30 YEARS OF THE MINERALOCORTICOID RECEPTOR

Mineralocorticoid receptor and NaCl transport mechanisms in the renal distal nephron

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Abstract

A key role of aldosterone and mineralocorticoid receptor is to regulate fluid volume and K⁺ homeostasis in the body by acting on the renal distal nephron. Global responses of the kidney to elevated aldosterone levels are determined by the coordinate action of different constituent tubule cells, including principal cells, intercalated cells and distal convoluted tubule cells. Recent studies on genetic mutations causing aldosterone overproduction have identified the molecules involved in aldosterone biosynthesis in the adrenal gland, and there is also increasing evidence for mechanisms and signaling pathways regulating the balance between renal NaCl reabsorption and K⁺ secretion, the two major effects of aldosterone. In particular, recent studies have demonstrated that mineralocorticoid receptor in intercalated cells is selectively regulated by phosphorylation, which prevents ligand binding and activation. Moreover, the ubiquitin ligase complex composed of Kelch-like 3 and Cullin 3 acts downstream of angiotensin II and plasma K⁺ alterations, regulating Na–Cl cotransporter independently of aldosterone in distal convoluted tubule cells. These and other effects are integrated to produce appropriate kidney responses in a high-aldosterone state, and are implicated in fluid and electrolyte disorders in humans. This review summarizes the current knowledge on mechanisms modulating mineralocorticoid receptor and its downstream effectors in the distal nephron.

Key Words

- ▶ angiotensin
- ▶ plasma K
- ▶ KLHL3
- ▶ ubiquitin proteasome system
- ▶ phosphorylation

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Introduction

The main function of the kidney is to maintain constant fluid volume and composition by altering the functioning of various cells lining the nephron. Changes in electrolyte balance are transmitted to individual cells through multiple signaling pathways, inducing appropriate responses to physiological perturbations and altered intake. The steroid hormone aldosterone is produced in zona glomerulosa

cells of the adrenal cortex and it regulates Na⁺, K⁺ and Cl[–] balance in the body mainly by acting on the distal part of the renal nephron (which normally includes distal convoluted tubules (DCTs), connecting tubules and collecting duct). Although NaCl reabsorbed in the distal segment represents only a fraction of that handled by the nephron, fine tuning occurs in this segment, which determines total body salt

and water content. Cells of the distal nephron also play important roles in controlling extracellular K^+ levels, and dysregulation of transporters and channels in these segments is a major cause of altered blood pressure and plasma K^+ levels (Lifton *et al.* 2001, Boyden *et al.* 2012).

Aldosterone is produced in two distinct physiological states, hypovolemia (intravascular volume depletion) and hyperkalemia. In the former, activation of the renin-angiotensin system induces angiotensin II (AngII) type 1 receptor signaling in glomerulosa cells, stimulating aldosterone biosynthesis via Ca^{2+} signaling (Spat & Hunyady 2004, Boulkroun *et al.* 2015). On the other hand, hyperkalemia stimulates aldosterone production by directly depolarizing the cells and activating voltage-gated Ca^{2+} channels (Spat & Hunyady 2004, Boulkroun *et al.* 2015). Aldosterone, in turn, increases mineralocorticoid receptor (MR) transcriptional activity in the distal nephron and regulates the activity of electrolyte transport mediators, which maximizes either NaCl reabsorption or K^+ secretion depending on the physiological context. Recent genetic studies of familial electrolyte imbalances as well as biochemical analyses of renal electrolyte flux regulators and upstream signaling pathways have provided insights into the molecular mechanisms that modulate the action of aldosterone in the distal nephron. In this review, the current knowledge on mechanisms that regulate electrolyte flux mediators in cells lining the distal nephron is summarized (i.e., principal cells, intercalated cells, and DCT cells (Fig. 1)). Accumulating evidence indicates that both MR-dependent and -independent factors are involved. In addition, how these mechanisms are integrated to produce appropriate

responses upon physiological perturbations and under pathological conditions is also discussed.

