Hypertrophy in the Distal Convoluted Tubule of an 11β-Hydroxysteroid Dehydrogenase Type 2 Knockout Model

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ABSTRACT

Na⁺ transport in the renal distal convoluted tubule (DCT) by the thiazide-sensitive NaCl cotransporter (NCC) is a major determinant of total body Na⁺ and BP. NCC-mediated transport is stimulated by aldosterone, the dominant regulator of chronic Na⁺ homeostasis, but the mechanism is controversial. Transport may also be affected by epithelial remodeling, which occurs in the DCT in response to chronic perturbations in electrolyte homeostasis. Hsd11b2⁻⁄⁻ mice, which lack the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) and thus exhibit the syndrome of apparent mineralocorticoid excess, provided an ideal model in which to investigate the potential for DCT hypertrophy to contribute to Na⁺ retention in a hypertensive condition. The DCTs of Hsd11b2⁻⁄⁻ mice exhibited hypertrophy and hyperplasia and the kidneys expressed higher levels of total and phosphorylated NCC compared with those of wild-type mice. However, the striking structural and molecular phenotypes were not associated with an increase in the natriuretic effect of thiazide. In wild-type mice, Hsd11b2 mRNA was detected in some tubule segments expressing Slc12a3, but 11βHSD2 and NCC did not colocalize at the protein level. Thus, the phosphorylation status of NCC may not necessarily equate to its activity in vivo, and the structural remodeling of the DCT in the knockout mouse may not be a direct consequence of aberrant corticosteroid signaling in DCT cells. These observations suggest that the conventional concept of mineralocorticoid signaling in the DCT should be revised to recognize the complexity of NCC regulation by corticosteroids.

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Na⁺ reabsorption in the renal distal convoluted tubule (DCT) determines total body Na⁺ and hence body fluid volume and BP. Dysfunction of the NaCl cotransporter (NCC) in the DCT perturbs BP not only in the extreme phenotypes of patients with Gitelman’s and Gordon’s syndromes but also in healthy individuals, and the inhibition of NCC by thiazide diuretics has a potent antihypertensive effect. In the short term, NCC activity is regulated at the molecular level; neurohormonal inputs converge on intracellular signaling networks (WK-SPA/OSR15 and SGK1/Nedd4-2 pathways) that shuttle NCC to and from the plasma membrane and induce post-translational protein modifications that modify transport function (phosphorylation and ubiquitylation). Sustained physiologic perturbations promote structural remodeling of DCT epithelium. In rodents, the DCT becomes hypertrophied and hyperplastic in response to Na⁺ loading, loop diuretic therapy, and genetic ablation of ROMK, as well as in models of Gordon’s syndrome. Conversely, DCT atrophy has been observed in rats treated with thiazide diuretics.

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diuretics and in mice after the overexpression of wild-type WNK4 or after the genetic ablation of NCC or SPAK. In some cases, structural remodeling correlates with changes in Na+ transport function: DCT hypertrophy is associated with increased Na+ transport capacity in micropерfused distal tubules in rats exposed to chronic furosemide and with enhanced thiazide-sensitive Na+ excretion in a mouse model of Bartter’s syndrome. The cause of DCT remodeling is not fully understood; it has been hypothesized that increased luminal Na+ delivery stimulates a hypertrophic response dependent on proportional changes in [Na+]i. In the presence of a tonic stimulus to NaCl reabsorption in the DCT, such a mechanism could result in a positive-feedback loop whereby excessive NaCl reabsorption begets epithelial hypertrophy and yet more NaCl reabsorption.

We hypothesized that such a phenomenon contributes to the pathogenesis of hypertension in salt-retaining states. Such positive feedback would ordinarily be suppressed by an intact renin-angiotensin-aldosterone system exerting negative feedback control of net urinary Na+ excretion. However, our work on a mouse model of apparent mineralocorticoid excess (AME) provided an opportunity to study the consequences of epithelial remodeling when this negative feedback loop is interrupted. AME comprises low-renin hypertension, Na+ retention, hypokalemic alkalosis, and polyuria in rare human kindreds carrying homozygous null mutations in the gene encoding 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2). This enzyme is expressed in mineralocorticoid target tissue, where it inactivates glucocorticoids, thus preserving specificity at the mineralocorticoid receptor (MR). Hypertension in AME is predominantly renal in origin and can be reversed by kidney transplantation. It is widely presumed that the Na+ retention results from enhanced epithelial sodium channel (ENaC) activity in the connecting tubule (CNT) and collecting ducts, but several observations suggest a contribution from NCC activation. First, the ENaC inhibitor amiloride does not completely ameliorate the hypertension in AME and is often used at high doses that may exert off-target effects. Second, thiazide diuretics can normalize BP in some patients with AME. Third, a gene variant associated with low 11βHSD2 activity was associated with increased thiazide sensitivity in BP in a hypertensive Sardinian population. Finally, a mouse model of AME (Hsd11b2−/−) exhibits hypertrophy in a tubular segment, tentatively identified using positional and morphologic criteria as the DCT.

