Direct and Indirect Mineralocorticoid Effects Determine Distal Salt Transport

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ABSTRACT
Excess aldosterone is an important contributor to hypertension and cardiovascular disease. Conversely, low circulating aldosterone causes salt wasting and hypotension. Aldosterone activates mineralocorticoid receptors (MRs) to increase epithelial sodium channel (ENaC) activity. However, aldosterone may also stimulate the thiazide–sensitive Na⁺-Cl⁻ cotransporter (NCC). Here, we generated mice in which MRs could be deleted along the nephron to test this hypothesis. These kidney–specific MR–knockout mice exhibited salt wasting, low BP, and hyperkalemia. Notably, we found evidence of deficient apical orientation and cleavage of ENaC, despite the salt wasting. Although these mice also exhibited deficient NCC activity, NCC could be stimulated by restricting dietary potassium, which also returned BP to control levels. Together, these results indicate that MRs regulate ENaC directly, but modulation of NCC is mediated by secondary changes in plasma potassium concentration. Electrolyte balance and BP seem to be determined, therefore, by a delicate interplay between direct and indirect mineralocorticoid actions in the distal nephron.


Patients with low aldosterone, as in Addison disease, exhibit salt wasting, hypotension, and hyperkalemia. In contrast, primary aldosteronism is characterized by hypertension and hypokalemia.¹ The steroid hormone aldosterone exerts its actions by binding to mineralocorticoid receptors (MRs) in multiple tissues.² Although actions outside the kidney have received attention recently, aldosterone was first recognized for its effects on sodium (Na⁺) and potassium (K⁺) transport by the kidney.³ MRs are expressed from the thick ascending limb to the collecting duct (CD),⁴ but physiologic effects of aldosterone on ion homeostasis occur predominantly along the aldosterone–sensitive distal nephron (ASDN), comprising the connecting tubule (CNT) and CD and in some species, the late distal convoluted tubule (DCT2).⁵,⁶

Although the canonical action of aldosterone is to increase epithelial Na⁺ channel (ENaC) activity, it is accepted that aldosterone also stimulates the thiazide–sensitive Na⁺-Cl⁻ cotransporter (NCC).⁷,⁸ Aldosterone infusion increases NCC activity⁹ and NCC abundance.¹⁰ Dietary salt restriction increases the abundance of phosphorylated (activated) Na⁺-Cl⁻ cotransporter (pNCC).¹¹,¹² Although several hormonal systems are likely activated in these models, aldosterone has been reported to play a key role.¹¹

Schütz and colleagues¹³ reported that constitutive MR knockout mice died in the neonatal period; when rescued by salt administration, however, they exhibited profound salt wasting and
hyperlakemia.\textsuperscript{14} Surprisingly, mice with MR deleted along much of the ASDN (the CNT and CD) exhibited only a mild phenotype.\textsuperscript{15} The failure to delete MR along more proximal segments was suggested to explain the difference. Here, we generated mice in which MR could be deleted along the nephron to test the hypothesis that renal MR regulation is essential. The results confirm that MRs regulate ENaC directly but indicate that effects on NCC are secondary to metabolic changes.

\section*{RESULTS}

We used the Pax8/LC1 CRE/Lox system to generate mice in which MR could be deleted along the entire nephron using doxycycline (documented by RT-PCR in Supplemental Figure 1). After MR gene deletion at 8 weeks, kidney-specific MR knockout (KS MR\textsuperscript{−−/−}) mice survived, but body weight was lower than that in controls (Figure 1A). Even on a normal diet, daily urine volume and Na\textsuperscript{+} excretion rates were higher in KS MR\textsuperscript{−−/−} mice than in controls, although urine K\textsuperscript{+} excretion rates were similar (Figure 1, B–D). The BP was substantially lower in the knockout mice than in control animals (Figure 1, E and F), and knockout mice had lower plasma [Na\textsuperscript{+}], higher plasma [K\textsuperscript{+}], and greater food intake (Figure 1, G–I). Plasma aldosterone was also higher in knockout mice (Figure 1J).

Although the data above suggest that the phenotype is similar to that of complete rescued MR knockout mice,\textsuperscript{14} we confirmed complete functional MR deletion by infusing aldosterone in control and KS MR\textsuperscript{−−/−} mice. In control animals, aldosterone infusion increased plasma [Na\textsuperscript{+}], decreased plasma [K\textsuperscript{+}] and [Cl\textsuperscript{−}], and tended to increase plasma [CO\textsubscript{2}] as expected; in contrast, KS MR\textsuperscript{−−/−} animals were resistant to these effects (Figure 2).

