U.S. Department of Veterans Affairs
Public Access Author manuscript
*Kidney Blood Press Res*. Author manuscript; available in PMC 2024 March 18.

Published in final edited form as:
*Kidney Blood Press Res*. 2017 ; 42(3): 444–455. doi:10.1159/000479296.

# Ablation of the $Cl^-/HCO_3^-$ Exchanger Pendrin Enhances Hydrochlorothiazide-Induced Diuresis

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## Abstract

**Background/Aims:** The $Cl^-/HCO_3^-$ exchanger pendrin and the thiazide-sensitive Na-Cl cotransporter NCC are expressed in the kidney distal nephron and mediate salt absorption. We hypothesized that deletion of pendrin leaves NCC as the major salt absorbing transporter in the distal nephron and therefore enhances salt excretion by hydrochlorothiazide (HCTZ).

**Methods:** Metabolic cage studies were performed in wild type, pendrin KO and NCC KO mice at baseline and following HCTZ treatment. In parallel studies, systemic blood pressure was measured in mice treated with HCTZ with the tail cuff method.

**Results:** Urine output, salt excretion and water intake were comparable in all groups under baseline condition. Urine output and water intake increased significantly only in pendrin KO mice in response to HCTZ, but not in WT or NCC KO mice. Sodium and chloride excretion increased in HCTZ-treated pendrin KO mice, but they remained unchanged in WT or NCC KO mice. Pendrin KO mice treated with HCTZ developed volume depletion, as determined by increased expression of renin mRNA and protein. The expression of ENaC and pendrin increased in HCTZ-treated WT mice. HCTZ treatment did not significantly modify blood pressure in any of the experimental group.

**Conclusion:** The ablation of the $Cl^-/HCO_3^-$ exchanger Pendrin enhances the magnitude of salt wasting by HCTZ.

### Keywords

Hypertension; Kidney; Pendrin; NCC; Thiazides



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Disclosure Statement
The authors declare no competing financial interests.

## Introduction

Hydrochlorothiazide is a specific inhibitor of the $Na^+$ - $Cl^-$ cotransporter (NCC) in the distal convoluted tubule, where ~7% of the filtered salt is reabsorbed [1, 2]. Despite being a very strong inhibitor of NCC, the magnitude of diuresis subsequent to HCTZ is very mild, raising the possibility that other salt transporters may be activated and enhance compensatory salt absorption in the connecting tubule, the collecting duct, and possibly other nephron segments. Both the epithelial sodium channel ENaC and the $Cl^-/HCO_3^-$ exchanger pendrin are expressed on the apical membrane of principal cells and B-intercalated cells, respectively, in the connecting tubule and the cortical collecting duct, and mediate salt reabsorption [3-7].

Genetic deletion of NCC does not cause significant salt wasting or volume depletion in NCC KO mice under baseline condition [8]. Both ENaC and pendrin are upregulated in kidneys of NCC KO mice and presumed to play critical roles in salt reabsorption in the setting of NCC deletion [9]. Indeed, inactivation of pendrin in the setting of NCC deletion caused massive diuresis in NCC/pendrin double KO mice [10]. Similarly, inhibition of ENaC with amiloride increased salt excretion in NCC KO mice [9].

While these studies strongly suggest that pendrin, working in tandem with ENaC plays a major role in blunting the diuretic effect of HCTZ, direct evidence supporting the role of pendrin in compensatory salt absorption in response to HCTZ remains lacking. A report showing a profound sensitivity to chlorthalidone, a thiazide derivative, was published in 2008 in a patient who showed massive volume depletion and hypokalemic alkalosis after receiving chlorthalidone for inner ear maladies [11]. The patient was diagnosed with Pendred Syndrome, an autosomal recessive disorder caused by inactivating mutation of pendrin and manifested with deafness and goiter [12].

To directly examine the role of pendrin in compensatory salt absorption following HCTZ treatment, we used pendrin KO mice that were treated with daily injection of HCTZ. Metabolic cage studies were performed to estimate the magnitude of salt excretion in experimental animals. In parallel studies, systemic blood pressure was measured in WT and pendrin KO mice. The expression of ENaC and pendrin in WT mice and AQP-2 in Pendrin KO mice was measured at baseline and following HCTZ treatment.

## Materials and Methods

### Animal Husbandry

Both male and female pendrin KO, NCC KO and wild type mice, on C57BL/6 background, were used for these studies. All animals were housed and cared for in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati. All animal handlers were IACUC-trained. Animals had access to food and water ad libitum, were housed in humidity, temperature, and light/dark controlled rooms, and were inspected daily. Animals were euthanized with the use of excess anesthetics (pentobarbital sodium) according to institutional guidelines and approved protocols. NCC KO and pendrin KO mice were genotyped as described in previous work [10].