Role of principal cells in NaCl and K^+ regulation

Principal cells express epithelial Na^+ channel (ENaC), a heterotrimeric protein composed of α , β and γ subunits (Pearce *et al.* 2015). While most Na^+ transport occurring in the renal tubules is coupled to the transport of other anions or cations (e.g., Na^+-H^+ exchanger 3 or Na^+ -glucose cotransporters in the proximal tubules, $Na^+-K^+-2Cl^-$ cotransporter 2 in the thick ascending limb and $Na-Cl$ cotransporter (NCC) in the DCT), only Na^+ ions are transported through ENaC. This electrogenic Na^+ reabsorption generates a lumen-negative potential, and establishes an electrochemical gradient that drives Cl^- and K^+ transport. Loss-of-function mutations in this channel not only affect Na^+ reabsorption but also perturb Cl^- and K^+ transport (Lifton *et al.* 2001). Conversely, gain-of-function mutations in this channel cause high blood pressure and hypokalemia (Table 1) (Shimkets *et al.* 1994, Staub *et al.* 1996, Kamynina & Staub 2002). As discussed later, the most important regulator of ENaC is aldosterone.

Apical K^+ secretion in principal cells is a critical determinant of the amount of K^+ excreted in urine and it is regulated by renal outer medullary K^+ (ROMK) channel. Like other inwardly rectifying K^+ channels, ROMK forms a tetramer and is highly selective for K^+ over Na^+ and other ions. The ROMK gene contains several exons that produce alternatively spliced transcripts, and principal cells of the cortical collecting duct express ROMK1 and

Figure 1

Cells comprising the renal distal nephron. Distal convoluted tubule (DCT) cells express thiazide-sensitive $Na-Cl$ cotransporter (NCC) at the apical membrane. Principal cells of the collecting duct express epithelial Na^+ Channel (ENaC) and renal outer medullary K^+ channel (ROMK) at the apical membrane. Intercalated cells are divided into three types; α -intercalated cells express H^+ -ATPase, whereas β -intercalated cells express Cl^-/HCO_3^- exchanger pendrin at the apical membrane. Non- α , non- β intercalated cells are characterized by the expression of both pendrin and H^+ -ATPase at the apical membrane.

Table 1 Human conditions related to dysregulation of MR or downstream effectors in the distal nephron.*

Disease or disorder	Clinical characteristics	Genetic cause or suggested mechanism
Pseudohypoaldosteronism type 1 (PHA1)	Neonatal salt wasting, hyperkalemia, metabolic acidosis	Loss-of-function mutation in MR or ENaC
Hypertension exacerbated by pregnancy	Early onset hypertension, markedly accelerated in pregnancy	MR mutation leading to activation by steroids lacking 21-hydroxyl (e.g., progesterone)
Apparent mineralocorticoid excess (AME)	Early onset hypertension, hypokalemia, metabolic alkalosis	MR activation by cortisol due to mutation in 11 β HSD2
Liddle syndrome	Early onset hypertension and hypokalemic alkalosis	Mutation in ENaC β or γ resulting in reduced clearance by Nedd4-2
Gitelman syndrome	Hypokalemia, metabolic alkalosis, mild hypotension	Loss-of-function mutation in NCC
Pendred syndrome	Hearing loss and thyroid abnormality; potential for hypokalemia, metabolic alkalosis and fluid loss	Loss-of-function mutation in pendrin
Pseudohypoaldosteronism type 2 (PHAII or FHHT)	Hypertension, hyperkalemia and metabolic acidosis	NCC overactivity due to mutation in KLHL3/CUL3 or WNK
Licorice-induced hypertension	Hypertension, hypokalemia, metabolic alkalosis	Inhibition of 11 β HSD2 by glycyrrhetic acid
CNI-induced hypertension	Hypertension associated with the use of CNIs such as tacrolimus and cyclosporine	NCC activation via WNK-SPAK pathway

*Disorders resulting from the increased circulating mineralocorticoids are not included in the table.

11 β HSD2, 11 β -hydroxysteroid dehydrogenase 2; CNI, calcineurin inhibitor; CUL3, cullin-3; ENaC, epithelial Na channel; FHHT, familial hyperkalemic hypertension; KLHL3, Kelch-like 3; MR, mineralocorticoid receptor; NCC, Na-Cl cotransporter; SPAK, STE20/SPS1-related kinase; WNK, with-no-lysine.