The observed phenotype suggested that KS MR\textsuperscript{−−/−} animals exhibit altered Na\textsuperscript{+} and K\textsuperscript{+} transport along the nephron. Therefore, we investigated the effects of MR deletion on renal ion transporters and channels. The abundance of α-ENaC was lower in KS MR\textsuperscript{−−/−} mice than in controls, whereas β- and γ-ENaC were similar (Figure 3, A–D). Because proteolytic cleavage of the α- and γ-subunits increases ENaC function,\textsuperscript{16} we also determined effects of renal MR deletion on the abundance of cleavage products. The ratios of cleaved to uncleaved α- and γ-ENaC were both lower in the knockout animals than in controls (Figure 3, B–D). Compared with controls, α-ENaC and to a lesser extent, γ-ENaC also exhibited a less apical orientation in the knockouts (Figure 3E). Neither prostasin (Figure 3E), which colocalized with α-ENaC (Supplemental Figure 2), nor SGK1 abundance (Figure 3, F and G), however, was different. NCC and pNCC
abundances were also lower in knockouts than in controls (Figure 3, H–J).

To determine whether the ability to tolerate Na⁺ deprivation and K⁺ loading was impaired in renal MR knockout mice, we treated control and KS MR⁻/⁻ animals with Na⁺-deplete and high-K⁺ diets. Mice were treated with an Na⁺-deplete diet for 5 days to allow for reestablishment of electrolyte balance. This caused significant weight loss in KS MR⁻/⁻ mice but not in control animals (Figure 4A). The urine volume tended to increase in the knockout mice (Figure 4, A and B), and the KS MR⁻/⁻ mice had persistently elevated Na⁺ excretion compared with controls (Figure 4, C and D). Although the interaction between genotype and time was not quite statistically significant for urinary K⁺ excretion (P=0.05 by two-way ANOVA with repeated measures), analysis of cumulative K⁺ excretion indicated that the KS MR⁻/⁻ animals failed to maintain adequate K⁺ excretion on the low-Na⁺ diet (Figure 4, E–G). The KS MR⁻/⁻ mice showed greater weight loss compared with control animals (Figure 3, H–J).

Consumption of an Na⁺-deplete diet increased the abundance and ratio of cleaved to uncleaved α- and γ-ENaC in control mice but not in KS MR⁻/⁻ animals (Figure 5, A–D). Surprisingly, consumption of an Na⁺-deplete diet further reduced pNCC abundance in the KS MR⁻/⁻ mice (Figure 5, E–G). Although the low pNCC in KS MR⁻/⁻ mice suggested that MRs regulate NCC, these mice also have elevated plasma [K⁺]. Because plasma [K⁺] has a direct effect on NCC,17 we prevented this rise in plasma [K⁺] by treating KS MR⁻/⁻ animals for 5 days with an Na⁺-deplete diet that is also deplete in K⁺ (Figure 5H). pNCC abundance in KS MR⁻/⁻ mice that consumed this diet was greater than it was in control mice that consumed a low-salt diet (Figure 5, I–K). This indicated that reduced pNCC abundance in KS MR⁻/⁻ mice resulted predominantly from the rise in plasma [K⁺] and not the lack of MR.

Treatment with a high-K⁺ diet produced similar but more dramatic effects in KS MR⁻/⁻ mice compared with the low-salt diet. The KS MR⁻/⁻ mice did not tolerate this diet well, so we performed a shorter experiment (3 days) to obtain data before the mice became sick. In control mice, K⁺ loading did not significantly affect body weight or urinary Na⁺ excretion, but it did produce a diuresis and a kaliuresis (Figure 6, A–D). Plasma [Cl⁻] and hematocrit increased in both genotypes compared with their baseline values, whereas plasma [CO₂] trended toward being reduced in the knockouts but not in control animals (Supplemental Figure 3).

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MR\(^{-/-}\) mice, whereas it was increased in controls (Figure 6, E and F). Plasma [K\(^+\)] and [Cl\(^-\)] increased in knockouts and plasma [CO\(_2\)] declined in response to high-K\(^+\) diet (Figure 6, F–H). Lastly, hematocrit rose substantially when the KS MR\(^{-/-}\) mice were fed high-K\(^+\) chow (Figure 6I).

Similar to Na\(^+\) deprivation, K\(^+\) loading increased total and cleaved \(\alpha\)- and \(\gamma\)-ENaC in control animals (Supplemental Figure 4), but this effect was completely absent in KS MR\(^{-/-}\) animals. As expected,\(^{17}\) pNCC abundance decreased in K\(^+\)-loaded control mice (Supplemental Figure 4). This effect was similar yet more dramatic in KS MR\(^{-/-}\) animals (Supplemental Figure 4).