### Metabolic cages studies

Mice (3-4 months of age) were placed in metabolic cages for two days for acclimation before the experiments. Cages were cleaned, and the consumption of water and food, as well as production of urine (collected under mineral oil to avoid volume loss due to evaporation) were monitored and collected daily during the course of the study. Each study consisted of measurements over 5 days of baseline (untreated) followed by 3 consecutive days of subcutaneous (s.c.) HCTZ (Sigma-Aldrich, St Louis, USA) at 40 mg/kg per day dissolved in propylene glycol/ethyl alcohol in a 4:1 ratio. This dose was chosen based on published reports, which have used HCTZ concentrations that are either similar or higher than the dose in our studies [13, 14]. Two recent reports have used HCTZ at 50 mg/kg [14, 15].

### Blood pressure measurement

Mice were restrained in special holding tube, and two cuffs placed on their tails and placed on the warming pad until their tails temperature reached 30-32 °C. Systolic blood pressure was measured and recorded using CODA Non-Invasive Blood Pressure software (Kent Scientific Corporation, CT, USA). At least 5 consecutive readings were recorded for each animal.

### RNA extraction and northern blot analysis

Northern blot was used to assist the expression of pendrin and renin mRNA and preformed as previously described [10]. Briefly, total cellular RNA was extracted from kidneys, quantitated spectrophotometrically, and stored at −80 °C. Total RNA samples (30 μg/lane) were fractionated on a 1.2% (g/dl) agarose-formaldehyde gel, transferred to Magna NT nylon membranes, cross-linked by UV light, and baked. Hybridization was performed as described [10]. The membranes were washed, blotted dry, and exposed to a Phosphor Imager screen (Molecular Dynamics). PCR-amplified mouse and rat cDNA fragments were used as specific probes for renin, pendrin and sodium channel gamma subunits respectively. The cDNA fragments encoding nucleotides 291–600 for renin (GenBank accession no. NM_031192), nucleotides 819 to 1509 for pendrin (Genebank # NM_011867) and nucleotides 135–790 for γ subunit of sodium channel were used for labeling.

### Protein extraction and western blot analysis

Plasma membrane proteins were prepared from kidney tissues as described before [16]. The protein contents of kidney tissues were determined by BCA assay (Thermo Scientific, Rockford, IL). For western blot analysis, 30-50μg of each sample was size fractionated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and subjected to western blot analysis as previously described, using antibodies against renin (MyBioSource), AQP2 (Santa Cruz Biotechnology), $^{Ser256}$p-AQP2 (Assay Biotech) and β-actin (Santa Cruz Biotechnology). Appropriate secondary antibodies conjugated to horseradish peroxidase (Thermo Scientific, Rockford, IL) were used. The bands were visualized by chemiluminescence method (Invitrogen, Carlsbad, CA) and captured on light-sensitive imaging film (Denville Scientific Inc, Metuchen, NJ). The bands densities were determined using ImageJ software.

### Immunofluorescence labeling studies

Animals were euthanized with an overdose of pentobarbital sodium and perfused through the left ventricle with 0.9% saline followed by cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Kidneys were removed, cut in tissue blocks, and fixed in formaldehyde solution overnight at 4°C. The tissues were fixed in paraffin, and 5-βm sections were cut and stored until used. Double-immunofluorescence labeling with pendrin and AQP2 antibodies was performed as described [16].

### Urine electrolyte and urine osmolality measurements

Urine electrolyte (sodium and chloride) levels were measured using a urine electrolyte analyzer (EasyLyte Urine Analyzer). Urine osmolality was measured by The Advanced Instruments Model 3300 Micro-Osmometer (Advanced Instruments, Norwood, MA), as before [10].

### Statistical analysis

Results are presented as means ± SEM. Statistical significance ($P<0.05$) between samples was determined by Student's paired t-test for metabolic cage studies before and after HCTZ treatment. Blood pressure data were tested by one-way ANOVA repeated measurements.

## Results

### The role of pendrin in HCTZ diuresis.

To test the role of pendrin in response to HCTZ, metabolic cage studies were performed using WT, pendrin KO and NCC KO mice. Urine volume and urine osmolality remained comparable in WT and NCC KO mice before and after HCTZ treatment, as displayed in Fig. 1A-B. However, pendrin KO mice exhibited significant diuresis in response to HCTZ, as shown by increased urine output and reduction in urine osmolality.

