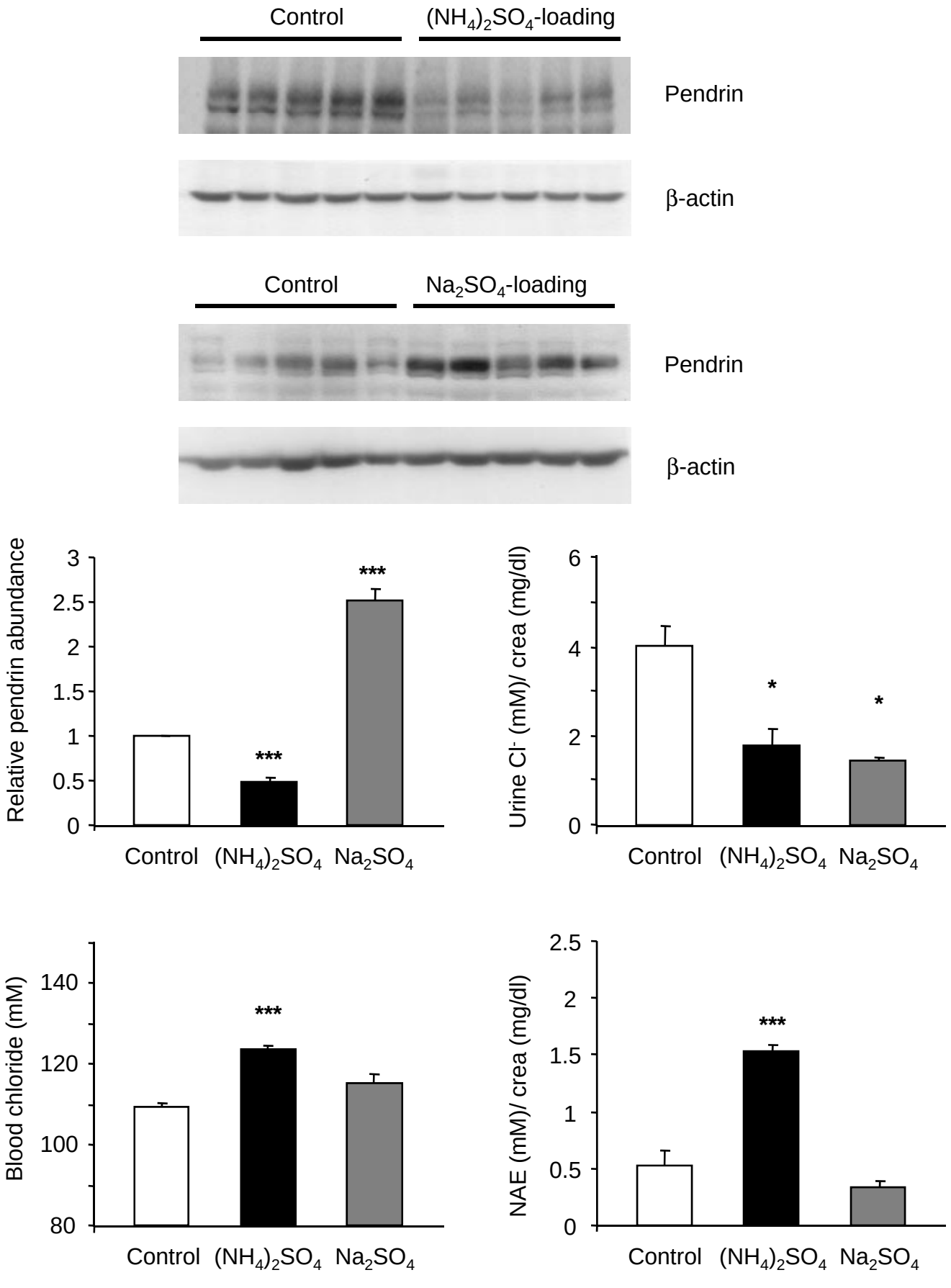
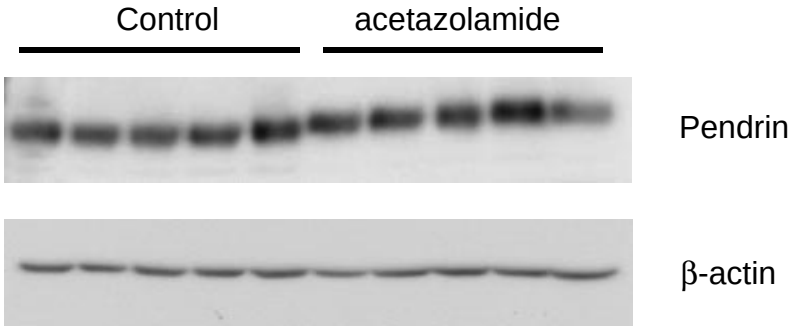
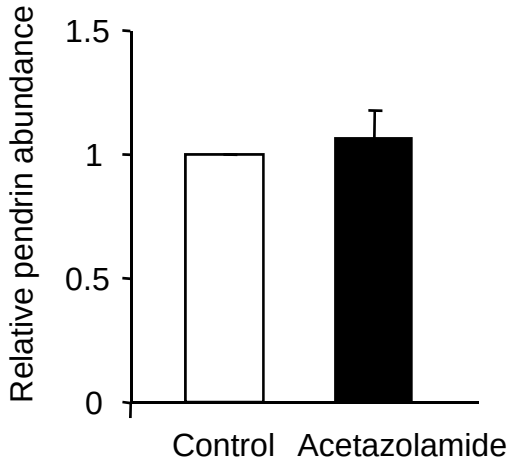
