Failure to Downregulate the Epithelial Sodium Channel Causes Salt Sensitivity in Hsd11b2 Heterozygote Mice

Eilidh Craigie, Louise C. Evans, John J. Mullins, Matthew A. Bailey

Abstract—In vivo, the enzyme 11β-hydroxysteroid dehydrogenase type 2 influences ligand access to the mineralocorticoid receptor. Ablation of the encoding gene, HSD11B2, causes the hypertensive syndrome of apparent mineralocorticoid excess. Studies in humans and experimental animals have linked reduced 11β-hydroxysteroid dehydrogenase type 2 activity and salt sensitivity of blood pressure. In the present study, renal mechanisms underpinning salt sensitivity were investigated in Hsd11b2+/− mice fed low-, standard-, and high-sodium diets. In wild-type mice, there was a strong correlation between dietary sodium content and fractional sodium excretion but not blood pressure. High sodium feeding abolished amiloride-sensitive sodium reabsorption, consistent with downregulation of the epithelial sodium channel. In Hsd11b2+/− mice, the natriuretic response to increased dietary sodium content was blunted, and epithelial sodium channel activity persisted. High-sodium diet also reduced renal blood flow and increased blood pressure in Hsd11b2+/− mice. Aldosterone was modulated by dietary sodium in both genotypes, and salt sensitivity in Hsd11b2+/− mice was associated with increased plasma corticosterone levels. Chronic administration of an epithelial sodium channel blocker or a glucocorticoid receptor antagonist prevented salt sensitivity in Hsd11b2+/− mice, whereas mineralocorticoid receptor blockade with spironolactone did not. This study shows that reduced 11β-hydroxysteroid dehydrogenase type 2 causes salt sensitivity of blood pressure because of impaired renal natriuretic capacity. This reflects deregulation of epithelial sodium channels and increased renal vascular resistance. The phenotype is not caused by illicit activation of mineralocorticoid receptors by glucocorticoids but by direct activation of glucocorticoid receptors. (Hypertension. 2012;60:000-000.) • Online Data Supplement

Key Words: glucocorticoid receptor • RU486 • spironolactone • renin-angiotensin system

Hypertension remains a significant public health burden worldwide, being a major risk factor for cardiovascular mortality and chronic kidney disease.1 Although specific causes of hypertension are often difficult to resolve, salt sensitivity of blood pressure (BP) is a contributory mechanism in a number of patient subgroups.2 Salt sensitivity is also an independent risk factor for adverse cardiovascular events in normotensive individuals3 and is a negative prognostic indicator for clinical progression toward hypertension, microalbuminuria, and endothelial dysfunction.4 The underlying mechanisms of salt sensitivity are not well defined, but subclinical renal impairment reducing the natriuretic efficiency of the kidney may be contributory. Abnormal modulation of the renin-angiotensin-aldosterone system by dietary salt has been linked to salt sensitivity and cardiorenal damage in both patients5 and in experimental models.6 Mineralocorticoid receptor (MR) blockade is cardioprotective, even when aldosterone levels are low or normal,7 and pathophysiological activation of MR by alternative ligands has been found in rodent models of salt-sensitive hypertension.8,9

Cross-talk at the receptor level between the renin-angiotensin-aldosterone system and the hypothalamic-pituitary-adrenal axis is normally prevented by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2). This enzyme protects MR directly by restricting the local availability of active glucocorticoids10 and may also confer indirect protection by locking glucocorticoid-occupied MR in a transcriptionally inactive state.11 Null mutations in the encoding gene (HSD11B2) cause the syndrome of apparent mineralocorticoid excess (AME; Online Mendelian Inheritance in Man +218030), an autosomal recessive disorder presenting with hypertension, severe hypokalemia, and low aldosterone. Hsd11b2 null (Hsd11b2−/−) mice have a similar phenotype to AME patients: unregulated activation of MR by glucocorticoids appears to be causative,12 and hypertension is associated with transient activation of the epithelial sodium channel (ENaC) in the aldosterone-sensitive distal nephron (ASDN).13

A type 2 variant of AME (Online Mendelian Inheritance in Man 207765) presents in adults as essential hypertension with mild abnormalities in steroid metabolism.14,15 With a strong