ROMK2 (Hebert *et al.* 2005). ROMK is sensitive to changes in pH, and is inhibited by the decrease in intracellular pH levels (Hebert *et al.* 2005). Several other upstream regulators are known to regulate ROMK such as serum and glucocorticoid-induced kinase 1 (Sgk1) and Src family protein tyrosine kinase. Sgk1 facilitates ROMK activity by phosphorylating S44 (Yoo *et al.* 2003). On the other hand, Src phosphorylates ROMK at Y337, which facilitates ROMK internalization (Lin *et al.* 2004). ROMK is also known to be inhibited by serine threonine kinase WNK4 (With-No-Lysine 4) via kinase-independent mechanism (Kahle *et al.* 2003), which acts in concert with Sgk1 and Src (Lin *et al.* 2015).

MR signaling in principal cells of the collecting duct

MR is abundantly expressed in principal cells of the collecting duct and the role of aldosterone and MR in these cells has been extensively characterized (Pearce *et al.* 2015). The most important downstream effector of MR signaling in these cells is the amiloride-sensitive epithelial Na⁺ channel (ENaC). Principal cells highly express 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2), which catalyzes the conversion of cortisol to inactive

cortisone and allows selective MR activation by aldosterone (Funder *et al.* 1988). In apparent mineralocorticoid excess (AME), decreased 11 β HSD2 activity due to a hereditary defect or pharmacological inhibition causes cortisol accumulation in these cells, resulting in MR activation by cortisol. The most common cause of secondary AME is the intake of licorice, whose active ingredient glycyrrhizic acid inhibits 11 β HSD2 activity (Table 1).

Once activated, MR controls ENaC function at multiple levels mainly by regulating the Ser/Thr kinase Sgk1 and the ubiquitin ligase Nedd4-2 (Pearce *et al.* 2015). Firstly, MR controls the stability of ENaC at the plasma membrane. Sgk1 transcription is rapidly induced by aldosterone, and is then activated by phosphatidylinositol 3-kinase and mammalian target of rapamycin complex 2 (Lang & Pearce 2016). Phosphorylated active Sgk1 in turn phosphorylates Nedd4-2, a homologous to the E6-AP carboxyl terminus (HECT) domain-containing E3 ubiquitin ligase that binds the C-terminal PY motif of ENaC to induce its degradation. Sgk1 phosphorylation of Nedd4-2 triggers the binding of 14-3-3 proteins, thereby inhibiting the association between ENaC and Nedd4-2 (Bhalla *et al.* 2005, Lang & Pearce 2016). This prevents ubiquitination, internalization and degradation, resulting in increased plasma membrane levels of ENaC. In Liddle syndrome, nonsense or

frameshift mutations in *SCNN1B* (encoding ENaC β) and *SCNN1G* (encoding ENaC γ) eliminate the C-terminal PY motif, abrogating the Nedd4-2–ENaC association similar to biological effects of Nedd4-2 phosphorylation by Sgk1 (Shimkets *et al.* 1994, Staub *et al.* 1996, Kamynina & Staub 2002). Besides these mechanisms, ENaC stability at the plasma membrane is controlled by the aldosterone-induced proteins Glucocorticoid-induced leucine-zipper protein 1 and the connector enhancer of kinase suppressor of Ras isoform 3, both of which form the multiprotein ENaC regulatory complex (Pearce *et al.* 2015).

Secondly, aldosterone regulates ENaC gene expression. The promoter of *SCNN1A* (encoding ENaC α) contains a glucocorticoid-responsive element, with gene transcription induced by aldosterone (Mick *et al.* 2001). Interestingly, this effect appears to be partly mediated by Sgk1. Acute lymphocytic leukemia 1-fused gene from chromosome 9 (Af9), a putative transcription factor, binds to the histone H3 Lys-79 methyltransferase Dot1a to regulate the histone methylation of the *SCNN1A* promoter. Sgk1 phosphorylates Af9 and prevents the Dot1a–Af9 interaction, leading to histone hypomethylation and increased *SCNN1A* transcription (Zhang *et al.* 2007).

Thirdly, aldosterone increases ENaC open probability via the regulation of serine proteases. Among the three subunits of ENaC, α and γ subunits are activated by cleavage of the inhibitory domain in the extracellular loop, which is mediated by cell-surface or soluble extracellular serine proteases such as prostasin, plasmin and kallikrein (Vallet *et al.* 1997, Narikiyo *et al.* 2002, Svenningsen *et al.* 2015). For example, prostasin promotes the cleavage of the ENaC γ protein inhibitory domain (Narikiyo *et al.* 2002). Importantly, urinary prostasin levels are increased in subjects with primary aldosteronism, and is reduced after adrenalectomy (Narikiyo *et al.* 2002), suggesting that hyperaldosteronism promotes ENaC activity via protease induction and cleavage of the inhibitory domain. Urine from patients with nephrotic syndrome shows increased plasmin levels and serine protease activity, which contributes to increased ENaC by directly cleaving ENaC γ (Svenningsen *et al.* 2009).