NCC activity is regulated by several neurohormonal systems, including the sympathetic nerves, angiotensin II, glucocorticoids, sex steroids, vasopressin, and insulin. There is controversy regarding the influence of mineralocorticoids, the dominant regulators of chronic Na+ balance. Aldosterone can activate the NCC; therefore, the DCT is classically considered to form part of the aldosterone-sensitive distal nephron (ASDN). However, it is not known whether this effect is a direct consequence of MR signaling in DCT cells or a secondary phenomenon resulting from changes in electrolyte transport in other tubular segments.

Here we show that Hsd11b2−/− mice exhibit hypertrophy and hyperplasia in the DCT and express phosphorylated NCC (pNCC) in greater abundance. In wild-type mice, the DCT does not express 11βHSD2 protein. We therefore conclude that the structural and function changes in the DCT of Hsd11b2−/− mice were the indirect consequence of activated mineralocorticoid signaling pathways in other nephron segments.

RESULTS

Hypertrophy and Hyperplasia of the DCT in Hsd11b2−/− Mice

The structure of the distal renal tubule in Hsd11b2−/− and wild-type mice was evaluated by immunofluorescence. The thick ascending loop of Henle (TALH) and DCT were recognized by apical immunoreactivity to NKCC2 and NCC antisera, respectively. AQP2 antisera labeled a pool of cortical tubules comprising CNT and cortical collecting duct (CCD). In Hsd11b2−/− mice, DCT cross-sections were larger in diameter and circumference and contained more cell nuclei than in the wild-type mice (Figure 1). The DCT epithelium was thicker, reflecting increased cell height and nuclear crowding (Figure 1). Epithelial hyperplasia was most striking in those tubular cross-sections that were found in close proximity to glomeruli, suggesting that the remodeling was more pronounced in the proximal portion of the DCT. Hsd11b2−/− kidneys had fewer tubular cross-sections per unit area that expressed

Figure 1. DCTs labeled with anti-NCC. Tiled fluorescence micrographs showing DCTs in wild-type (A) and Hsd11b2−/− (B) kidney cortex. Each image is taken from a different animal (55–65 days of age). Bar, 50 μm.
NKCC2 and AQP2 than in the wild-type mice, but this was not the case for the DCT. Similarly, Hsd11b2<sup>−/−</sup> kidneys had fewer tubular cells per unit area expressing NKCC2 and AQP2 than in the wild types but a greater density of tubular cells expressing NCC (Figure 2, Supplemental Tables 1 and 2). Although these data were not obtained using formal morphometric methodology, they are nevertheless consistent with the relative expansion of the DCT within the distal nephron.

**Accelerated Epithelial Proliferation in the DCT**

A continuous infusion of bromodeoxyuridine (BrdU) was used to label the nuclei of cells that had divided during a 7-day period. Immunostaining for NKCC2, NCC, or AQP2 permitted the calculation of the “BrdU index” (the proportion of cell nuclei that had incorporated BrdU) within each segment of the distal nephron (Figure 3). The BrdU index was higher in the DCT of Hsd11b2<sup>−/−</sup> kidneys than in the wild types at 60 days of age (Figure 3), suggesting that progressive DCT hyperplasia results from accelerated epithelial proliferation that continues throughout early adulthood. The BrdU index was also elevated in AQP2-expressing cortical tubules but was suppressed in the TALH of Hsd11b2<sup>−/−</sup> mice, demonstrating that the proliferative phenotype extended into the CNT and/or collecting duct but did not reflect a global change in cell proliferation in the renal tubule (Figure 3). We therefore concentrated our studies on mice at 60 days of age, reasoning that any process contributing to DCT hypertrophy remained active at this age and that previous work had confirmed that hypertension in our model is largely driven by renal Na<sup>+</sup> retention at this age.31

**Increased Total and Phosphorylated NCC in the Plasma Membrane**

Immunoblotting of whole-kidney homogenates was used to estimate the abundance of NCC and of the specific phosphorylated forms of NCC that are associated with NaCl transport activity. Total NCC and the phosphorylated forms pT53, pT58, and pS71 were more abundant in Hsd11b2<sup>−/−</sup> kidneys than in the wild types (Figure 4A). Differential centrifugation was used to prepare protein fractions enriched for plasma membranes (P1) or subapical membrane vesicles (P2). Total NCC was more abundant in both fractions prepared from Hsd11b2<sup>−/−</sup> kidneys than in the wild types (Figure 4B).

Our antibody to total NCC raised bands of three distinct molecular masses; the lowest of these (at approximately 110 kD) was not present in plasma membranes but was enriched in subapical membrane vesicles, nor was it recognized by antibodies to pNCC (Figure 4, Supplemental Figures 1 and 2). These findings suggest that the lower band may represent a form of NCC (either immature or partially degraded) that does not participate in active transport. However, given the uncertainty regarding the
molecular identity of each band, all three were evaluated in the densitometry analyses.