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correlation between the severity of the AME phenotype and the underlying \textit{HSD11B2} mutation. \textit{HSD11B2} is an attractive candidate gene for salt sensitivity. Indeed, \textit{HSD11B2} polymorphisms associated with either raised BP, per se, or salt sensitivity of BP have been found in several populations, \textsuperscript{16–19} although not all studies report a positive correlation. \textsuperscript{20,21}

We recently demonstrated a causal link between \textit{11BHS2D} activity and salt sensitivity in \textit{Hsd11b2} heterozygote null (\textit{Hsd11b2} \textsuperscript{+/-}) mice. \textsuperscript{22} These mice were found to have salt-sensitive BP and electrolyte abnormalities consistent with mineralocorticoid excess. AME is classically considered a renal disease, and we have, therefore, analyzed renal sodium handling in \textit{Hsd11b2} \textsuperscript{+/-} mice. We find that sodium excretion is abnormally modulated by dietary salt in these mice, dependent on a dysregulation of ENaC. Our studies also point toward a major role for the glucocorticoid receptor (GR) and not the MR in the salt-sensitive phenotype.

**Methods**

Experiments on age-matched cohorts of \textit{Hsd11b2} \textsuperscript{+/+} and \textit{Hsd11b2} \textsuperscript{+/-} mice were performed under a United Kingdom Home Office License, following ethical review by the university.

**Renal Clearance**

Renal function and BP were measured in anesthetized \textit{Hsd11b2} \textsuperscript{+/+} and \textit{Hsd11b2} \textsuperscript{+/-} mice, maintained on either a low- (LS; 0.03%; \textit{n}=7 and 8), standard- (SS; 0.25%; \textit{n}=8 and 8), or high-sodium (HS; 2.5%; \textit{n}=9 and 11) diet. After baseline measurements, amiloride (2 mg/kg; IV) was injected to measure ENaC activity. (online-only Data Supplement).

**Chronic Inhibitor Administration**

Renal function was measured mice maintained on an HS diet, receiving 1 of 3 cotreatments, spironolactone, RU486 (\textit{n}=6 and 8), RU486 (\textit{n}=9 and 10), or benzamil (\textit{n}=6 and 7).

**Sodium Balance in Conscious Mice**

Mice were housed continuously in metabolic cages. Cumulative sodium balance was measured over cycles of 3 days. Mice were first fed SS diet, after which benzamil or vehicle was administered. After another balance period, the HS diet was given for 3 days, this being the period of sodium retention. \textsuperscript{22}

**Quantitative PCR**

mRNA abundance was measured using a Universal Probe Library kit (Roche) and primers designed for the following targets, \textit{nr3c1}, \textit{nr3c2}, \textit{scnn1a}, \textit{scnn1b}, and \textit{sgk1} (see Table S1).

**Statistics**

All of the data are presented as mean\pmSE. Statistical comparisons were made using Prism 5 (GraphPad Software).

**Results**

**Salt-Sensitive BP and Renal Hemodynamics in \textit{Hsd11b2} \textsuperscript{+/-} Mice**

BP was measured in groups of \textit{Hsd11b2} \textsuperscript{+/+} and \textit{Hsd11b2} \textsuperscript{+/-} mice maintained on an LS, SS, or HS diet. On the LS and HS diets, BP was comparable between genotypes. HS diet caused a significant increase in BP in \textit{Hsd11b2} \textsuperscript{+/-} mice (Figure 1A). Overall, there was a significant correlation between mean arterial BP and dietary sodium content in \textit{Hsd11b2} \textsuperscript{+/-} mice (Pearson \textit{r}=0.67; \textit{P}<0.001); no such relationship was observed in \textit{Hsd11b2} \textsuperscript{+/+} mice.

Glomerular filtration rate was higher in \textit{Hsd11b2} \textsuperscript{+/-} mice than in controls, but there was no relationship between this and dietary salt (Figure 1B). Renal blood flow (RBF) was higher in \textit{Hsd11b2} \textsuperscript{+/-} mice than \textit{Hsd11b2} \textsuperscript{+/-} on LS and SS diets (Figure 1C). Dietary salt loading decreased RBF in \textit{Hsd11b2} \textsuperscript{+/-} mice; filtration fraction and renal vascular resistance were both significantly elevated (Table).