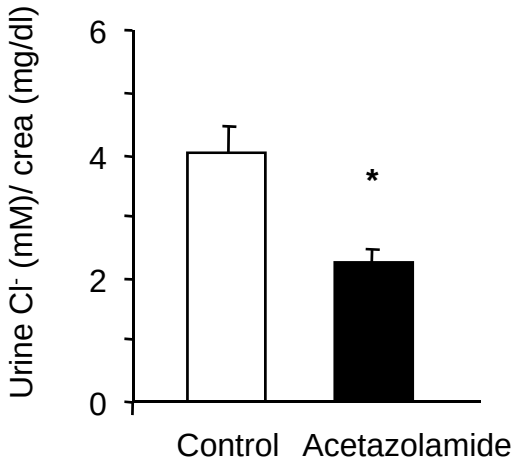
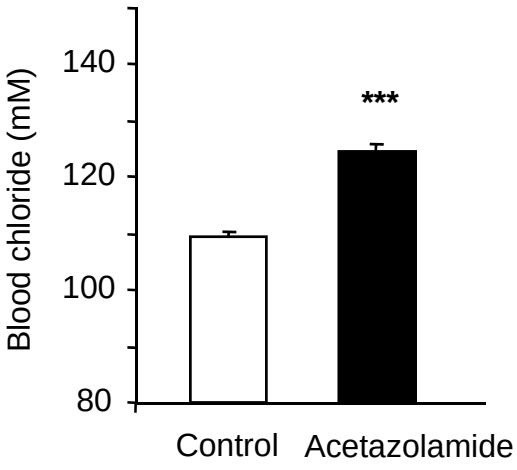