**Molecular Adaptation in the Distal Renal Tubule of Hsd11b2<sup>2/2</sup> Mice**

To assess global changes in the molecular phenotype of the renal tubules in Hsd11b2<sup>2/2</sup> mice, we used quantitative real-time PCR (Q-PCR) to estimate the abundance of mRNA transcripts known to be expressed in defined tubular segments. Transcripts expressed in the distal tubule were present in greater abundance in Hsd11b2<sup>2/2</sup> kidneys than in the wild types (Table 1). Transcripts expressed in the DCT (NCC, parvalbumin, L-SPAK, and KS-WNK1<sup>36,37</sup>) were more abundant, as were those expressed predominantly in the CNT or CCD (ENaC, NCX1, Trpv5, and calbindin-D28k), indicating that a molecular adaptation to increased solute transport extended into these segments.

**Mechanisms Causing DCT Remodeling**

To explore the mechanisms driving DCT hypertrophy in Hsd11b2<sup>2/2</sup> kidneys, we tested the hypothesis that the structural phenotype was a direct consequence of the lack of 11βHSD2 in DCT cells. We used double-label immunofluorescence to determine the sites of 11βHSD2 expression in male C57BL6/J kidneys (used as the wild-type controls for the present experiments) relative to the following markers of known tubular segments: NKCC2, NCC, AQP2, parvalbumin, and calbindin-D28k (Figure 5, Supplemental Figure 3). The fluorescence signals associated with NCC and 11βHSD2 did not colocalize; in some sections, it was possible to observe the discontinuation of NCC expression and the start of 11βHSD2 expression in a continuous stretch of tubule (Figure 5). In microdissected tubular segments, Hsd11b2 mRNA could be detected by PCR in the CCD, but was not consistently detected in the DCT (Figure 6). Our results support a model in which 11βHSD2 is expressed in the CNT and CCD, but not the DCT (Figure 7).

Because DCT hypertrophy in Hsd11b2<sup>2/2</sup> mice did not result directly from the loss of 11βHSD2 from DCT cells, we sought a mechanism whereby changes in a different nephron segment might influence DCT structure. Epithelial proliferation was suppressed within the TALH of Hsd11b2<sup>2/2</sup> mice, resulting in relative atrophy of this segment. Accordingly, Hsd11b2<sup>2/2</sup> mice exhibited a progressive reduction in the abundance of NKCC2 as they aged (Figure 8), suggesting that diminished Na<sup>+</sup> reabsorption in the TALH provides greater delivery of Na<sup>+</sup> to the DCT: a potent stimulus to DCT hypertrophy/hyperplasia<sup>9,12,21</sup>. However, changes in NKCC2 expression were not sufficient to account for the DCT phenotype; the expression of NKCC2 in Hsd11b2<sup>2/2</sup> mice at 30 days of age was no different from the wild-type mice, whereas NCC was more abundant.

**Thiazide-Sensitive Na<sup>+</sup> Transport in Hsd11b2<sup>2/2</sup> Mice**

We hypothesized that as a consequence of DCT hypertrophy and increased NCC expression, a greater proportion of filtered sodium would be reabsorbed through thiazide-sensitive pathways in Hsd11b2<sup>2/2</sup> mice. We therefore measured the acute...
Figure 4. Total and phosphorylated forms of NCC in wild-type and Hsd11b2−/− kidneys. Immunoblots of whole-kidney homogenates from wild-type (Hsd11b2, +) or knockout (−) mice culled at 55–65 days of age (n=5). (A) Total cellular protein (fraction S0; 12 µg loaded per lane). Similar results are obtained in mice aged 30, 60, and 120–150 days (Supplemental Figure 1). (B) Protein fractions are enriched by differential centrifugation for plasma membranes (P1, 6 µg per lane) or subapical membrane vesicles (P2, 30 µg per lane). Size markers are 117 kD (blots) and 55 kD (Coomassie gels). Signal densities are in arbitrary units (AU) relative to the wild-type group (mean and 95% confidence interval). *P<0.05 (genotypes compared by the unpaired t-test). The specificity of these phosphoantibodies for NCC is confirmed by the presence of a signal in immunoblots of the kidney cortex but not medulla (Supplemental Figure 2). AU, arbitrary unit; SAV, subapical membrane vesicle.

The acute natriuretic response to hydrochlorothiazide (HCTZ) in conscious mice housed in metabolic cages. The magnitude of the thiazide-induced natriuresis in Hsd11b2−/− mice did not differ from that in wild-type mice (Figure 9, A and B). However, this experiment may have been confounded by thiazide-induced changes in glomerular filtration or Na+ reabsorption via the ENaC in the CNT and collecting ducts. Therefore, the acute response to HCTZ was evaluated by renal clearance in anesthetized mice receiving a constant, volume-expanding infusion of a solution containing insulin (permitting the calculation of a thiazide-induced increment in the fractional excretion of Na+) and during the constant infusion of the ENaC inhibitor benzamil. The magnitude of the thiazide-induced natriuresis was no greater in Hsd11b2−/− mice than in the wild-type mice (Figure 9, C and D). Similar results were obtained in mice aged >120 days, when ENaC activity is downregulated in Hsd11b2−/− mice (Supplemental Figure 4). In this age group, the natriuresis induced by chronic treatment with HCTZ in wild-type mice was blunted in Hsd11b2−/− mice (Figure 9E).

Urinary excretion of HCTZ did not differ between genotypes (Supplemental Figures 5). The acute natriuretic response in Hsd11b2−/− mice was no different from the wild type when assessed using an alternative thiazide diuretic (bendroflumethiazide) lacking carbonic anhydrase activity (Figure 9F).