**\textit{Hsd11b2} \textsuperscript{+/-} Mice Have Impaired Fractional Sodium Excretion**

Sodium excretion was lower in \textit{Hsd11b2} \textsuperscript{+/-} mice than wild-types on both SS and HS diets (Table), and fractional sodium excretion (\textit{FENa}) was calculated to assess tubular function. In \textit{Hsd11b2} \textsuperscript{+/-} mice, there was an appropriate increase in \textit{FENa} with increasing dietary sodium (Figure 2A; Pearson \textit{r}=0.67; \textit{P}<0.01). This relationship was blunted in \textit{Hsd11b2} \textsuperscript{+/-} mice and on the SS or HS diet; \textit{FENa} was significantly lower than in \textit{Hsd11b2} \textsuperscript{+/-} mice (\textit{P}<0.01). Although \textit{Hsd11b2} \textsuperscript{+/-} mice were not able to adapt their renal sodium excretion to their dietary sodium load as effectively as \textit{Hsd11b2} \textsuperscript{+/-} mice, plasma sodium concentration was not affected (Table). Potassium excretion was elevated in \textit{Hsd11b2} \textsuperscript{+/-} mice but only on an SS diet (Table), and fractional potassium excretion was comparable across all of the dietary regimens (Figure 2B).

**Amiloride-Sensitive Sodium Reabsorption in \textit{Hsd11b2} \textsuperscript{+/-} Mice**

The natriuretic effect of amiloride (\textit{Δamiloride} \textit{CNa}) was used to quantify ENaC-mediated sodium reabsorption. In \textit{Hsd11b2} \textsuperscript{+/-} mice, there was a significant correlation between mean arterial BP and dietary sodium content in \textit{Hsd11b2} \textsuperscript{+/-} mice (Pearson \textit{r}=0.67; \textit{P}<0.001); no such relationship was observed in \textit{Hsd11b2} \textsuperscript{+/-} mice.

**Renal Function**

Renal function was measured mice maintained on an HS diet, receiving 1 of 3 cotreatments, spironolactone, RU486 (\textit{n}=6 and 8), RU486 (\textit{n}=9 and 10), or benzamil (\textit{n}=6 and 7).

**Sodium Balance in Conscious Mice**

Mice were housed continuously in metabolic cages. Cumulative sodium balance was measured over cycles of 3 days. Mice were first fed SS diet, after which benzamil or vehicle was administered. After another balance period, the HS diet was given for 3 days, this being the period of sodium retention. \textsuperscript{22}

**Quantitative PCR**

mRNA abundance was measured using a Universal Probe Library kit (Roche) and primers designed for the following targets, \textit{nr3c1}, \textit{nr3c2}, \textit{scnn1a}, \textit{scnn1b}, and \textit{sgk1} (see Table S1).
significantly natriuresis on the HS diet, suggesting that plasma concentration of corticosterone; Paldo, plasma concentration of aldosterone.

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<td>P₉ ad., pmol/L</td>
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<td>537±164</td>
<td>421±94</td>
<td>332±59</td>
<td>39±0.6**</td>
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Hsd11b2+/+ and Hsd11b2+/− mice were maintained on a low-sodium (LS; n=7 and 8), standard-sodium (SS; n=8 and 8), or high-sodium (HS; n=9 and 11) diet. Data are mean±SEM. Statistical comparisons were made using ANOVA with Bonferroni post hoc test. FF indicates filtration fraction; RVR, renal vascular resistance; E₄Na, urinary excretion of sodium; E₅ K, urinary excretion of potassium; P₄Na, plasma concentration of sodium; P₅ K, plasma concentration of potassium; P₉, plasma concentration of corticosterone; P₉ ad., plasma concentration of aldosterone.

*P<0.01 vs wild-type.
**P<0.001 vs wild-type.

mice (Figure 2C), there was an inverse relationship between dietary sodium content and ΔamilorideNa₄ (Pearson r = −0.74; P<0.001), consistent with downregulation of functional ENaC after increased sodium intake.