Fig. 3


Control
acetazolamide
Pendrin
β-actin


Relative pendrin abundance
1.5
1
0.5
0
Control
Acetazolamide


Urine Cl- (mM)/ crea (mg/dl)
6
4
2
0
*
Control
Acetazolamide


Blood chloride (mM)
140
120
100
80
***
Control
Acetazolamide

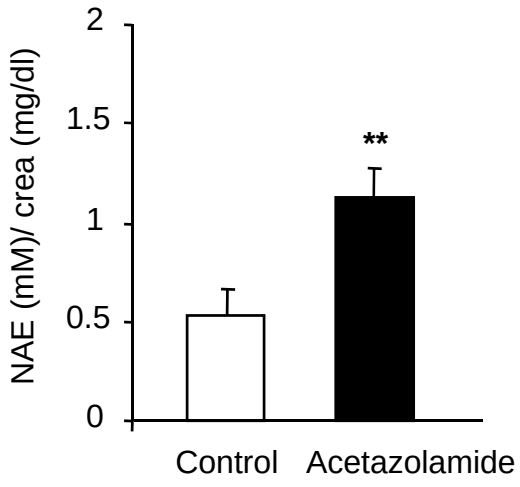
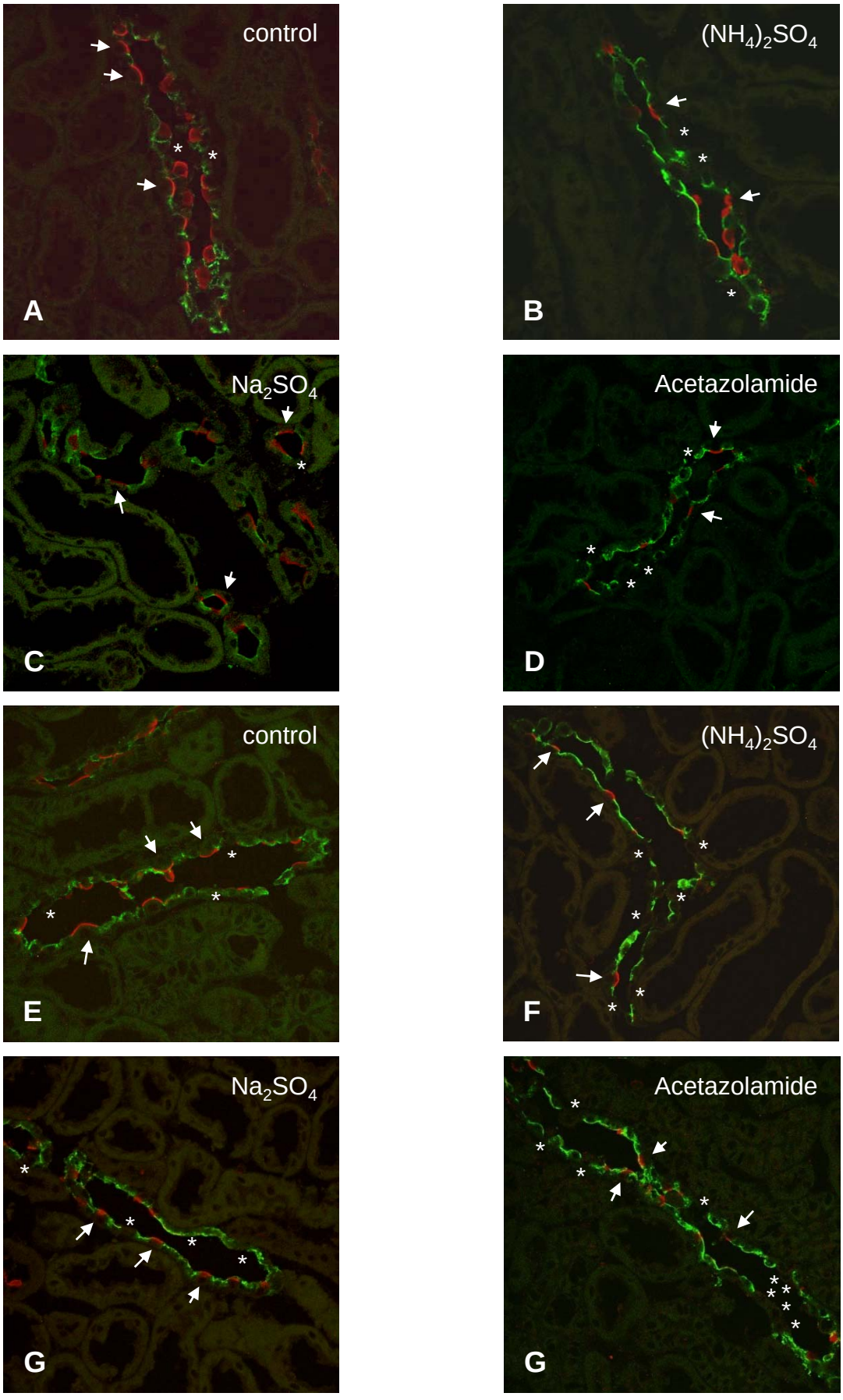