Na⁺ reabsorbed through ENaC is transported to the extracellular fluid via Na⁺/K⁺-ATPase, a heteromeric protein composed of α , β and γ subunits. The activity of Na⁺/K⁺-ATPase in principal cells is increased in response to aldosterone, and several lines of evidence suggest that both direct and indirect effects are involved. Studies have shown that the induction of Na⁺/K⁺-ATPase activity by aldosterone was inhibited by amiloride

(Hayhurst & O'Neil 1988). Consistent with these findings, a recent study reported that ENaC induction increased Na⁺/K⁺-ATPase levels and activity in mouse cortical collecting duct cells (Wang *et al.* 2014). This effect was regulated at the post-transcriptional levels through the inhibition of p38 kinase-mediated endocytosis of Na⁺/K⁺-ATPase (Wang *et al.* 2014). Besides these mechanisms, aldosterone can increase the transcription of *ATP1A1* and *ATP1B1* subunits (Tsuchiya *et al.* 1996, Setiawan *et al.* 2002, Alvarez de la Rosa *et al.* 2006). Several studies have also suggested that Sgk1 is involved in the regulation of Na⁺/K⁺-ATPase (Verrey *et al.* 2003a,b). Thus, the available evidence indicates that Na⁺/K⁺-ATPase activity is coordinately regulated by aldosterone and by the Na⁺ entry through ENaC.

Role of intercalated cells in fluid and electrolyte homeostasis

While principal cells constitute the majority of cells lining the collecting duct, distinct cell types are also present in this segment, which are grouped together and known as intercalated cells. These are further divided into α -, β -, and non- α , non- β intercalated cells according to the subcellular localization of H⁺-ATPase and Cl[−]/HCO₃[−]-exchangers. α -Intercalated cells express H⁺-ATPase and the Cl[−]/HCO₃[−] exchanger AE1 at the apical and basolateral membranes, respectively. In contrast, β -intercalated cells express the Cl[−]/HCO₃[−] exchanger pendrin and H⁺-ATPase at the apical and basolateral membranes, respectively. Non- α , non- β intercalated cells are characterized by the expression of both pendrin and H⁺-ATPase at the apical membrane.

Intercalated cells have long been recognized for their role in acid/base homeostasis; however, there is accumulating evidence to suggest that they also control extracellular fluid volume and K⁺ homeostasis. As mentioned earlier, the activation of ENaC in principal cells creates an electrochemical gradient that promotes either K⁺ secretion or Cl[−] reabsorption in the collecting duct (Welling 2013, Pearce *et al.* 2015). K⁺ is excreted via ROMK in principal cells, whereas Cl[−] ions are transported either para- or transcellularly (Hou *et al.* 2013, Wall & Weinstein 2013). In the former process, Cl[−] permeability of the cell–cell junction is determined by several claudins (Hou *et al.* 2013), whereas the latter process is primarily mediated by pendrin in β -intercalated cells (Royaux *et al.* 2001, Wall *et al.* 2004). Indeed, Cl[−] flux in the cortical collecting duct was abolished in mice lacking

pendrin, which resulted in a blunted pressor response to mineralocorticoids (Wall *et al.* 2004). Moreover, mice lacking both pendrin and NCC showed severe volume depletion (Soleimani *et al.* 2012), demonstrating the compensatory role of these Cl⁻ transporters. Conversely, pendrin overexpression induced salt-dependent hypertension (Jacques *et al.* 2013). These findings are also relevant to humans, because individuals with Pendred syndrome (who have loss-of-function mutations in *SLC26A4* (encoding pendrin)) are extremely sensitive to thiazide diuretics (Pela *et al.* 2008). In addition, several reports have shown that the individuals with Pendred syndrome can potentially develop life-threatening hypokalemia associated with volume depletion and metabolic alkalosis (Kandasamy *et al.* 2011, Sanei-Moghaddam *et al.* 2011), providing further evidence that pendrin indeed regulates electrolyte balance.