In Hsd11b2+/− mice, the inverse relationship was blunted (Pearson r = −0.42; P<0.05). Critically, amiloride evoked a significant natriuresis on the HS diet, suggesting that Hsd11b2+/− mice fail to regulate their ENaC activity appropriately in relation to sodium intake. This does not reflect altered gene transcription: mRNA abundance for ENaC subunits was not different between genotypes. Sgk1 expression was increased, which would promote ENaC retention in the apical membrane (see Figure S1) and maintain an electrophysiological driving force for potassium secretion. Indeed, despite hypokalemia, the potassium sparing effect of amiloride was sustained (Figure 2D) in Hsd11b2+/− mice on the HS diet, whereas wild-type mice maintained potassium homeostasis through processes independent of ENaC activity.

The effect of the specific ENaC antagonist, benzamil, on sodium balance was assessed in conscious mice. In untreated Hsd11b2+/− mice, the transition to the HS diet caused a positive sodium balance (Hsd11b2+/− = 104±15 versus Hsd11b2+/+.)

**Figure 2.** A, Fractional sodium excretion, (B) fractional potassium excretion, (C) amiloride-sensitive sodium reabsorption (ΔamilorideNa₄), and (D) amiloride sensitive potassium secretion (ΔamilorideK₄) in Hsd11b2+/− (□) and Hsd11b2+/− (○) mice after 3 weeks on low-sodium (LS; n=7 and 8), standard-sodium (SS; n=8 and 8), or high-sodium (HS; n=9 and 11) diet. Data are mean±SEM. Comparisons were made using ANOVA with Bonferroni post hoc test. *P<0.05; **P<0.01.
Roles of GR and MR

In the current study, increased sodium intake reduced aldosterone in both genotypes (Table). Nevertheless, Hsd11b2+/− mice had significantly lower levels than Hsd11b2+/- mice, suggesting tonic suppression of the renin-angiotensin-aldosterone system. It is, therefore, unlikely that aldosterone excess is responsible for the increased ENaC activity observed in Hsd11b2+/- mice.

Corticosterone was similar between genotypes on the LS and SS diets (Table) but was elevated in Hsd11b2+/- mice on the HS diet. To militate against confounding effects of anesthesia, corticosterone was measured in unrestrained conscious mice before and after HS feeding. The sodium-induced increase in corticosterone was confirmed in the 7 PM measurement, before the active phase: 7 AM corticosterone was not different between genotypes (Figure S2).

Because activation of MR and/or GR by glucocorticoids was likely to be causative to the salt-induced phenotype, the renal expression of both was measured: MR expression was similar in both genotypes, but GR expression was higher in Hsd11b2+/- mice (see Figure S3). To resolve the mechanisms for salt sensitivity, Hsd11b2+/- and Hsd11b2+/- mice on an HS diet were treated chronically with the following: (1) the ENaC blocker benzamil; (2) the MR antagonist spironolactone; or (3) the GR antagonist RU486. The salt-sensitive phenotype was ameliorated by either benzamil or RU486 treatment but not by spironolactone treatment (Figure 3A). The sodium-induced reduction in RBF was also ameliorated by benzamil treatment, and RBF was actually increased by RU486 treatment (Figure 3B). Spironolactone did not improve FENa (Figure 3C), and amilorideNa remained high. In contrast, both benzamil and RU486 treatment restored FENa in Hsd11b2+/- mice to Hsd11b2+/- levels and abolished genotype differences in amilorideNa (Figure 3D).