NAE (mM)/ crea (mg/dl)
2
1.5
1
0.5
0
**
Control
Acetazolamide

Fig. 4


control
A
$(NH_4)_2SO_4$
B
Na_2SO_4
C
Acetazolamide
D
control
E
$(NH_4)_2SO_4$
F
Na_2SO_4
G
Acetazolamide
G

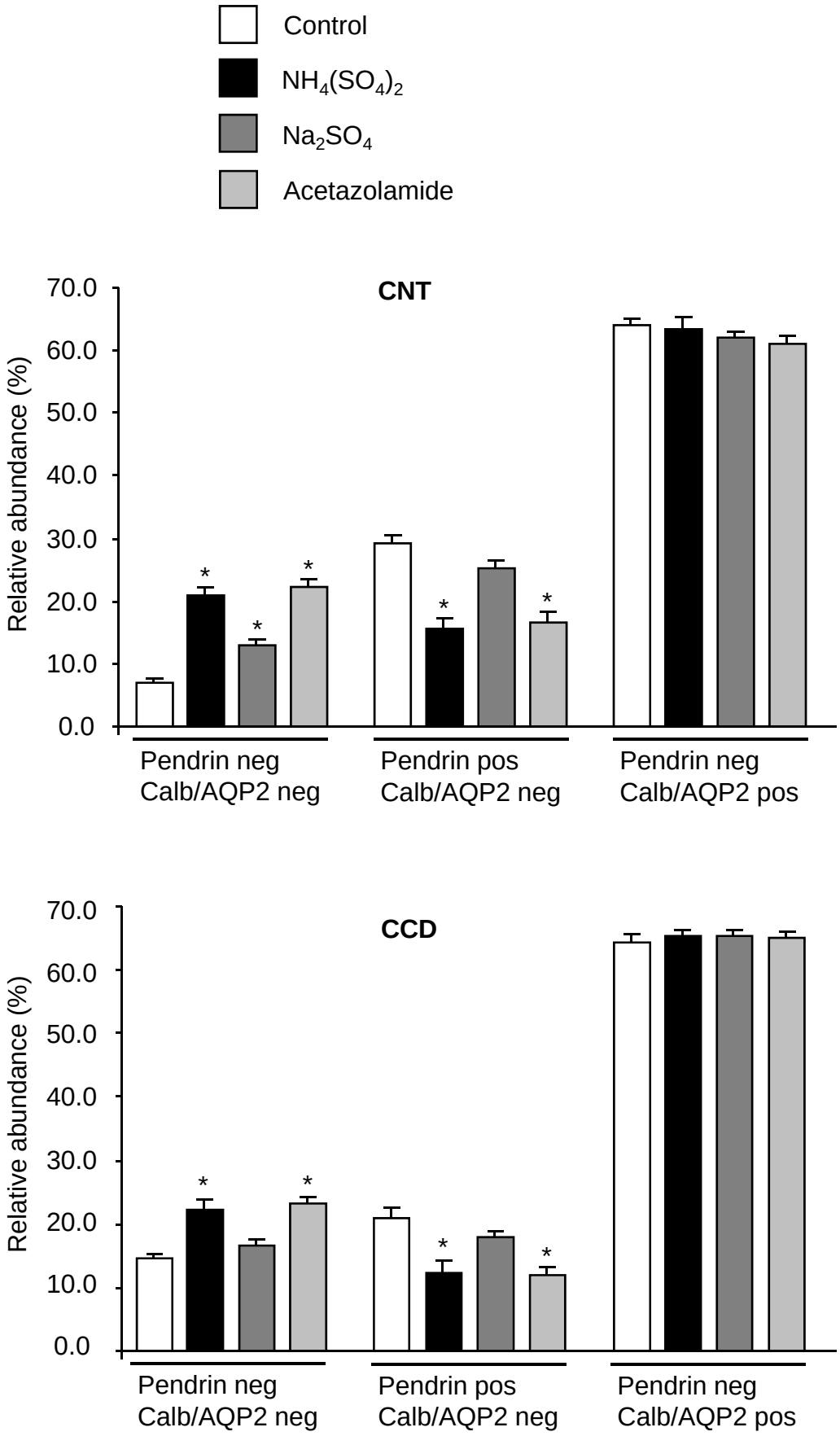

Control
$NH_4(SO_4)_2$
Na_2SO_4
Acetazolamide
CNT
Relative abundance (%)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