Studies have also suggested the role of H⁺-ATPase in intercalated cells in NaCl and K⁺ homeostasis. Unlike in other cell types, the resting membrane potential of intercalated cells is determined by the activity of H⁺-ATPase rather than that of Na⁺/K⁺-ATPase (Chambrey *et al.* 2013). The B1 subunit of H⁺-ATPase is selectively expressed in renal intercalated cells, and mutations in the gene encoding this protein (*ATP6V1B1*) cause metabolic acidosis and hypokalemia (Karet *et al.* 1999, Finberg *et al.* 2003). Although fluid status has not been extensively studied in humans, mice lacking B1 H⁺-ATPase showed a salt loss phenotype (Gueutin *et al.* 2013). Although the underlying mechanism is not entirely clear, B1 H⁺-ATPase regulates ENaC levels via prostaglandin E2 paracrine signaling (Gueutin *et al.* 2013). Other studies have shown that apical H⁺-ATPase activity provides a driving force for transcellular Cl⁻ transport in the collecting duct (Pech *et al.* 2007).

Although the primary target of thiazide diuretics is NCC in DCTs, it is thought that NaCl transport mechanisms sensitive to thiazide also exist in the cortical collecting duct (Terada & Knepper 1990). Since ENaC is not inhibited by thiazide, this mechanism was presumed to be independent of electrogenic Na⁺ transport in principal cells. Subsequent studies have characterized the mechanism as one involving Na⁺-driven Cl⁻/2HCO₃⁻-exchanger (NDCBE) in β -intercalated cells; it was proposed that parallel operation of this exchanger and pendrin results in electroneutral NaCl transport (Leviel *et al.* 2010). A recent follow-up study reported the physiological effects resulting from the loss of NDCBE. As the renal salt transport mechanism is highly redundant, the effects of NDCBE deletion are largely compensated by other NaCl

transport mechanisms along the nephron (Sinning *et al.* 2016). Double knockout of NDCBE and NCC resulted in hypokalemia and signs of volume contraction, although the phenotype was milder than that of pendrin and NCC double-knockout mice. These data support the role of NDCBE but also indicate that fluid volume regulation through pendrin is not solely dependent on NDCBE.

MR signaling in intercalated cells of the collecting duct

As stated above, recent studies have shown that membrane transporters present in intercalated cells regulate blood pressure and electrolyte balance. Although the detailed biochemical pathways regulating their function are less well characterized than those of ENaC in principal cells, it is known that intercalated cells express MR (Ackermann *et al.* 2010, Izumi *et al.* 2011). In addition, aldosterone as well as other mineralocorticoids regulates intercalated cell function. Aldosterone stimulates acid secretion in α -intercalated cells (Garg & Narang 1988). Pendrin in β -intercalated cells is also induced by chronic infusion of aldosterone (Verlander *et al.* 2003). However, the effects of mineralocorticoids in these cells may not be apparent depending on the physiological context (Mohebbi *et al.* 2013), indicating that modulation of MR signaling is complex and involves mechanisms that are distinct from those in principal cells.

Besides circulating ligands, there is increasing evidence to suggest that the function of steroid and other nuclear receptors are modulated at the receptor level by multiple factors. In particular, post-translational modification significantly alters nuclear receptor function (Gaillard *et al.* 2006, Choi *et al.* 2010, Kino *et al.* 2010). Several studies have shown that MR is phosphorylated, which is mediated by kinases such as cyclin-dependent kinase 5 and mitogen-activated protein kinase (Alnemri *et al.* 1991, Galigniana 1998, Kino *et al.* 2010, Faresse *et al.* 2012). However, the significance of phosphorylation in the regulation of MR function was unclear. Using a phospho-proteomics approach (Rinehart *et al.* 2009), we identified 16 phosphorylation sites in full-length human MR (Shibata *et al.* 2013a). Phosphorylation at S843 (the only site in the ligand-binding domain) had the greatest functional effect in a luciferase reporter assay, and previous studies have reported that this site is responsible for the difference in ligand selectivity between glucocorticoid receptor and MR (Ortlund *et al.* 2007). The importance of S843 for MR function was also suggested by the

observation that it is mutated (p.Ser843Pro) in subjects with pseudohypoaldosteronism type I (PHA1) (Fernandes-Rosa *et al.* 2011). A binding assay using a MR^{S843E} phosphomimetic revealed that phosphorylation severely impaired aldosterone binding; accordingly, cell culture experiments demonstrated that phosphorylated MR is exclusively cytoplasmic in the presence of physiological concentrations of its ligand aldosterone.