These data confirm that aldosterone activates ENaC through the MR, because activation of ENaC did not occur in KS MR\(^{-/-}\) mice. In contrast, these data also indicate that MRs are not necessary for NCC activation, because NCC and pNCC abundance increased with K\(^+\) restriction in KS MR\(^{-/-}\) mice. To test the converse hypothesis that aldosterone administration increases pNCC abundance only when it causes hypokalemia, we infused aldosterone into wild-type animals consuming either a normal or a high-K\(^+\) diet; the high-K\(^+\) diet prevented the reduction in plasma [K\(^+\)], which otherwise occurred (Figure 7A). As expected, aldosterone increased both \(\alpha\)- and \(\gamma\)-ENaC abundance (Figure 7, B–D) and pNCC when mice consumed the normal K\(^+\) diet (Figure 7, F–H). K\(^+\) loading of aldosterone-infused mice led to even greater changes in \(\alpha\)- and \(\gamma\)-ENaC (Figure 7, A–E). In striking contrast, high-K\(^+\) diet completely prevented a rise in NCC during aldosterone infusion (Figure 7, F–H). Thus, high-aldosterone

![Figure 4.](https://www.jasn.org/images/2016/03/Figure4.png)
concentrations are not sufficient to increase pNCC abundance. Similar results were obtained when plasma \([K^+]\) was maintained using amiloride rather than diet (Supplemental Figure 5). When data from control and KS MR\(^{-/-}\) mice were pooled, pNCC abundance and plasma \([K^+]\) seemed to describe a single curve (Figure 7I), further suggesting that plasma \([K^+]\) and not mineralocorticoid effect is the primary NCC driver.

These results suggest that combined inhibition of both NCC and ENaC may cause the profound salt wasting and low BP of MR deficiency. If the NCC inhibition, however, is indirect, then BP should normalize in KS MR\(^{-/-}\) mice when the rise in plasma \([K^+]\) is prevented. Figure 7J shows that arterial pressure in KS MR\(^{-/-}\) mice when they are fed low-K\(^+\) chow is essentially identical to that of control mice. In fact, low-K\(^+\) diet made KS MR\(^{-/-}\) mice susceptible to salt–induced BP rises, similar to those that occurred in control animals (Figure 7K).

**DISCUSSION**

Individuals with low–circulating aldosterone concentrations suffer from severe and potentially fatal electrolyte disorders and hypotension. Genetic deletion of MR throughout the body largely recapitulates this phenotype, but deletion of MR along the CNT and CD causes only mild salt wasting. The present results show that MRs along the DCT and proximal CNT account for this difference, because deletion of MR along the entire nephron recapitulates the effects of rescued total body knockout.

Although ENaC is the canonical MR target, it is now widely accepted that aldosterone also activates NCC. A first analysis of the current results seemed to confirm this view, because NCC and pNCC abundances were substantially reduced in the KS MR\(^{-/-}\) mice. However, plasma \([K^+]\), an important direct regulator of NCC, was also elevated in the knockout mice. Thus, we investigated whether the effects on NCC were secondary to the changes in plasma \([K^+]\) instead of lack of MR. Dietary K\(^+\) restriction of KS MR\(^{-/-}\) mice maintained the abundance of pNCC at a level similar to or greater than in control, indicating that the high plasma \([K^+]\), not MR deficiency, reduces NCC abundance. Although remaining MRs in the kidney might have played a role, the complete absence of ENaC activation, despite low-salt or high-K\(^+\) diets, argues strongly against this conjecture. Coupled with the observation that NCC abundance was suppressed by a high-K\(^+\)
diet, even during aldosterone infusion, these results make clear that MRs are neither necessary (Figure 4) nor sufficient (Figure 7) for NCC activation. Instead, plasma [K+] derangements induced by MR effects on ENaC are dominant. Most or all prior in vivo observations suggesting that aldosterone stimulates NCC9,10,19-21 can be explained by this K+ effect, likely combined with effects of angiotensin II,22 consistent with studies suggesting that NCC is regulated primarily by K+ status.12,17,23-25 These data do not exclude the previously suggested direct effect of aldosterone on NCC,9-11,19,20 but K+ effects seem predominant. Ko et al.21 used cultured cells to argue for a direct mechanism; their in vivo aldosterone infusion experiment, however, did not test the mechanism involved.21 Roy et al.26 used cell culture systems to suggest that aldosterone regulates WNK1 to stimulate NCC, but their work did not determine effects of aldosterone on NCC either in vitro or in vivo. Given our data, changes in WNK1 after aldosterone infusion might have resulted from changes in plasma [K+].