To test whether the observed increased urine production is associated with increased salt wasting, urine Na+ and Cl− excretion rates were determined in mice treated with HCTZ. Fig. 1 C and D demonstrates that HCTZ increased salt excretion only in pendrin KO mice but not in NCC KO or WT mice. The enhanced urine output was associated with increased water intake in pendrin KO mice (Fig. 1E). The food intake was reduced in WT and pendrin KO mice after HCTZ injection (Fig. 1F). These results indicate that the inhibition of NCC increases salt excretion in the presence of pendrin inactivation and/or down regulation and reveal the role of pendrin in blunting the diuretic response to HCTZ. Systemic acid base analysis and serum potassium concentration on blood harvested via cardiac puncture in WT and pendrin KO mice following HCTZ showed comparable values. The results were as follows: pH 7.38±0.03 in WT *vs.* 7.38±0.06 in pendrin KO; $pCO_2$ 36.3± 4.4 mmHg in WT *vs.* 43.2±5 in pendrin KO, and $HCO_3^-$ of 24.05±3.5 mEq/l in WT *vs.* 24±1.9 in pendrin KO mice. Serum $K^+$ concentration was 6.4±0.1 mEq/l in WT vs. 6.43 ± 0.6 in pendrin KO mice. Acid base parameters in WT and pendrin KO mice under baseline conditions have been reported and show either no difference or a mild alkalosis in pendrin KO mice [6, 22].

### Activation of renin angiotensin system in response to HCTZ.

To test if mice lacking pendrin have developed volume depletion and thus activated the renin/ angiotensin system in response to HCTZ, we examined the expression of renin in kidney tissues of WT and pendrin KO mice. Fig. 2A demonstrates enhanced mRNA expression of renin in kidneys of pendrin KO, but not WT mice treated with HCTZ. Western blot analysis indicated increased abundance of renin protein in kidneys of pendrin KO mice treated with HCTZ (Fig. 2B). These results suggest the development of volume depletion subsequent to increased salt excretion in response HCTZ in pendrin KO mice.

The reduction in urine osmolality and an increase in urine output in HCTZ-treated pendrin KO mice (Fig. 1) may point to defective water salvage mechanisms in the collecting duct and suggest compromised AQP2 expression and/or function. In the next series of experiments we examined the expression of AQP2 in kidneys of pendrin KO mice at baseline and following HCTZ treatment. Accordingly, the abundance of total AQP2 and the vasopressin-regulated, plasma membrane-targeted $^{Ser256}$p-AQP2 was assessed. Western blot analysis of kidney samples revealed that the expression levels of $^{Ser256}$p-AQP2, when normalized for B-actin, were significantly reduced in pendrin KO mice following treatment with HCTZ (Fig. 2 E and F). The abundance of total AQP-2 was mildly reduced (Fig. 2E and F). These findings are consistent with the impaired urine concentrating ability in the setting of vascular volume depletion in pendrin KO mice.

### Pendrin and ENaC expression in response to HCTZ

Pendrin and ENaC work in tandem to absorb salt (sodium and chloride), and both are upregulated in the setting of NCC inactivation in order to minimize the magnitude of salt wasting [9]. In the next series of experiments we examined the expression levels of pendrin and ENaC-γ subunit in kidneys of WT mice treated with HCTZ. Northern hybridizations showed that expression levels for ENaC-γ subunit and pendrin were increased significantly in response to HCTZ treatment in WT mice (Fig. 3 A-C). To better illustrate the effect of HCTZ on pendrin regulation, double immunofluorescence labeling with AQP-2 and pendrin antibodies was performed in kidneys of mice treated with HCTZ and copmpared to control. As indicated in Fig. 3D (low and high magnifications), immunofluorescence labeling studies corroborated the upregulation of pendrin in kidneys of HCTZ-treated mice (lower panels in Fig. 3D) by indicating increased number of pendrin positive cells as well as enhanced apical rim labeling pattern compared to subapical and/or cytoplasmic labeling in control animals. Taken together, these results indicate that pendrin and ENaC are activated and blunt the diuretic effects of HCTZ in WT mice.

The effect of HCTZ on systemic blood pressure in WT, NCC KO and pendrin KO mice.

In the next series of experiments we examined whether the significant diuresis in pendrin KO mice is associated with changes in systemic blood pressure. Therefore, we measured the systolic blood pressure of WT, NCC KO and pendrin KO mice at baseline and in response to HCTZ using a computerized tail cuff method (Fig. 4 A and B). As indicated, blood pressure recordings in WT, NCC KO, or pendrin KO mice did not change at 24 hrs (Fig. 4A) and 48 hrs (Fig. 4B) after HCTZ administration vs. baseline recordings.

## Discussion

The thiazide-sensitive NaCl cotransporter NCC (SLC12A3) and the Cl−/HCO3- exchanger pendrin (SLC26A4) are expressed on apical membranes of distal cortical nephron segments and mediate salt absorption, with pendrin working in tandem with the epithelial Na channel and NCC working by itself [9, 10, 17]. Pendrin is expressed on the apical membrane of non-A intercalated cells in the connecting tubule (CNT) and the cortical collecting duct (CCD) [5, 18, 19]. The thiazide-sensitive NaCl cotransporter NCC is primarily expressed on the apical membrane of distal convoluted tubule (DCT) cells [20, 21]. Single deletion of pendrin or NCC does not cause salt wasting or excessive diuresis under basal conditions [4, 6, 8, 22]. Indeed, even mild degree of salt wasting has not been found in these two genetically engineered mouse models at steady state [6, 8]. Kidney functions, including sodium and chloride excretion, urine output, and blood urea nitrogen (BUN) levels in mutant mice are comparable to wild type animals [6, 8, 22].