In the kidney, MR phosphorylated at S843 (MR^{S843-P}) is almost exclusively localized in the cytoplasm of renal intercalated cells (Shibata *et al.* 2013a), and is not detected in principal or DCT cells. Of note, hypovolemia via AngII signaling and K⁺ loading decreased and increased MR^{S843-P} levels, respectively. The former was associated with an increased nuclear accumulation of MR in intercalated cells, consistent with the *in vitro* finding that MR^{S843-P} regulates ligand binding and nuclear translocation. Moreover, in volume depletion, we found that dephosphorylation of MR^{S843-P} results in ligand-dependent increases in intercalated cell apical H⁺-ATPase and pendrin. Thus, the ligand-binding ability of MR is selectively regulated by phosphorylation in intercalated cells, which controls Cl⁻ reabsorption mechanisms involving intercalated cells while minimizing changes in acid/base balance.

Interestingly, we recently found that these mechanisms also regulate plasma K⁺ concentrations

(Xu *et al.* 2017). The decrease in plasma K⁺ levels dephosphorylates MR^{S843-P}, which facilitates ligand-dependent MR activation and increases pendrin levels (Fig. 2). This mechanism promotes Cl⁻ reabsorption but instead reduces K⁺ secretion in the distal nephron. Indeed, genetic ablation of pendrin aggravates hypokalemia in aldosterone excess (Xu *et al.* 2017), demonstrating that intercalated cell Cl⁻ flux through pendrin regulates not only blood pressure but also plasma K⁺ levels. Thus, hypokalemia dephosphorylates MR^{S843-P}, which promotes MR activation and pendrin expression, counteracting the progression of hypokalemia but instead promoting hypertension. The proposed mechanism may also explain why individuals with Pendred syndrome have the potential to develop severe hypokalemia and show the impaired ability to reduce K⁺ excretion in the face of reduced total body K⁺ (Pela *et al.* 2008, Kandasamy *et al.* 2011, Sanei-Moghaddam *et al.* 2011).

MR^{S843-P} has been detected in the kidney but not in other tissues known to express MR, including brain, heart, colon and vasculature. However, considering the low expression levels of total MR in these organs relative to the renal tubules, it is still possible that MR^{S843-P} is expressed outside the kidney. Given that signaling cross-talk between MR and AT1R has been reported in vascular smooth muscle cells, a role in the cardiovascular system

Figure 2

Regulation of MR in β -intercalated cells. In principal cells, aldosterone binds to MR and activates ENaC, which drives K⁺ secretion through apical K⁺ channel ROMK. The resultant decrease in plasma K⁺ levels causes MR^{S843-P} dephosphorylation in β -intercalated cells, allowing aldosterone binding to MR in these cells and increasing pendrin at the plasma membrane. Reproduced, with permission, from Xu N, Hirohama D, Ishizawa K, Chang WX, Shimosawa T, Fujita T, Uchida S & Shibata S (2017) Hypokalemia and pendrin induction by aldosterone. *Hypertension* [in press].

may be worth investigating. Studies on the role of cortisol in regulating MR in intercalated cells are also the areas of future investigation (Funder 2013).

Mechanisms regulating NCC in DCT cells

Thiazide-sensitive NCC regulates salt transport in DCTs, contributing to fluid volume homeostasis. The key regulatory mechanism of NCC was elucidated by genetic and biochemical analyses of PHAI pathogenesis. PHAI, also known as Gordon syndrome or familial hyperkalemic hypertension, is characterized by pathological NCC hyperactivity; hypertension and hyperkalemia in these patients can be reversed by thiazide treatment. Mutations in four genes are known to cause PHAI (Wilson *et al.* 2001, Boyden *et al.* 2012, Louis-Dit-Picard *et al.* 2012). Two of these encode the serine threonine kinases With-no-lysine (WNK) 1 and WNK4, whereas the other two genes encode Cullin 3 (CUL3) and Kelch-like 3 (KLHL3), both of which are components of a Really Interesting New Gene (RING) E3 ubiquitin ligase complex.