*
Pendrin neg
Calb/AQP2 neg
Pendrin pos
Calb/AQP2 neg
Pendrin neg
Calb/AQP2 pos
CCD
Relative abundance (%)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
*
Pendrin neg
Calb/AQP2 neg
Pendrin pos
Calb/AQP2 neg
Pendrin neg
Calb/AQP2 pos

Fig. 6

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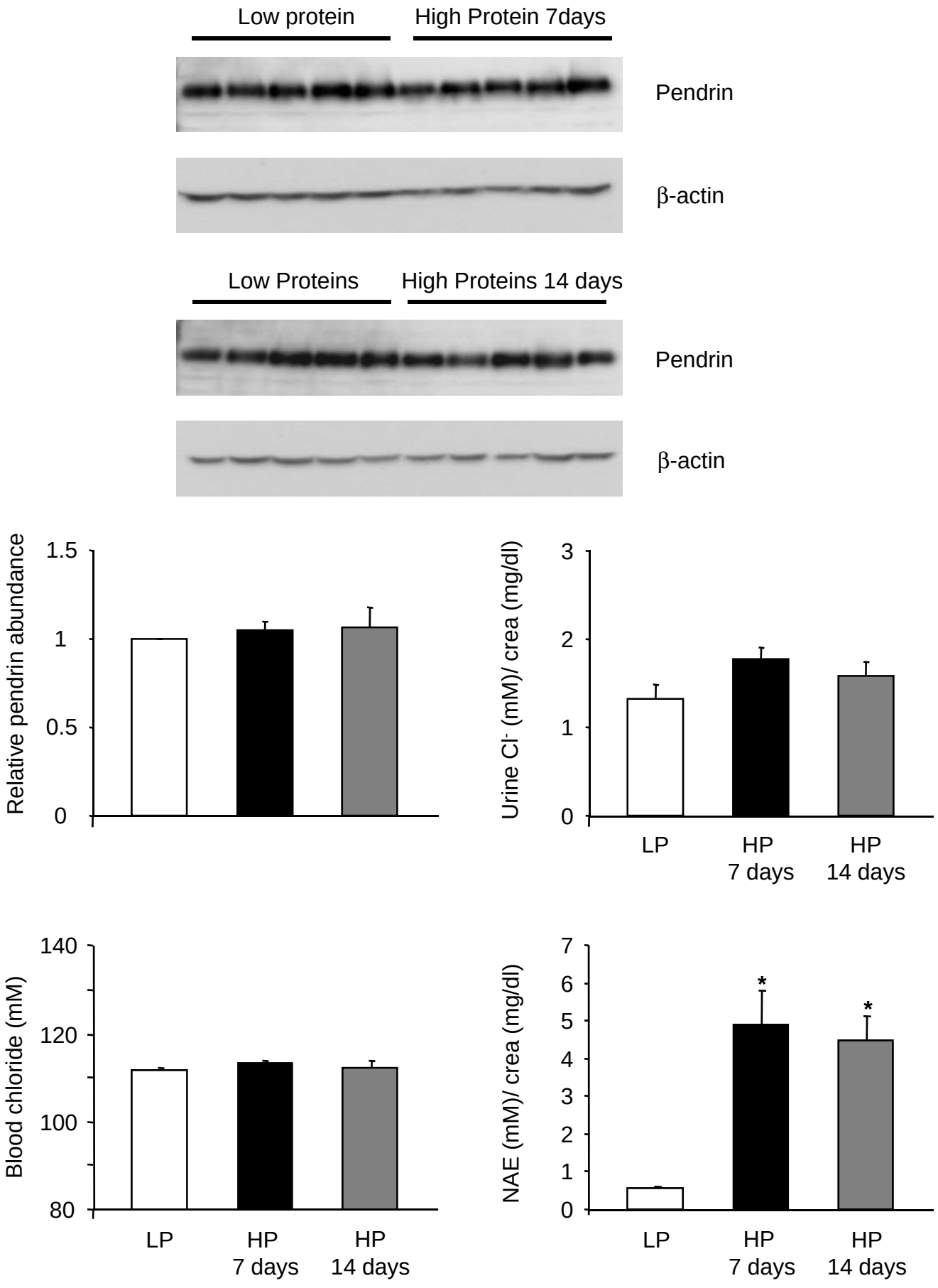