Both pendrin KO and NCC KO mice, however, show signs of volume depletion or develop hypotension during salt restriction [8, 22]. These findings have led investigators to conclude that pendrin and NCC are predominantly active during salt depletion (or in response to aldosterone excess), and their contribution to salt reabsorption at baseline conditions is small. However, recent published studies suggest that pendrin plays an important role in the maintenance of vascular volume, specifically in the setting of NCC inhibition or inactivation. Mice with double knockout of NCC and pendrin exhibited profound polyuria (increased urine output) and polydipsia (increased water intake) [10]. There was no polyuria or polydipsia in single NCC or pendrin KO or WT mice [10]. Pendrin/NCC KO mice showed sharp increases in sodium and chloride excretion and developed severe volume depletion as demonstrated by renin/angiotensin system activation, kidney hypoperfusion and hypotension [10].

In the present studies the role of pendrin in compensatory salt absorption following NCC inhibition was tested in pendrin deficient mice. By comparing the magnitude of HCTZ-induced diuresis in WT and pendrin KO mice, we wanted to directly assess the contribution of pendrin to salt reabsorption consequent to NCC inhibition. Our results demonstrated that HCTZ caused a significant diuresis in pendrin KO, but not WT mice. The magnitude of urine output as well as salt excretion was significantly pronounced in pendrin KO mice compared to WT mice in response to HCTZ. While salt wasting in pendrin KO mice treated with HCTZ mirrors the salt wasting phenotype in pendrin/NCC dKO mice, Pendrin KO mice did not develop any noticeable reduction in their systemic blood pressure following HCTZ, whereas pendrin/NCC dKO mice exhibited significant hypotension. It should be noted that pendrin/NCC dKO mice exhibited severe salt wasting and volume depletion over a long period before their blood pressure was recorded, whereas pendrin KO mice were examined less than 3 days after HCTZ treatment. As a result, the magnitude of volume depletion was milder in pendrin KO mice treated with HCTZ compared to pendrin/NCC dKO mice, as judged by a more profound upregulation of renin in kidneys of pendrin/NCC dKO mice [10] vs. current studies (Fig. 2). The milder systemic vascular volume depletion in pendrin KO mice treated with HCTZ is reflected in the absence of a significant drop in their systemic blood pressure. Our results further indicated enhanced expression of ENaC

and pendrin in WT mice treated with HCTZ, suggesting that these two transport proteins play critical roles in blunting the diuretic effect of HCTZ. Our current results also mimic the impaired urine concentrating ability in the setting of salt wasting and vascular volume depletion in pendrin/NCC dKO mice [10].

A recent study showed that treatment with chlorthalidone, a thiazide derivative, resulted in severe volume depletion, profound metabolic alkalosis and decreased kidney function in a patient with Pendred syndrome [11]. There were no documents verifying the presence of severe salt wasting [11]. However, given the presence of new onset hypotension, kidney hypoperfusion, metabolic alkalosis and hypokalemia, it was reasonable to conclude that the signs and symptoms of volume depletion were consequent to thiazide derivative treatment [11]. An extremely unusual picture for thiazides, this presentation [11] mimics the picture in pendrin/NCC double KO mice [10], with respect to salt wasting and hypotension, and supports the conclusion that pendrin blunts the diuretic effect of NCC inhibition consequent to thiazides by compensatory absorption of salt in the distal nephron.

In detailed studies examining the effect of HCTZ on healthy hypertensive volunteers, investigators identified 2 groups of individuals, based on their blood pressure response and the magnitude of salt excretion following HCTZ administration, with one group significantly increasing their diuresis along with more volume depletion and greater activation of renin/angiotensin system whereas the other group did not exhibit significant salt wasting [24]. Interestingly, the group that exhibited more salt excretion by HCTZ did not display a significant reduction in their blood pressure, apparently due to the activation of renin angiotensin system [24]. Although enhanced thiazides-induced diuresis may have several explanations, one possibility could be decreased pendrin activity. While purely speculative, it is interesting to entertain the possibility that a number of SNPs in the coding or non-coding regions of SLC26A4 gene may be associated with altered pendrin activity in individuals with normal hearing. The presence of allelic variants in SLC26A4 gene in individuals with normal or impaired hearing has been well documented [25, 26,27], and specifically points to several SNPs in a group of individuals with normal hearing. Future studies examining the gene-function relationship of allelic variants in SLC26A4 in individuals with enhanced or reduced thiazide-induced diuresis should answer the association of pendrin and thiazides sensitivity.