Kinase-active WNKs phosphorylate the downstream kinases STE20/SPS1-related proline-alanine-rich protein kinase (SPAK) and oxidative stress-responsive (OSR) 1 (Vitari *et al.* 2005), which in turn phosphorylate NCC at the C terminus and thereby activate the transporter. WNK1 has a Cl⁻ binding pocket formed by Leu369, Leu371 and surrounding amino acids (Piala *et al.* 2014). Binding of Cl⁻ to WNK1 precludes auto-phosphorylation and activation. Similarly, WNK4 is sensitive to Cl⁻ and is inactivated by elevations in intracellular Cl⁻ levels and by the mutation of Leu322 (which corresponds to Leu369 in WNK1) (Piala *et al.* 2014, Bazua-Valenti *et al.* 2015). Sensitivity to Cl⁻ differs among WNKs, with WNK4 having the highest sensitivity (Terker *et al.* 2016). The KLHL3/CUL3 complex binds and targets WNK1 and WNK4 for proteasomal degradation; disease-causing mutations impair this interaction (Ohta *et al.* 2013, Shibata *et al.* 2013b, Wakabayashi *et al.* 2013). In particular, PHAI mutations in the Kelch domain of KLHL3 and in an acidic domain of WNK4 both prevent KLHL3–WNK4 binding.

DCT cells express MR, and 11 β HSD2 is also present in the distal portion of DCTs (DCT2, which co-expresses NCC and ENaC) (Campean *et al.* 2001). Aldosterone infusion increases NCC activity, and endogenous aldosterone production induced by diuretic treatment also leads to NCC upregulation (Velazquez *et al.* 1996, Kim *et al.* 1998). This is likely controlled at the post-translational level, since NCC induction is not accompanied by an increase in

transcript abundance (Abdallah *et al.* 2001). Recent studies have indicated that the ubiquitin ligase Nedd4-2 and Ser/Thr kinase WNK1 mediate the effects of aldosterone on NCC. Although Nedd4-2 is a regulator of ENaC in principal cells, accumulating evidence suggests that it also regulates NCC (Arroyo *et al.* 2011, Ronzaud *et al.* 2013). Nedd4-2 co-immunoprecipitates with NCC and its deficiency in DCT cells results in NCC upregulation (Arroyo *et al.* 2011). Accordingly, total and phosphorylated NCC levels are increased in the kidney of tetracycline-inducible, nephron-specific Nedd4-2 knockout mice (Ronzaud *et al.* 2013). Unlike for ENaC, regulation of NCC by Nedd4-2 does not require the PY-like motif of NCC, suggesting that it may occur via an indirect mechanism.

A later study identified alternatively spliced exons embedded within a proline-rich region of WNK1 that contains PY motifs; these can be bound by Nedd4-2, which targets PY motif-containing WNK1 isoforms for proteasomal degradation (Fig. 3) (Roy *et al.* 2015). Because WNK1 is a potent activator of the SPAK/OSR–NCC signaling cascade, these data suggest a mechanism whereby Nedd4-2 inhibits NCC. Moreover, aldosterone can regulate this process via the induction of Sgk1, which inhibits WNK1 degradation by inactivating Nedd4-2 and also induces WNK1 activity by phosphorylating its N terminus (Roy *et al.* 2015). Thus, the regulation of PY-containing WNK1 isoforms by Sgk1 and Nedd4-2 serves as the link between MR signaling and NCC induction in DCT cells (Fig. 3).

On the other hand, there is increasing evidence suggesting that NCC function in DCT cells is regulated independently of aldosterone. For example, AngII signaling regulates NCC via a mechanism that does not require aldosterone (van der Lubbe *et al.* 2013). In an effort to identify physiological mechanisms whereby AngII regulates NCC, we have identified phosphorylation of S433 in the Kelch (substrate-binding) domain of KLHL3 that turns off the function of KLHL3/CUL3 ubiquitin ligase (Shibata *et al.* 2014). S433 is shown to be repeatedly mutated in PHAI families, supporting its functional importance in substrate binding; in these cases, either KLHL3^{S433N} or KLHL3^{S433G} substitution was sufficient to cause NCC activation, resulting in blood pressure elevation and hyperkalemia (Boyden *et al.* 2012, Louis-Dit-Picard *et al.* 2012).