In order to provide a positive control cohort for the above studies of Na+ transport, wild-type mice were subjected to a continuous furosemide infusion for 7 days (a maneuver expected to augment Na+ reabsorption in the DCT). Phosphorylated forms of NCC (pT53 and pT58) were detected in greater abundance in the kidneys of furosemide-treated mice (Figure 10, A and B), consistent with activation of Na+ transport in the DCT as a consequence of greater Na+ delivery. These mice also exhibited an increase in the thiazide-induced increment in the fractional excretion of Na+(Figure 10, C and D), demonstrating that in this context, increased expression of pNCC did correlate with greater thiazide-sensitive NaCl transport. Chronic furosemide induced significant rises in plasma osmolality, plasma [Na+], and hematocrit of a similar magnitude to those detected in Hsd11b2−/− mice (Supplemental Tables 3).

DISCUSSION

Hsd11b2−/− mice exhibit hypothyphy and hyperplasia of the DCT, with increased expression of total and phosphorylated NCC. 11βHSD2 was not universally expressed in the DCT of wild-type mice. We conclude that the structural remodeling observed in the Hsd11b2−/− mouse may be a secondary, rather than primary, response and we propose a modification to the conventional concept of the “aldosterone-sensitive” distal nephron.

Molecular Definition of the ASDN

The ASDN can be defined as that part of the nephron where 11βHSD2 is coexpressed with the MR. 11βHSD2 restricts the bioavailability of glucocorticoids; thus, aldosterone is the dominant corticosteroid regulating Na+ transport in this region. This paradigm of corticosteroid signaling enjoys robust empirical support in the CNT and CCD. ENaC expression/activity increases when 11βHSD2 is inhibited by pharmacologic38,39 or genetic31 means, as a result of MR activation by glucocorticoids.

Whether the DCT should be included in the ASDN is less clear and may depend on the species (Supplemental Table 5). The unambiguous identification of DCT cells is critical; it is necessary to show coexpression of 11βHSD2 with NCC. This has been shown in the rabbit,40 so the ASDN comprises DCT, CNT, and CCD in this species. The presumption that the DCT

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J Am Soc Nephrol 26: 1537–1548, 2015

DCT in the Hsd11b2 Null Condition

1541
Table 1. Estimate of transcript abundance by Q-PCR in whole-kidney homogenates

| Gene               | Expression Relative to the Wild Type | P Value     |
|--------------------|--------------------------------------|-------------|
|                    | Wild Type                           | Hsd11b2−/−  |             |
| Negative and positive controls |                                     |             |             |
| 11βHSD2            | 1.00 (0.89 to 1.11)                  | 0.00 (0.00 to 0.00) | 3.02E-12a |
| SGK1               | 1.00 (0.82 to 1.18)                  | 1.31 (1.10 to 1.51) | 1.91E-02b |
| Na⁺ transporters   |                                     |             |             |
| NHE3               | 1.00 (0.84 to 1.17)                  | 0.78 (0.63 to 0.93) | 3.61E-02b |
| NKCC2 (total)      | 1.00 (0.87 to 1.13)                  | 0.86 (0.68 to 1.03) | 1.48E-01  |
| NCC                | 1.00 (0.87 to 1.13)                  | 1.77 (1.24 to 2.31) | 5.03E-03b |
| ENaC               | 1.00 (0.92 to 1.08)                  | 1.22 (1.06 to 1.38) | 1.09E-02b |
| NDCBE              | 1.00 (0.86 to 1.15)                  | 1.17 (0.87 to 1.48) | 2.45E-01  |

Other genes in the distal tubule

| Parvalbumin        | 1.00 (0.78 to 1.22)                  | 2.04 (1.83 to 2.25) | 1.20E-06a |
| Calbindin D28k     | 1.00 (0.87 to 1.13)                  | 1.94 (1.67 to 2.20) | 2.95E-06a |
| NCX1               | 1.00 (0.87 to 1.13)                  | 1.36 (1.05 to 1.67) | 2.33E-02b |
| TRPV5              | 1.00 (0.89 to 1.11)                  | 2.02 (1.78 to 2.26) | 3.12E-07a |
| WNK4               | 1.00 (0.95 to 1.06)                  | 1.31 (1.04 to 1.58) | 1.94E-02b |
| WNK1 (total)       | 1.00 (0.85 to 1.15)                  | 1.23 (0.89 to 1.56) | 1.69E-01  |
| WNK1-L             | 1.00 (0.82 to 1.18)                  | 1.10 (0.89 to 1.30) | 4.15E-01  |
| WNK1-KS            | 1.00 (0.86 to 1.14)                  | 1.56 (1.17 to 1.95) | 6.70E-03b |
| SPAK (total)       | 1.00 (0.81 to 1.19)                  | 1.45 (1.23 to 1.66) | 2.33E-03a |
| SPAK-L             | 1.00 (0.91 to 1.10)                  | 1.35 (1.19 to 1.52) | 6.82E-04a |
| SPAK-KS            | 1.00 (0.74 to 1.26)                  | 1.46 (0.86 to 2.07) | 1.19E-01  |
| OSR1               | 1.00 (0.92 to 1.08)                  | 1.09 (0.96 to 1.22) | 2.00E-01  |
| PP4                | 1.00 (0.93 to 1.07)                  | 1.00 (0.90 to 1.10) | 9.73E-01  |

Data are presented as the mean (95% confidence interval). Mice were culled at 55 to 65 days of age (n=8 for each genotype). For each gene, transcript abundance was expressed relative to the mean abundance of three endogenous control genes (18S rRNA, TBP, and cyclophilin A) and then normalized relative to the wild-type group. P values are from unpaired t tests.

aP<0.003, which is the individual error rate required to maintain the family error rate at 0.05 for 20 independent tests.
bP<0.05.