Discussion

In Hsd11b2+/- mice, BP is directly influenced by dietary sodium intake. This strong salt sensitivity of BP was not observed on the parental C57BL/6J background and, thus, the HSD11B2 locus is a plausible candidate gene for salt sensitivity in humans. Because gene defects associated with monogenic BP disorders affect the renal handling of sodium,23 we hypothesized that salt sensitivity in Hsd11b2+/- mice may also reflect abnormal renal function. Data obtained across a regimen encompassing dietary sodium restriction and sodium loading supported this hypothesis, indicating that abnormal renal sodium homeostasis is driven by activation of GR and not MR in this model.
Renal sodium handling is the major determinant of long-term BP control, and Hsd11b2+/− mice had lower FEK, than Hsd11b2+/+ mice. Elevated tubular reabsorption is likely to be the major factor for impaired natriuresis and salt-sensitive BP in Hsd11b2+/− mice. Because 11βHSD2 governs ligand access to MR in vivo, we focused on the classic MR target ENaC.24 Hsd11b2+/− mice failed to downregulate amiloride-sensitive sodium reabsorption with increasing dietary sodium, and impaired natriuresis therefore reflects an inability to regulate ENaC activity appropriately for sodium intake. We recognize that amiloride can also inhibit other sodium transport proteins, notably the sodium-hydrogen exchanger 3 in the proximal tubule.25 Two lines of evidence argue against a major role here for the sodium-hydrogen exchanger 3. First, the potassium-sparing effect of amiloride localizes the natriuretic effect to the ASDN.26 Second, chronic administration of the ENaC selective amiloride analog benzamil attenuated the ENaC.24

Control by MR of ENaC and its regulatory proteins is well documented.24 In the current study, the physiological ligand aldosterone was appropriately modulated by dietary sodium in both groups of mice. However, there was a tonic suppression of the renin-angiotensin-aldosterone system in Hsd11b2+/− mice across all of the dietary regimens, consistent with MR activation by glucocorticoids after reduced 11βHSD2. To investigate the contribution of the MR, mice fed a HS diet were chronically treated with spironolactone. MR antagonism caused a small reduction in BP in both groups of mice, but the pressure differential between the genotypes persisted, as we have reported previously.22 Critically, spironolactone treatment did not normalize ENaC-mediated sodium reabsorption in Hsd11b2+/− mice. The lack of sensitivity to spironolactone may reflect the increased abundance of GR, relative to MR, and it is possible that a higher dose would uncover an MR-mediated effect. Nevertheless, the current dose was found previously to be effective against similar concentrations of glucocorticoid,8 and although spironolactone treatment can improve both the hypertension and hypokalemia observed in AME,27 it is of variable benefit in long-term treatment.28

We found that chronic GR blockade normalized ENaC activity and increased RBF. RU486 also prevented the salt-induced rise in BP, consistent with our previous findings.22 Quantitatively, the effect of RU486 was similar to that of chronic benzamil administration. Together, these data indicate that the cluster of ENaC-related phenotypes in the Hsd11b2+/− mice are mediated via GR, not MR. Regulation of ENaC by GR-dependent pathways has been documented in renal cell lines,29,30 in dexamethasone-treated adrenalec-tomized rats,31 and in a mouse model of Cushing syndrome.8 A recent study immunolocalized both MR and GR to 11βHSD2-expressing cells in the rat ASDN, demonstrating that physiological variations in circulating aldosterone regulated the translocation of GR and not MR between the nucleus and the cytoplasm.32 This challenges the conventional view of ASDN regulation by corticosteroids but is consistent with our data and suggests a mechanism for salt sensitivity in this model.

The infusion of the dexamethasone can increase the abundance of renal αENaC mRNA and protein expression. This does not automatically equate to an increased physiological activity for ENaC.33 and in Hsd11b2+/− mice, ENaC transcription was comparable to controls. Additional ENaC regulatory pathways may be critical, and our data in Hsd11b2+/− mice, as well as other models,4 suggest that dietary salt and/or activation of the hypothalamic-pituitary-adrenal axis play important roles. A realistic point of convergence under these circumstances may be WNK4, which exerts a negative regulatory effect on ENaC activity.34 WNK4 is physiologically regulated by dietary sodium status35 and by glucocorticoids via a negative glucocorticoid response element in the promoter region of the gene.36 In addition, β2-receptor activation has been demonstrated to induce salt-sensitive hypertension in mice because of GR-mediated inhibition of WNK4 expression.37 Conversely, Cre-lox technology has suggested that GR expression in the ASDN is not critical to dexamethasone-induced hypertension when salt intake is normal.38 However, mice lacking GR in the ASDN had elevated BP before dexamethasone treatment, and the effect of an HS diet was not assessed in this study.