Suppl. Fig. 1

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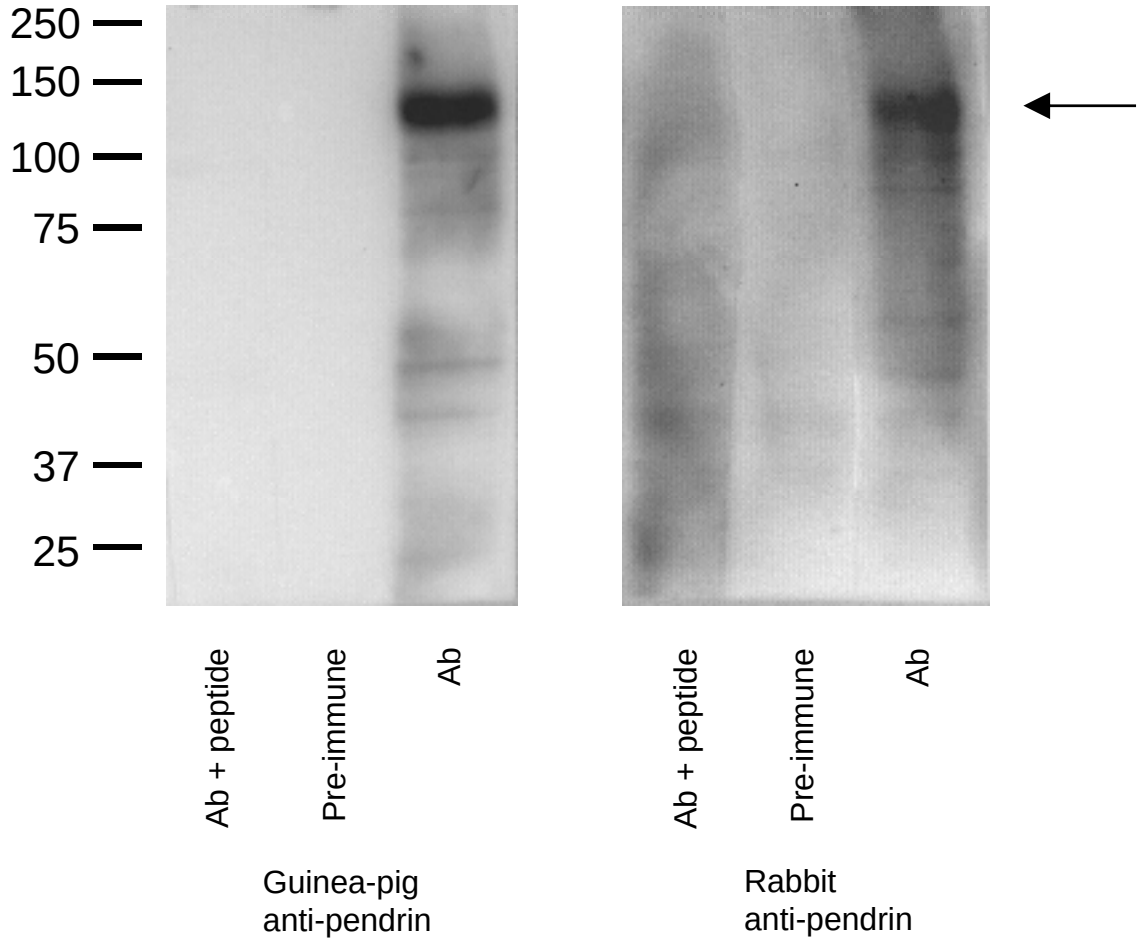