Figure 5. Expression of 11βHSD2 in the renal tubule of male C57BL/6J mice at 55–65 days of age. (A) 11βHSD2 (red) and NCC (green) are not expressed in a common set of cortical tubules. Similar results are observed at 30 days of age. (B) High-power views of the boxed regions in A, showing transitions between tubule segments expressing NCC and those expressing 11βHSD2. Bar, 200 μm in A; 100 μm in B.
would increase Na+ delivery out of Henle we have implicated diminished NKCC2 expression, which aberrant electrolyte transport elsewhere in the kidney. Herein, in some microdissected DCT segments, but not in others.

Figure 6. Hsd11b2 expression in microdissected wild-type tubular segments. Tubular segments are microdissected from male C57BL/6J kidneys, and cDNA prepared. Specific transcripts are detected by using intron-spanning primer pairs in end-point PCR. Each lane represents a different microdissected tubule segment; the positive control template is total kidney cDNA (+), and the negative control templates are RT-negative control cDNA preparation from total kidney RNA (RT−) and water (aq). The Hsd11b2 transcript is detected in the CCD, but is not consistently detected in the DCT. Slc12a3 (encoding NCC) is detected only in the DCT, verifying the purity of the tubule preparations. Scnn1g (encoding ENaC) is detected in the CCD, but also at lower levels in the DCT.

Figure 7. Expression of 11βHSD2 protein in the wild-type renal tubule. Solid black indicates strong expression, whereas hatched gray indicates weak expression. The discontinuous expression of AQP2 and 11βHSD2 in the CNT and CCD reflect the presence of these antigens in principal but not intercalated cells. We are unable to detect 11βHSD2 protein in DCT cells. The Hsd11b2 transcript is detected at low levels in some microdissected DCT segments, but not in others.

knockout mice, demonstrating that K+ status can influence pNCC expression via aldosterone-independent pathways.

What Drives DCT Remodeling in Hsd11b2−/− Mice?
With a lack of consistent 11βHSD2 expression in the DCT, the striking epithelial remodeling and increased expression of pNCC observed in Hsd11b2−/− mice is most likely to reflect aberrant electrolyte transport elsewhere in the kidney. Herein, we have implicated diminished NKCC2 expression, which would increase Na+ delivery out of Henle’s loop and into the DCT. In support of this, the remodeling appeared more pronounced in the proximal portion of the DCT and chronic administration of a loop diuretic evoked a similar increase in pNCC expression. Other facets of the AME phenotype might also activate NCC (e.g., hypokalaemia, alkalosis, chloride depletion, or sympathetic activation). In this constitutive knockout, developmental effects are possible, including those resulting from increased glucocorticoid exposure in vivo as a result of loss of the placental 11βHSD2 barrier. Our finding that there were fewer glomeruli per unit area in sections from Hsd11b2−/− kidneys suggests developmental nephron loss.

What Is the Functional Consequence of DCT Hypertrophy?
DCT hypertrophy is associated with enhanced NaCl transport in salt-losing states, contributing to a homeostatic defense against salt and water loss. We found that the increase in pNCC evoked by chronic furosemide was associated with enhanced thiazide-sensitive Na+ reabsorption. DCT hypertrophy has also been observed in mouse models of Gordon’s syndrome. This hypertensive phenotype was substantially reversed by thiazide diuretics or by crossbreeding to NCC knockout mice, indicating a close relationship between DCT structure and function.

However, in Hsd11b2−/− mice, DCT hypertrophy and increased expression of pNCC were not associated with an increased natriuretic response to thiazide either acutely or chronically. It is not easy to account for such dissociation, because the observed reduction of NHE3 and NKCC2 expression should preserve Na+ delivery to the DCT. We can only speculate that Na+ transport in the DCT of Hsd11b2−/− mice might be limited by pathologic structural remodeling, resulting in an epithelium that is not adapted for efficient solute transport. Abnormalities are found in subcellular (binucleation, nuclear hyperchromasia, and apical blebbing) and tissue structure (multiple layers of cells on a single basement membrane; Figure 1).