The HS diet also increased renal vascular resistance in Hsd11b2+/− mice, which would reduce natriuretic capacity, particularly if the medullary vasa recta were constricted. 11βHSD2 is expressed in both arteriole smooth muscle39 and the vascular endothelium.40 On a mixed MF1 background, Hsd11b2+/− mice had endothelial dysfunction and enhanced vasocostriction to norepinephrine.41 This may relate to the genetic background; vascular function was normal on a congenic C57Bl/6J strain.13 Nevertheless, we cannot discount a vascular component of salt-sensitive BP in Hsd11b2+/− mice, ENaC in the vascular endothelium is stimulated by aldosterone excess and high sodium,42 and this is associated with reduced NO release.43 Notably, in Hsd11b2+/− mice, chronic ENaC blockade prevented the salt-induced increase in renal vascular resistance.

Perspectives

Failure to regulate ENaC activity with sodium status underpins salt sensitivity in Hsd11b2+/− mice. This is a GR, rather than an MR-mediated phenotype. HS feeding increased corticosterone in Hsd11b2+/− mice, and it is notable that salt-sensitive individuals display both an enhanced stress-induced activation of the hypothalamic-pituitary-adrenal axis44 and attenuated glucocorticoid clearance.45 Because renal enzyme activity is not influenced by dietary sodium in Hsd11b2+/− mice,22 impaired peripheral metabolism cannot fully explain this phenomenon, and we suggest that dietary salt activates the hypothalamic-pituitary-adrenal axis. Indeed, 11βHSD2 is also expressed in cardiovascular control centers of the brain influencing sympathetic outflow.46 Central mechanisms are, therefore, likely to contribute to salt-sensitive BP, at least in the stable phase when sodium balance is restored.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- Reduced activity of a steroid-processing enzyme impairs the efficiency of salt excretion by the kidney.
- The protein causing sodium retention was identified and a new role for the regulation of sodium transport in the kidney by steroid hormones revealed.
- High intake of salt in the diet increases glucocorticoids in the blood, which increases salt retention by the kidney. This causes blood pressure to rise.

**What Is Relevant?**

- In some people, BP rises with HS intake, and this increases the risk for developing hypertension and cardiovascular and renal diseases.

- High levels of glucocorticoids are common in stress and metabolic disorders and could reduce the excretion of salt by the kidney.
- The mechanisms for salt retention could, therefore, be potential targets for antihypertensive therapy.

**Summary**

Reduced activity of a glucocorticoid-metabolizing enzyme in the kidney and brain causes increased levels of steroid in the blood, impairs the ability of the kidney to excrete sodium, and causes BP to rise.
Failure to Downregulate the Epithelial Sodium Channel Causes Salt Sensitivity in \textit{Hsd11b2} Heterozygote Mice

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Failure to down-regulate the epithelial sodium channel causes salt-sensitivity in Hsd11b2 heterozygote mice

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Supplementary Methods

Experiments were carried out in Hsd11b2 wild-type (Hsd11b2+/+) and Hsd11b2+/− littermates generated from Hsd11b2+/− x Hsd11b2+/− crosses. Mice were genotyped by Southern hybridization, as described1 and were used between 100-200 days of age.

Renal Clearance Experiments: Renal function was measured in age-matched cohorts of Hsd11b2+/− or Hsd11b2+/+ mice, maintained on either a low (LS; 0.03 %), standard (SS; 0.25 %) or high sodium (HS; 2.5 %) diet (Special Diet Services, Essex, UK) for 18-21 days. Mice were then anesthetised (Thiobutabarbitral sodium (Inactin), 100 mg/kg; IP) and cannulae placed in the jugular vein (for IV infusion) and carotid artery (for measurement of BP and blood sampling). A tracheotomy was performed to maintain a clear airway and a catheter placed in the bladder. Urine was collected directly into pre-weighed tubes under mineral oil. Mice were infused throughout (0.2 ml/h/10 g bodyweight IV) with a saline solution (120 mM NaCl, 15 mM NaHCO3, 5 mM KCl) containing 0.5 % FITC-labeled inulin and 2% p-aminohippurate acid (PAH). After a 40-minute equilibration period, a 40-minute control urine collection was made. A bolus dose of amiloride (2 mg/kg; IV) was injected 10 minutes before the second 40-minute collection was started. Arterial blood samples of ~50 µl were taken at the beginning and end of each urine collection period. Mean arterial BP was measured continuously (PowerLab, AD Instruments, UK) via a carotid cannula. At the end of the experiment, a ~500 µl sample of arterial blood was taken for measurement of plasma sodium, potassium, aldosterone and corticosterone concentrations.