A recent publication suggested that pendrin can work in tandem with the sodium-dependent $Cl^-/HCO_3^-$ exchanger (NDCBE; also referred to as Slc4a8 in Genebank) in B intercalated cells to absorb sodium [14]. Such a coordinated mechanism would be electroneutral and could provide an ENaC-independent salt reabsorption pathway in the collecting duct [14, 28]. Whereas the pendrin-dependent, ENaC-mediated salt absorption is associated with a net $K^+$ secretion, the NDCBE-mediated salt absorption was presumed to be working independent of $K^+$ transport [14, 28]. Immunolocalization studies showing the expression of NDCBE on the apical membrane of B-intercalated cells or *in situ* hybridizations and/or nephron segment RT-PCR demonstrating the presence of NDCBE transcripts in cortical collecting duct are lacking. Further, there are no data available demonstrating the impairment of pendrin function in mice lacking NDCBE.

## Conclusion

We conclude that pendrin, working in tandem with ENaC, blunts hydrochlorothiazide-induced diuresis, suggesting that the diuretic effect of thiazide derivatives is largely determined by the functional state of the $Cl^-/HCO_3^-$ exchanger pendrin and ENaC.

We suggest that inhibitors of pendrin will enhance the diuretic effect of thiazide derivatives. A recent study reported the identification of a pendrin specific inhibitor which enhanced the diuresis caused by furosemide in mice [29]. We suggest that the combination of thiazide derivatives and pendrin inhibitors can be used as novel diuretic regimens for the treatment of fluid overloaded states, such as congestive heart failure. The schematic diagram in Fig. 5 summarizes the role of pendrin in the setting of NCC inhibition.

## Acknowledgements

This work was supported by Merit Review 5 I01 BX001000-06 award from the Department of Veterans Affairs, and by funds from the Center on Genetics of Transport and Epithelial Biology at the University of Cincinnati (to M. S). Saeed Alshahrani was supported by a Predoctoral scholarship from Jazan University, Saudi Arabia. The authors greatly acknowledge the contributions of Sharon Barone and Kamyar Zahedi to these studies.

## Abbreviations

| | |
|---|---|
| **NCC** | Na-Cl cotransporter |
| **HCTZ** | hydrochlorothiazide |
| **ENaC** | epithelial sodium channel |
| **SNPs** | single nucleotide polymorphisms |

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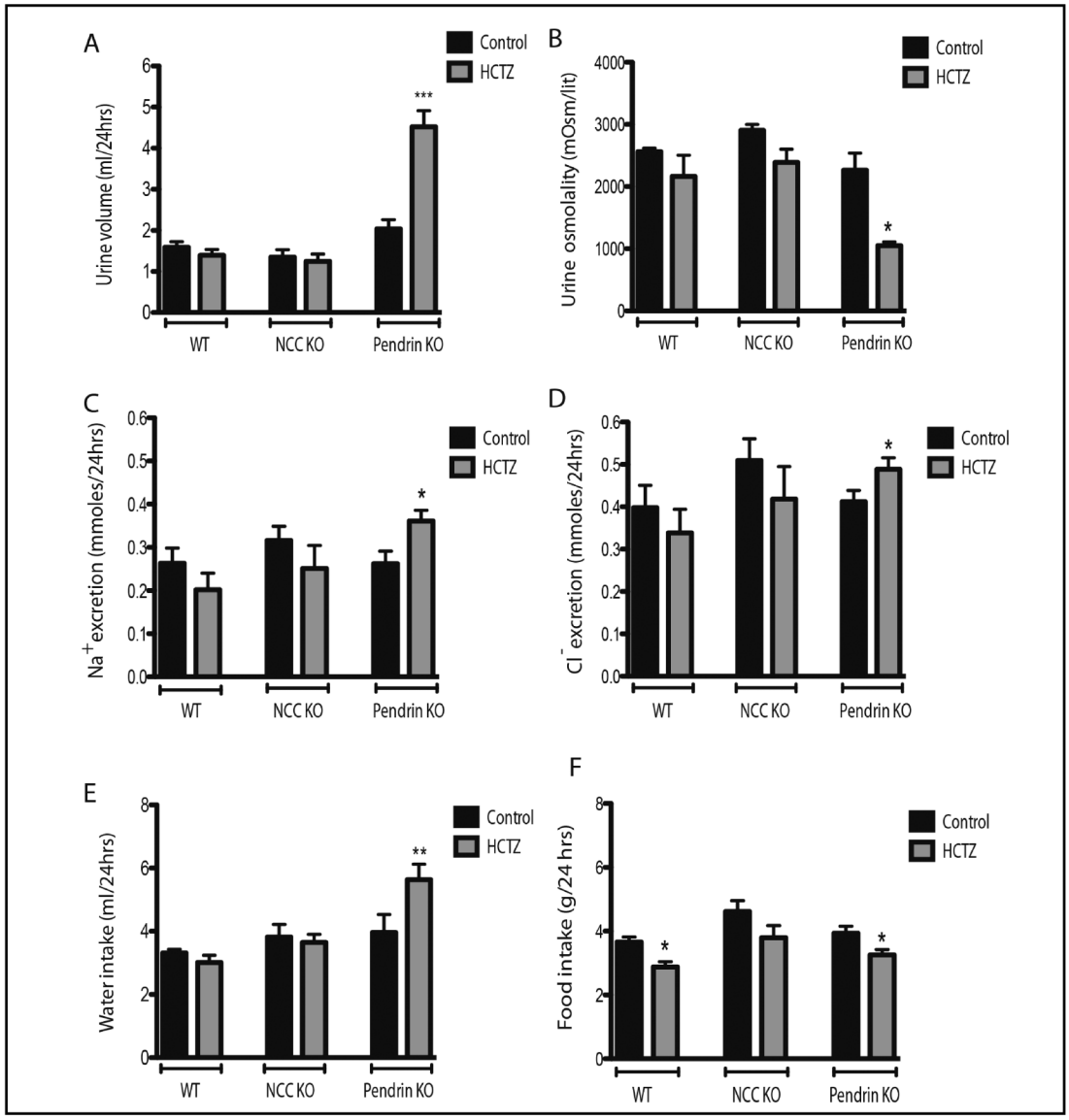