Our data show that NCC phosphorylation status does not always correlate with a whole-organ measure of transport activity. This dissociation has also been reported in SPAK heterozygote null mice, which have reduced phosphorylation of NCC compared with wild-type mice but no difference in the magnitude of the thiazide-induced natriuresis. We attempted to minimize confounding hemodynamic and off-target effects in the design of our renal clearance protocol. We do not think hemodynamic changes exerted a major effect on outcome in this study because our protocol achieves maximal NCC blockade without affecting renal blood flow or GFR. It is unlikely that the thiazide readout in Hsd11b2−/− mice was masked by increased Na+ transport in the downstream tubule because we obtained the same results during concomitant ENaC blockade. We also discount a major contribution to the net natriuretic response of off-target thiazide effects. Inhibition of Na+ reabsorption in the PCT after carbonic anhydrase inhibition is unlikely
be sure of the true functional consequence of DCT hypertrophy by thiazides would increase renal Na⁺ excretion but sodium-dependent chloride/bicarbonate exchanger (NDCBE) which does not inhibit this enzyme. Similarly, blockade of the 1544 gene on the C57BL/6JOlaHsd background.31 Experimental animals were

All experiments were conducted in accordance with UK Home Of Animals

Complete methods are provided in the Supplemental Methods.

The increased abundance of pNCC suggests that Na⁺ trans-

port in the DCT may be upregulated in Hsd11b2⁻/⁻ mice; this is not supported by the renal clearance thiazide test. However, both of these measures provide only an indirect assessment of NCC transport activity. This study does not provide a direct measure of Na⁺ transport in the DCT and therefore we cannot be sure of the true functional consequence of DCT hypertrophy in Hsd11b2⁻/⁻ mice.

In conclusion, Hsd11b2⁻/⁻ mice exhibit hypertrophy and hyperplasia of the DCT, with increased expression of total and phosphorylated NCC. In our study, 11βHSD2 protein did not colocalize with NCC in the wild-type renal tubule. Thus, the structural remodeling of the DCT observed in Hsd11b2⁻/⁻ mice and the increased expression/phosphorylation of NCC may be a secondary consequence of unregulated MR activation elsewhere in the nephron. This study contributes to a growing recognition that aldosterone is not necessarily the dominant controller of Na⁺ transport in the DCT, especially in the mouse.

**CONCISE METHODS**

Complete methods are provided in the Supplemental Methods.

**Animals**

All experiments were conducted in accordance with UK Home Office regulations and the Animals (Scientific Procedures) Act 1986. Hsd11b2⁻/⁻ mice were derived from a colony carrying a null mutation in the Hsd11b2 gene on the C57BL/6JOlaHsd background.31 Experimental animals were the male offspring of parents that were both homozygous for the null mutation; C57BL/6JOlaHsd wild-type controls were matched for age and sex. We confirmed that 11βHSD2 protein and enzyme activity were absent from Hsd11b2⁻/⁻ kidneys (Supplemental Figure 6).

**Immunofluorescence**

Administration of BrdU

Wild-type and Hsd11b2⁻/⁻ mice were implanted with an osmotic pump (model 1007D; Alzet) containing 50 mg/ml BrdU in 50% DMSO, which had been primed in 0.9% NaCl at 37°C overnight. Pumps remained in situ for 7 days before the animals were culled by perfusion fixation.

**Perfusion Fixation**

Kidneys were fixed in situ using a perfusion fixation protocol adapted from that described by Lofﬁng and Kaissling.11,37 Under terminal anesthesia, the infrarenal aorta was cannulated and retrograde perfusion with a 4% paraformaldehyde solution was initiated immediately by 50 ml of 4% paraformaldehyde in PBS, pH 7.4, delivered at a rate of 15 ml/min. The right kidney was removed, sectioned, and immersed in 4% paraformaldehyde at 4°C for 24 hours.

**Immunofluorescence**

Indirect immunofluorescence detection of target antigens was performed using a Leica BOND-MAX robot, after an antigen retrieval step. Primary antibodies and their dilutions were as follows: sheep anti-11βHSD2 (AB1296; EMD Millipore) at 1:6000, rabbit anti-NCC (AB3553; EMD Millipore) at 1:2000, sheep anti-NKCC2 (Division of Signal Transduction Therapy, Dundee University) at 1:4000, goat anti-AQP2 (sc-9882; Santa-Cruz Biotechnology) at 1:4000, rabbit anti-parvalbumin (PV25; Swant) at 1:6000, rabbit anti-calbindin D-28k (CB38; Swant) at 1:100,000, and sheep anti-BrdU (20-BS17; Fitzgerald) at 1:250. Secondary antibodies and their dilutions were as follows: goat anti-rabbit IgG-horseradish peroxidase (HRP) (AB6112; Abcam, Inc.) at 1:500, rabbit anti-sheep IgG-HRP (Nordic Immunology) at 1:500, and rabbit anti-goat IgG-HRP (AP106P; EMD Millipore) at 1:500. The binding sites of HRP-conjugated secondary antibodies were detected using a tyramide-labeled fluorophore (either Cy3 or Cy5).

**Semiquantitative Immunoblotting**

**Protein Sample Preparation**

Kidneys were homogenized in a buffer containing phosphatase, kinase, and protease inhibitors (250 mM sucrose, 10 mM triethanolamine, 2 mM EDTA, 50 mM NaF, 25 mM Na β-glycerophosphate, 5 mM Na pyrophosphate, 1 mM Na orthovanadate, and 1% Protease Inhibitor Cocktail Set III [Calbiochem], pH 7.6). Supernatants from two 15-minute centrifugations at 4000g were pooled to form a total cellular protein fraction (50), free from gross cellular and nuclear debris. This fraction was subject to a further centrifugation at 16,000g for 32 minutes to pellet a fraction.
enriched in plasma membranes; the supernatant from this spin was centrifuged at 200,000 g for 60 minutes to pellet a fraction (P2) enriched in subapical membrane vesicles.