Suppl. Fig. 2

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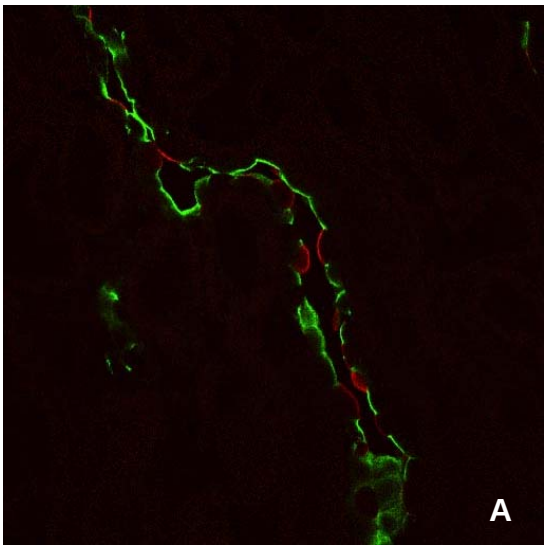
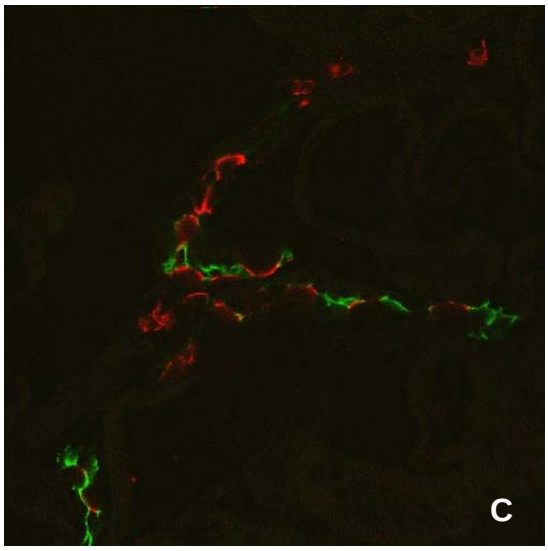
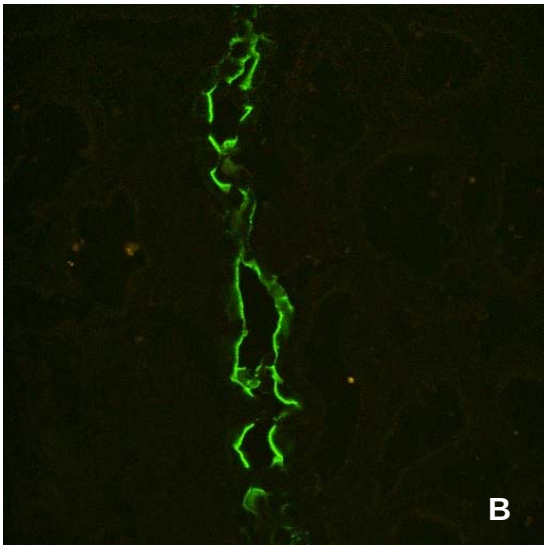
Rabbit
anti-pendrin



Guinea-pig
anti-pendrin



Rabbit
anti-pendrin +
Immunizing peptide



Guinea-pig
anti-pendrin +
Immunizing peptide