**Fig. 1.**

Comparison of the effects of HCTZ on urine volume and salt excretion in WT, NCC KO and pendrin KO mice. (A) Comparison of the averages of 24 hour urine volume at baseline and after 3 days of HCTZ treatment in WT, NCC KO and pendrin KO mice indicates that while there were no changes in the urine output of WT and NCC KO mice, the pendrin KO mice urine output was significantly increased. (B) Comparison of urine osmolality over a 24 hour period in WT, NCC KO and pendrin KO mice at baseline and in response to HCTZ treatment. Pendrin KO mice showed significant reduction in urine osmolality. (C and D) Comparison of the salt excretion (sodium and chloride) in WT, NCC KO and PDS KO after HCTZ treatment indicates increase in salt excretion only in pendrin KO mice. (E and F) Water and food intake studies before and after HCTZ show increase in water intake in pendrin KO but not in NCC and WT mice. (n=5/group); * P<0.05; ** P<0.01; *** P<0.001 for Baseline vs. HCTZ treatment.

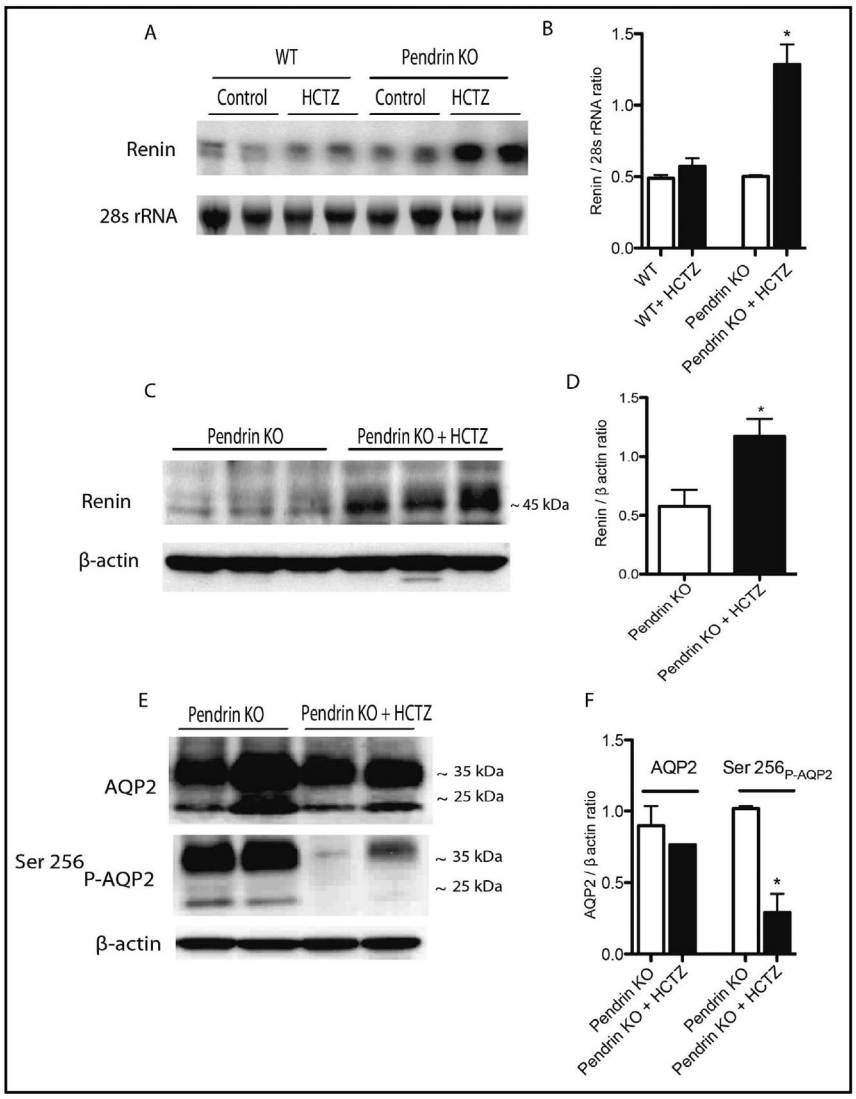