**SDS-PAGE and Immunoblotting**

Samples were resolved by SDS-PAGE on NuPAGE Novex 3%–8% Tris-acetate gels (Invitrogen) and then transferred to an Amersham Hybond-P PDVF membrane (GE Healthcare). Primary antibodies and their dilutions were as follows: rabbit anti-NCC (AB3553; EMD Millipore) at 1:1000; sheep anti-pT53-NCC, anti-pT58-NCC, and anti-pS71-NCC (Division of Signal Transduction Therapy, Dundee University), each at 1:500 (0.2–1.2 μg/ml); and sheep anti-NKCC2 (DSTT, Dundee) at 1:10,000. For Western blots designed to recognize specific phosphorylated forms of NCC, the corresponding nonphosphorylated peptide was included in the solution of primary antibody at a final concentration of 10 μg/ml. Secondary antibodies and their dilutions were as follows: goat anti-rabbit IgG-HRP (sc-2030; Santa-Cruz Biotechnology) at 1:2000, and donkey anti-sheep IgG-HRP (A3415; Sigma-Aldrich) at 1:20,000. Peroxidase activity was revealed using SuperSignal West Pico Chemiluminescent Substrate to expose photographic film. During densitometry analysis, the density of each band was divided by that of a band from the same sample stained with Coomassie blue.

**Q-PCR**

RNA was extracted from frozen mouse kidneys using the QIAGEN RNeasy Mini Kit, using a protocol that included an on-column treatment with DNase I. cDNA was transcribed from 500 ng of total RNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. Q-PCR assays made use of the Roche Universal ProbeLibrary (Supplemental Table 4). Reactions were run on a Roche LightCycler 480. 18S rRNA, TBP, and cyclophilin A were selected as endogenous control genes because these transcripts did not differ in abundance between Hsd11b2<sup>−/−</sup> and wild-type kidneys. All other assays were expressed relative to the mean of the endogenous control genes.

**Tubule Segment Microdissection**

**Microdissection and Preparation of RNA and cDNA**

Wild-type male C57BL/6 mice were subjected to terminal anesthesia. The left kidney was perfused in situ though a catheter placed in the abdominal aorta with 1 ml ice-cold heparinized 0.9% NaCl

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**Figure 9.** Natriuretic response to thiazide in mice at 55–65 days of age. (A) Natriuretic response to HCTZ (20 mg/kg body wt intraperitoneally) administered to conscious mice in metabolic cages (wild-type mice, n=11; Hsd11b<sup>−/−</sup> mice, n=10). (B) Metabolic cage data are presented as the thiazide-induced increment in urinary Na<sup>+</sup> excretion for each mouse. There is no significant difference between genotypes (P>0.05 by the unpaired t test). (C) The F<sub>eNa</sub> in anesthetized mice in a renal clearance study (wild-type mice, n=9; Hsd11b<sup>−/−</sup> mice, n=8) is determined at baseline, after BZM and after BZM plus HCTZ (2 mg/kg intravenously). Neither the diuretic responses to BZM, nor the response to HCTZ, differs between genotypes (P>0.05 by two-way repeated measures ANOVA and post hoc Bonferroni tests). (D) Clearance data are presented as the thiazide-induced increment in F<sub>eNa</sub> for each mouse (i.e., F<sub>eNa</sub> during HCTZ and BZM minus F<sub>eNa</sub> during BZM only). There is no significant difference between genotypes (P>0.05 by the unpaired t test). (E) U<sub>Na</sub>V in mice aged >120 days treated with chronic HCTZ. Mice receive daily intraperitoneal injections of vehicle during the baseline period (days −2 to 0) and then daily injections of HCTZ (20 mg/kg intraperitoneally) for 7 days (wild-type mice, n=5; Hsd11b<sup>−/−</sup> mice, n=5). P<0.05 (for genotype, time, and interaction by two-way ANOVA). *P<0.05 (for comparison between genotypes by post hoc Bonferroni test). (F) F<sub>eNa</sub>, in a renal clearance study (wild-type mice, n=5; Hsd11b<sup>−/−</sup> mice, n=3 aged >120 days) determined at baseline and after BFZ (12 mg/kg intravenously). There is no significant difference between genotypes. All data are means and 95% confidence intervals. BFZ, bendroflumethiazide; BW, body weight; BZM, benzamil; F<sub>eNa</sub>, fractional excretion of Na<sup>+</sup>; U<sub>Na</sub>V, urinary sodium excretion.
followed by 2 ml HBSS (in mM: NaCl 140, KCl 5, MgSO4 0.8, MgCl2 1, NaH2PO4 0.33, NaH2PO4 0.44, CaCl2 0.5, glucose 5, HEPES 10, and citrate, pH 7, 0.5% sarcosyl, and 100 mM MgCl2, 0.025 U/μl Taq DNA polymerase (Thermo Fisher Scientific), and template cDNA from approximately 25 μm of tubule. PCR conditions were as follows: 95°C for 3 minutes, then 33 cycles of 94°C for 20 seconds, 59°C for 30 seconds, 72°C for 45 seconds, and then 72°C for 7 minutes. Intron-spanning primers were designed to amplify gene products from Hsd11b2 (5'-ctggctgctgcttcagtg and 5'-ccaaacaggattgtagtttttc), Slc12a3 (5'-ctcaactggctaatagcag and 5'-taggtagagttgtcaggta), and Scnn1g (5'-ccaaacaggccaataaaacag and 5'-gggagggcgaatagaga).