Urine flow rate was calculated by weight, assuming a density equal to that of water. FITC-inulin and PAH in urine and plasma were used to calculate glomerular filtration rate (GFR) and effective renal plasma flow (eRPF), respectively, by standard clearance equations. The urinary and plasma concentrations of sodium and potassium were measured by ion selective electrode (Electrolyte analyzer 9180, Roche, UK) and multiplied by urine flow to calculate excretion rates. Fractional excretion of electrolytes is the urinary excretion expressed as a percentage of filtered load. The effect of amiloride on sodium and potassium excretion was taken as the difference between excretion rates in the first and second urine collections, as described 2.

Chronic Inhibitor Administration: These experiments were performed on mice maintained on a HS diet and receiving one of three co-treatments: spironolactone, RU486 or benzamil.

Spironolactone and RU486 were mixed into a Silastic matrix (a gift from Dow-Corning, USA) and formed into pellets, which were cured overnight at 37°C. Two pellets, each containing ~30mg of the drug were implanted subcutaneously under isofluorane anesthesia, five days before feeding HS diet. In vitro studies confirmed that drug release from the matrix was at a constant rate over the experimental period.
The concentration of drug in terminal plasma was measured by mass-spectrometry. The active metabolite of spironolactone, canrenone, was \(~75 \text{ nmol/l}\); that of RU486 was \(~100 \text{ nmol/l}\). These concentrations were previously shown to exert a hypotensive effect against concentrations of corticosterone similar to that reported here \(^3\).

Benbamil was administered via an osmotic minipump (model 2004, Alzet, Charles River, UK) at a dose rate of 0.7 \(\mu\)g/g body weight/day. Minipumps were implanted subcutaneously under isoflurane gas anesthesia and IV buprenorphine analgesic and surgical wounds were closed with auto-wound clips. The mice were allowed five days to recover from the effects of surgery before experimental measurements commenced.

**Sodium Balance in Conscious Mice:** Mice were housed continuously in metabolic cages (Techniplast, Italy). During the equilibration and control periods, mice were fed SS. After three consecutive days of stable sodium balance (control period), mice underwent surgery for minipump implantation to allow for the chronic administration of benbamil (described above) or vehicle. Sodium excretion was again measured over three consecutive days before the diet was changed to HS. Sodium balance was measured over a 3-day period as before. Water and food intake was monitored daily, as was urine and fecal output and mouse body weight. Urinary sodium concentration was measured by flame photometry (BWB-1, BWB technologies, UK) and used to calculate urinary sodium excretion over a 24h period. Fecal sodium concentration was also measured by flame photometry, following extraction into nitric acid. Daily sodium balance was calculated as dietary sodium intake – (fecal sodium excretion + urinary sodium excretion). The data are expressed a cumulative balance over a 3-day period.

**Steroid Measurements:** Plasma aldosterone concentrations were measured in 100\(\mu\)l of plasma, collected under terminal anesthesia, by radioimmunoassay (Coat-A-Count, DPC, CA, USA). Plasma corticosterone concentrations were measured as follows: briefly, plasma samples were diluted 1 in 10 in borate buffer (0.133 M boric acid, 67.5 mM NaOH, 0.5 % BSA, 1 % methanol, 0.1 % ethylene glycol, pH7.4 with HCl). Diluted plasmas were then incubated at 80°C for 30 minutes. Aliquots of the diluted plasma were mixed in duplicate in a 96 well plate (BD Falcon, 96 well flexible plate) with 1:4000 dilution of sheep antibody to corticosterone (Micropharm Ltd, UK), 1.5 M \(^3\)H-labelled corticosterone, and anti sheep scintillation proximity assay reagent (1:4 dilution; GE Healthcare). This mixture was incubated overnight at room temperature, and sample wells were counted in a liquid scintillation counter (1450 Microbeta Plus model, Wallac).