**Fig. 2.**

Kidney expression of renin and AQP-2 in response to HCTZ. (A and B) Northern blot analysis of renin mRNA expression in WT and pendrin KO mice kidney after HCTZ treatment indicate significant increase of renin expression in pendrin KO post-treatment of HCTZ compared to WT mice. (C and D) Western blot analysis of renin expression in pendrin KO mice before and after HCTZ indicates an increase in renin expression following HCTZ treatment (n=3/group); * $P<0.05$. Western blot analysis of total AQP2 and $^{Ser256}$p-AQP2 in kidneys of experimental animals indicates a significant reduction in $^{Ser256}$P-AQP2 in pendrin KO mice treated with HCTZ vs. untreated pendrin KO mice (E and F).

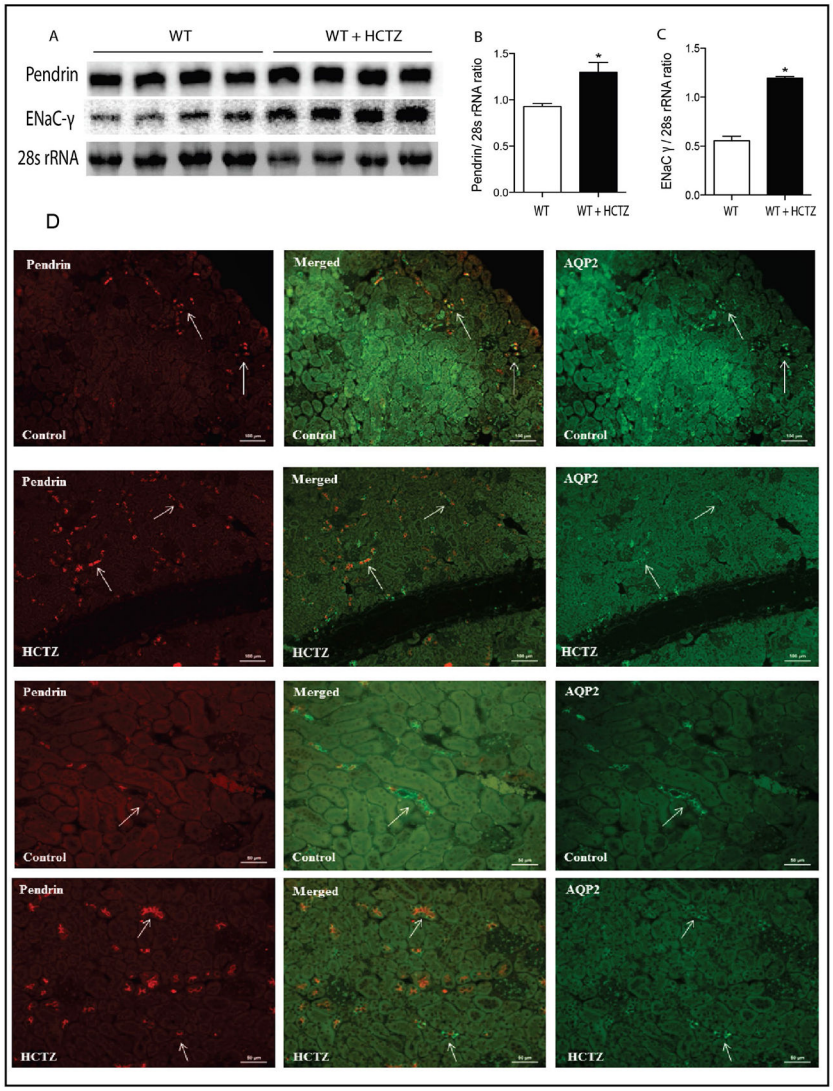


**Fig. 3.**

Kidney expression of Pendrin and ENaC in response to HCTZ in WT mice. (A) Northern blot analysis of pendrin and ENaC-γ subunit in kidneys of WT mice at baseline and following HCTZ treatment for 3 days. The results indicate a significant increase in the expression of both pendrin and ENaC-γ subunit (B and C) (n=4/group); * P<0.05. (D) Double immunofluorescence labeling of pendrin and AQP2 shows significant increase in the number of pendrin positive cells in kidneys of HCTZ treated WT mice vs. control WT mice (low and high magnification images are shown in top and bottom panels, respectively).