**Thiazide-Sensitive Tubular Na+ Reabsorption**

**Acute HCTZ**

Mice were housed individually in metabolic cages that enabled the independent collection of urine and feces and were left to acclimatize for 4 days before any experimental data were obtained. Each mouse was given an intraperitoneal injection of vehicle (approximately 0.2 ml 2% DMSO), immediately followed by a 6-hour urine collection. Mice received an injection of HCTZ (20 mg/kg body wt) 24 hours later, followed by a further 6-hour urine collection. Urine collections were timed to fall during the animal’s active phase (i.e., the dark phase of a 24-hour dark/light cycle).

**Chronic HCTZ**

Mice were housed individually in metabolic cages and acclimatized as above. Each mouse was given an intraperitoneal injection of vehicle (approximately 0.2 ml 2% DMSO) daily for 3 days during the baseline period and then an injection of HCTZ (20 mg/kg body wt) daily for 7 days during the test period. Urine samples were collected over 24 hours.

**Renal Clearance**

Renal clearance experiments were performed as previously described.31 Mice were anesthetized with thiobutabarbital sodium salt hydrate (Inactin; Sigma-Aldrich) and catheters were inserted into the trachea, jugular vein, carotid artery, and bladder. A bolus dose (0.1 ml/10 g body wt) of physiologic saline solution was given via the jugular catheter as soon as intravenous access was established, followed by a continuous infusion of 0.2 ml/10 g per hour. This infusion comprised 100 mM NaCl, 5 mM KCl, 15 mM NaHCO3, and 0.25% FITC-inulin (pH 7.4). A 60-minute equilibration period was followed by three 40-minute collection periods, during which urine was collected under water-saturated mineral oil. After the first control collection, a bolus dose of bendazam (2 mg/kg body wt) was administered intravenously, followed by a continuous infusion (1 mg/kg per hour for the remainder of the experiment). A 20-minute period of re-equilibration was followed by a second 40-minute urine interval.

Figure 10. Molecular and functional adaptation to chronic furosemide in wild-type mice. (A and B) Immunoblots of total and phosphorylated NCC in wild-type mice treated with chronic furosemide (approximately 90 mg/kg per day for 7 days; n=5) and in untreated controls (n=5). Size markers are 117 kDa (blots) and 55 kDa (Coomassie gels). *P<0.05 (comparison between treated and untreated groups by the unpaired t test). (C) FENa in anesthetized mice (control mice, n=7; furosemide-treated mice, n=6). Data are presented as in Figure 9C. ***P<0.001 (comparison between control and furosemide-treated groups by two-way repeated-measures ANOVA and post hoc Bonferroni test). (D) Data are presented as the thiazide-induced increment in FENa for each mouse (as in Figure 9D). *P<0.05 (comparison between control and furosemide-treated groups by the unpaired t test). All data are means and 95% confidence intervals. AU, arbitrary unit; FENa, fractional excretion of Na+.
collection. Mice then received a bolus dose of HCTZ (2 mg/kg body wt), followed by another 20-minute re-equilibration and then a final 40-minute urine collection. Approximately 80-μL blood samples were obtained from the arterial line at the end of the first equilibration period and after each urine collection; these were used to determine plasma [inulin]. At the end of the protocol, a 1-ml sample of blood was obtained for the measurement of plasma [Na⁺], [K⁺], and osmolality.

A subset of mice was used in a renal clearance study to determine the acute natriuretic effect of bendroflumethiazide (12 mg/kg body wt intravenously). A 40-minute baseline collection period was followed by the bendroflumethiazide bolus, followed by 20 minutes of re-equilibration and then a second 40-minute collection period.

**Chronic Furosemide Treatment**

Mice were implanted with a subcutaneous osmotic pump (model 2001; Alzet) containing furosemide (80 mg/ml in 50% DMSO, pH 8.0), delivering a dose of approximately 90 mg/kg per day. The pumps remained in situ for 7 days, during which time the mice had access to both tap water and salt solution (0.8% NaCl, 0.1% KCl).

**Statistical Analyses**

Data are presented as the mean and 95% confidence interval for the mean. Experimental groups were compared by the unpaired t test or by ANOVA with post hoc Bonferroni or Dunnett’s tests as appropriate. For multiple comparisons by t test, the Dunn–Sidak method was used to apply a correction to maintain the family-wise error rate (α) at 0.05, where the individual error rate is α = 1 – (1 – α)^(1/n), for n independent tests.

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**DISCLOSURES**

None.

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