These results indicate that MRs along the nephron are essential for normal electrolyte and pressure homeostasis, even in fully developed mice. The results correspond with the observation that Addison disease is associated with hypotension or the resolution of hypertension.27 The results also model the hyponatremia typically observed in Addison disease, which is ascribed to nonosmotic vasopressin release.

A high-K+ diet was associated with lower blood [CO2] and higher hematocrit in KS MR<sup>2</sup>/2 mice, both of which were observed after total body MR knockout.13 Our knockout animals also ate more, suggestive of secondary hyperphagia, perhaps caused by salt craving. Thus, the reason for reduced body weights is not entirely clear.

Although K+ excretion is determined by many factors,28 these results argue that aldosterone plays an essential and
nonredundant role in daily K⁺ balance. We did not perform formal balance studies, however, so that MR effects on plasma [K⁺] may not reflect changes in total body K⁺ content as suggested. However, deletion of renal MR shifts the relationship between plasma [K⁺] and urinary K⁺ excretion to the right and downward (K⁺ excretion is essentially flat when plasma [K⁺] is raised in the absence of MR). This supports the elegant model of total body K⁺ regulation developed by Young and colleagues 30–33 30 years ago. It should be noted, however, that the effect of aldosterone (MR) is multiplicative with that of plasma [K⁺], so that the effects of aldosterone are increasingly apparent when mice or humans are challenged.

Young31 also suggested that aldosterone strongly affects internal K⁺ balance by enhancing K⁺ uptake into cells throughout the body. Our model provided one opportunity to test this directly, because an infusion of aldosterone should reduce the plasma [K⁺] under these conditions. Against predictions, aldosterone infusion at concentrations that were clearly effective in control mice did not alter plasma [K⁺] in KS MR⁻/⁻ mice. At least two issues, however, complicate the interpretation.

First, although the Pax8 system is relatively specific for kidney epithelia, substantial gene deletion also occurs in the liver, which may have contributed to extrarenal K⁺ uptake. Second, extrarenal MR may be stimulated in KS MR⁻/⁻ mice at baseline, because plasma [aldosterone] is elevated; the addition of supraphysiologic aldosterone, then, may have little additional effect.

As expected, MR deletion had a profound effect on ENaC activity (inferred from the Na⁺ wasting and K⁺ retention). Deletion of MR along the entire nephron led to a shift of ENaC away from the apical membrane, a reduction in α-ENaC abundance, and impaired cleavage; these observations generally concord with the work of others. On a low-salt diet, ENaC abundance and cleavage did not change further, suggesting that aldosterone itself is the dominant ENaC regulatory factor. Although angiotensin II has been reported to stimulate ENaC, the lack of ENaC stimulation observed here is consistent with evidence from angiotensin receptor knockout studies of Coffman and colleagues.36 Here, we also found a marked decrease in apical membrane γ-ENaC and nearly absent cleavage,

Figure 7. Aldosterone appears to stimulate ENaC directly, but NCC indirectly, to modulate blood pressure. (A) Effects of aldosterone and high-K⁺ diet (HK) on plasma [K⁺], (B) effect of aldosterone and diet on ENaC, (C–E) quantification of results in A, (F) effect of aldosterone and diet on NCC, (G and H) quantification of results in E, (I) relationship between plasma [K⁺] and pNCC in control and KS MR⁻/⁻ mice (data are pooled from all presented experiments, and some of control data have been published previously), (J) BP in control and KS MR⁻/⁻ mice on low-K⁺ (LK) diet (J, Inset shows mean values during the active period), and (K) effect of high-salt intake on BP in LK diet–treated mice (K, Inset shows mean values during the active period). Animals in A–H, n=3–4. For BP data, n=3 controls and n=4 knockouts. Mean arterial pressure (MAP) tracings are averages of all animals. For BP analyses, unpaired t tests performed on data displayed in bar charts were not statistically significant. *P<0.05.
even when mice were treated with low-salt or high-K⁺ diets. Prostasin plays an important role in γ-ENaC cleavage.⁴⁷ Although prostasin was strikingly enriched in the ASDN, MR deletion had little or no effect on either its distribution or abundance.