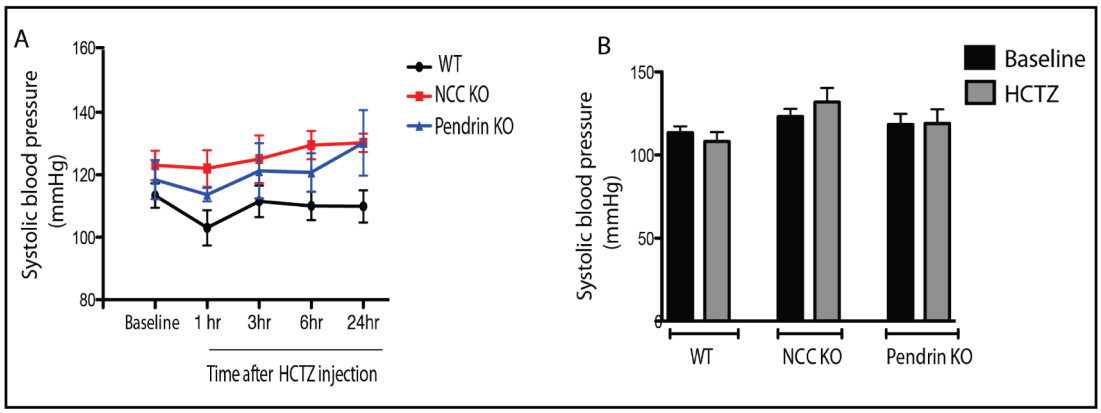


**Fig. 4.**

Blood pressure measurements after HCTZ treatment in WT, NCC KO, and pendrin KO mice. (A) Recordings of systolic blood pressure at 1, 3, 6, and 24 hours after HCTZ treatment in WT, NCC KO, pendrin KO mice (n=4/group). As shown, there is no significant change in systolic blood pressure among all three groups after treatment. (B) Systolic blood pressure measurements at 48 hours of HCTZ treatments demonstrate there is no change in blood pressure of all groups.

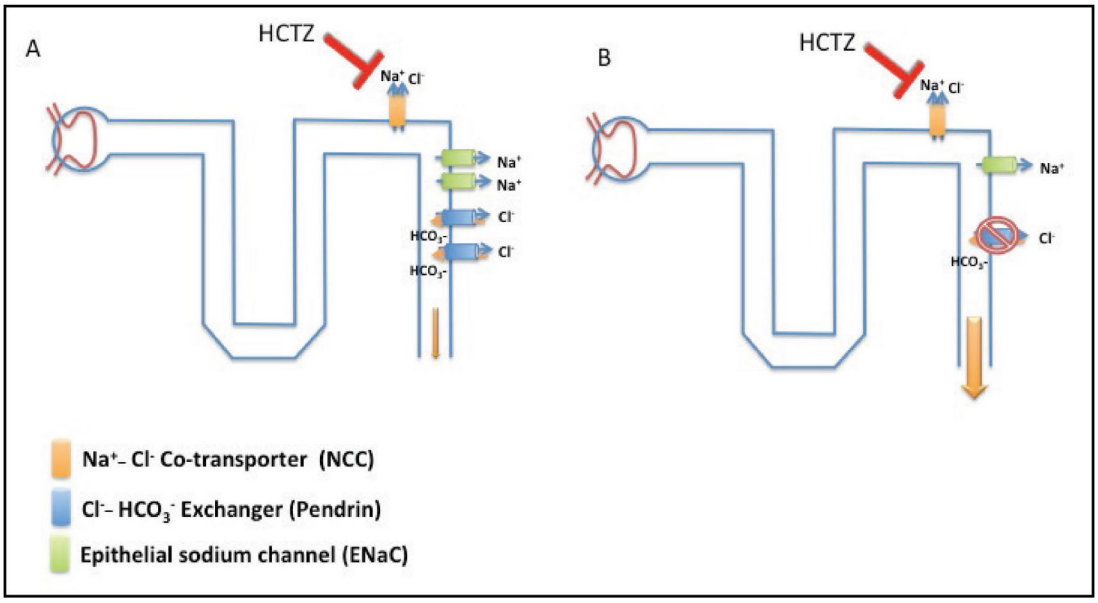


**Fig. 5.**

Schematic diagram depicting the synergistic effects of NCC and pendrin inhibition on salt excretion in the kidney. A (left panel). The inhibition of NCC by HCTZ activates the expression and activity of pendrin and ENaC, which blunt the diuretic effect of HCTZ. B (right panel). In the setting of pendrin downregulation or inactivation, the inhibition of NCC results in significant salt wasting due to the absence of pathways that can salvage salt reabsorption in the collecting duct.