**qPCR analysis of mRNA abundance:** RNA was isolated from homogenized kidney using an RNeasy mini kit (Qiagen, UK) and reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). Quantitative PCR was performed using a Universal Probe Library kit (Roche, UK) and primers designed using ProbeFinder version 2.45 for Mouse, (Roche Diagnostics, UK). The following target genes were assessed: \(nr3c1\), \(nr3c2\), \(scnn1a\), \(scnn1b\) and \(sgk1\). Quantification was performed using the second derivative maximum method and target gene expression was normalized to mean concentration values. Values were normalized to the abundance Ppia, which was not different between groups and had a similar CT (~25). 18S rRNA was also found not to significantly differ across groups but has a CT of ~8 and was therefore not used to normalize target gene expression. Primer sequences are detailed in Table S1.
References


**Table S1**: Target genes for qPCR, with primer sequences and the Roche UPL Probe number

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Genbank ID</th>
<th>Left Primer</th>
<th>Right Primer</th>
<th>Probe Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ppia</em> (Peptidylprolyl isomerase A)</td>
<td>NM_008907.1</td>
<td>acgcacctgtcgettttc</td>
<td>geaaacagctgaaggagac</td>
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<tr>
<td><em>Nr3c2</em> (Mineralocorticoid receptor)</td>
<td>NM_001083906.1</td>
<td>ccaagagcgtggaagg</td>
<td>tttcctgaatctatctaatgc</td>
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<tr>
<td><em>Nr3c1</em> (Glucocorticoid receptor)</td>
<td>NM_008173.3</td>
<td>ccaagattgcatctatgaa</td>
<td>ctggcttgcagctcctcc</td>
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<tr>
<td><em>Scnn1a</em> (Epithelial sodium channel subunit alpha)</td>
<td>NM_011324.2</td>
<td>ccaaggtgtaggtctgtga</td>
<td>agaaagccagctgcagttta</td>
<td>46</td>
</tr>
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<td><em>Scnn1b</em> (Epithelial sodium channel subunit beta)</td>
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</tr>
<tr>
<td><em>Sgk1</em> (Serum/glucocorticoid regulated kinase 1)</td>
<td>NM_001161845.2</td>
<td>gattgccagcaacacatag</td>
<td>ttaggtgtgagaggacagtg</td>
<td>91</td>
</tr>
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</table>
Supplementary Results

Figure S1: Quantitative PCR in kidney extracts. A) Scnn1a, which encodes for the αENaC subunit; C) Scnn1b, which encodes for the βENaC subunit and C) Sgk1, which encodes for serum and glucocorticoid-inducible kinase 1. Hsd11b2^{+/+} mice (open bars, n=6) and Hsd11b2^{−/−} mice (grey bars, n=7) were maintained on a high sodium (2.5%) diet for 3 weeks. Data are mean ± SEM of gene expression normalized to that of Ppia, **=P<0.01 by Student’s unpaired t-test.
Figure S2. Plasma corticosterone concentration in conscious, unrestrained mice. *Hsd11b2*+/+ mice (open bars, n=6) and *Hsd11b2*+/− mice (grey bars, n=6) were initially fed a control sodium (0.25%) diet and measurements were taken at 7am and 7pm. Mice were then fed a high sodium (2.5%) diet for three weeks, before measurements were again made. Data are mean ± SEM. Statistical analysis was by 2-way ANOVA with repeated measures, indicating a significant effect of genotype (P<0.001), diet (P<0.01) and interaction (P<0.01). Bonferroni post-hoc test P values are as shown.

![Graph showing plasma corticosterone concentration](image)

Figure S3: Quantitative PCR in kidney extracts. A) *Nr3c2*, encoding for the mineralocorticoid receptor and B) *Nr3C1*, encoding for the glucocorticoid receptor *Hsd11b2*+/+ mice (open bars, n=6) and *Hsd11b2*+/− mice (grey bars, n=7), were maintained on a high sodium (2.5%) diet for 3 weeks. Data are mean ± SEM of gene expression normalized to that of *Ppia*. **=P<0.01 by Student’s unpaired t-test.

![Bar graph showing quantitative PCR results](image)