Surprisingly, SGK1 abundance was also unaltered in the KS MR⁻/⁻ mice, suggesting that the chronic suppression of ENaC activity is not related to changes in SGK1 signaling. Although we measured only total kidney SGK1, our results concord with those in the work by Alvarez de la Rosa et al.,⁴⁸ which reported that low-salt diet does not affect the distribution or intensity of SGK1 in the kidney.

The low BP in KS MR⁻/⁻ mice resulted from the combined deficiency of NCC and ENaC activity in the setting in which transport proteins along more proximal nephron segments are likely activated by extracellular fluid volume depletion.⁴⁹ Soleimani and colleagues⁴⁰,⁴¹ have shown how defects in salt reabsorption along sequential nephron segments often lead to profound salt wasting, whereas defects along one of the segments do not. Deletion of renal MR seems to present an interesting variant on this model; although only ENaC seemed to be inhibited directly by MR deletion, NCC abundance and activity were suppressed secondarily by high plasma [K⁺]. Although we used pNCC abundance to estimate rather than measure NCC activity, Figure 7 shows that arterial pressure and pNCC abundance track together, suggesting that the biochemical effects reflect transporter activity.

These results, thus, elucidate the delicate interplay between aldosterone-regulated ENaC and K⁺-regulated NCC (Figure 8). When, for example, primary aldosteronism stimulates ENaC, this reduces plasma [K⁺], activating NCC and contributing to hypertension.⁴² When aldosterone secretion is deficient, such as in Addison syndrome, ENaC cannot be activated, plasma [K⁺] rises, and NCC is suppressed; this causes salt wasting and low BP.

In summary, these results show the essential role that MRs play in electrolyte balance and highlight the importance of the DCT in these effects. They indicate that MRs are not necessary for NCC activation but instead, act through ENaC and plasma [K⁺] to activate the transporter. The results show how diminution of both ENaC and NCC activity is required for the profound effects of MR deletion on arterial pressure. Finally, they indicate how the tight control of salt and K⁺ balance is dependent on both direct and indirect effects of the mineralocorticoid hormone, aldosterone.

**CONCISE METHODS**

**Animals**

Studies were approved by Oregon Health and Science University’s Animal Care and Usage Committee (Protocol IS3286). KS MR⁻/⁻ mice were generated through use of the Pax8-rtTA/LC1 system⁴⁴ as before.⁴⁵ Mice were genotyped as described in Supplemental Material. To induce Cre-mediated recombination, mice were treated with 2 mg/ml doxycycline hyclate in 5% sucrose drinking water for 2 weeks. All mice used for experiments were 12–24 weeks old.

**BP Measurement**

BP was measured by radiotelemetry. Two-hour averages were used to calculate mean arterial pressures.

**Metabolic Cage Studies**

Animals were acclimated to metabolic cages (Hatteras Instruments) for 2 days before urine collection. Animals were fed a gel diet with indicated mineral content (Harlan Industries, Indianapolis, IN) (Supplemental Material, Animal Diets and Measurement of Food Consumption). Urine was collected under water-saturated light mineral oil every 24 hours. Urine Na⁺ and K⁺ contents were determined by flame photometry.

**Blood Collection and Electrolyte Measurements**

Whole blood was collected by cardiac puncture under anesthesia. Plasma was removed and stored at −20°C.

**Kidney Western Blot**

Kidneys were removed, snap frozen, and homogenized.⁴³ Homogenate was centrifuged, and supernatant was transferred to a new tube. After total protein quantification, 40 μg protein per sample was separated on either a 4%–12% Bis-Tris or a 3%–8% Tris-acetate gel (Invitrogen) and transferred overnight to a PVDF membrane. Membranes were then washed, incubated with an HRP-coupled secondary
antibody (1:5000; Invitrogen, Carlsbad, CA), washed again, and imaged using a Western Lightning Kit (PerkinElmer, Waltham, MA).

Perfusion Fixation and Immunofluorescence Imaging

Kidneys were fixed in vivo as described; 5-μm sections were washed, blocked in 5% nonfat milk, and incubated overnight in primary antibody. The next day, sections were washed, incubated with Cy-3–coupled secondary antibody (Invitrogen), and embedded.

Aldosterone Infusion and Plasma Concentration Measurement

Aldosterone was infused at a concentration of 240 μg/kg per day for 7 days by osmotic minipump (Alzet, Cupertino, CA). Plasma aldosterone concentration was measured by ELISA (IBL America).

Statistical Analyses

The null hypothesis was tested using unpaired t tests, two-way ANOVA, or two-way ANOVA for repeated measures as indicated in the figures and text.

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DISCLOSURES

None.

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