A biochemical analysis revealed that KLHL3 phosphorylated at S433 (KLHL3^{S433-P}) was incapable of substrate binding, resulting in impaired degradation and accumulation of WNK4. Phosphorylation at this site was regulated by protein kinase C (PKC) acting

Figure 3

MR-dependent regulation of NCC in DCT cells. Aldosterone and MR increase serine/threonine kinase SGK1 abundance by regulating its transcription. SGK1 phosphorylates ubiquitin ligase Nedd4-2, which reduces its interaction with exons 11 and 12 of WNK1, preventing WNK1 degradation via the ubiquitin proteasome system (UPS). SGK1 then phosphorylates and increases WNK1 kinase activity. Active WNK1 in turn promotes SPAK and OSR1 activation, which directly stimulates NCC by phosphorylation.

downstream of AngII (Shibata *et al.* 2014). The resultant decrease in KLHL3/CUL3 ubiquitin ligase activity leads to accumulation of WNKs, activating the SPAK/OSR1-NCC cascade. This pathway does not require aldosterone or MR signaling to induce NCC. Besides these mechanisms, there is also evidence that AngII modulates WNK function (San-Cristobal *et al.* 2009). Of note, a recent study identified WNK4 sites that are phosphorylated downstream of AngII signaling (Castaneda-Bueno *et al.* 2017). Interestingly, AngII phosphorylation of WNK4 modulates WNK4 kinase activity by promoting autophosphorylation of S332 of the T-loop in the kinase catalytic domain.

Plasma K⁺ levels also alter NCC activity independently of aldosterone. K⁺ loading decreased NCC phosphorylation in DCTs even when plasma aldosterone was elevated (Sorensen *et al.* 2013, Rengarajan *et al.* 2014). In a model of PHAI lacking ENaCα in the kidney, hyperkalemia was shown to determine NCC activity regardless of salt wasting and high plasma aldosterone (Perrier *et al.* 2015). Conversely, K⁺ restriction increased NCC activity under aldosterone suppression (Castaneda-Bueno *et al.* 2014).

To evaluate the possible involvement of KLHL3/CUL3-based ubiquitin ligase in low-K⁺-mediated NCC induction, we recently analyzed KLHL3 levels in mice on a low-K⁺ diet (Ishizawa *et al.* 2016). Notably, we found that KLHL3^{S433-P} levels were increased whereas total KLHL3 levels were decreased in the kidney by K⁺ depletion. These data indicate that KLHL3/CUL3 ubiquitin ligase activity is suppressed by the net effect of KLHL3^{S433-P} induction and decrease in the total levels. In fact, these changes were accompanied by the accumulation of the target substrate WNK4 and activation of the downstream kinases SPAK and OSR1, resulting in NCC phosphorylation and plasma membrane accumulation. Increased S433 phosphorylation was attributed to higher levels of active, phosphorylated PKC. This was the first demonstration of the involvement of KLHL3/CUL3 ubiquitin ligase in aldosterone-independent, low-K⁺-mediated NCC induction (Fig. 4) (Ishizawa *et al.* 2016).

Besides the KLHL3/CUL3 complex, recent data indicate that the kinase activity of WNK is also regulated by changes in plasma K⁺ levels (Fig. 4) (Terker *et al.* 2015b, Hadchouel *et al.* 2016). Hypokalemia is thought to induce hyperpolarization of DCT cell membrane voltage and potentiate Cl⁻ efflux from the cells, resulting in reduced intracellular Cl⁻ concentration. This, in turn, increases WNK kinase activity, activating NCC via SPAK/OSR1. The modulation of plasma K⁺ levels altered NCC phosphorylation even in kidney-specific MR knockout mice, confirming that the proposed pathway is independent of MR signaling in these cells (Terker *et al.* 2015a). Thus, the increase in WNK levels by KLHL3 inactivation and WNK kinase activation induced by the change in intracellular Cl⁻ levels synergistically regulate NCC (Fig. 4) (Terker *et al.* 2015b, Ishizawa *et al.* 2016). A recent study also reported that a Cl⁻- and SPAK/OSR1-independent pathway activates NCC in hypokalemia (Penton *et al.* 2016).

NCC regulation through WNKs has also been implicated in hypertension associated with the use of calcineurin inhibitors (CNIs), which increase blood

Figure 4

Hypokalemia-dependent regulation of NCC in DCT cells. The decrease in plasma K^+ levels by aldosterone stimulates the WNK-SPAK/OSR1-NCC cascade via two distinct mechanisms. Firstly, hypokalemia reduces the activity of ubiquitin ligase complex composed of KLHL3/CUL3 by S433 phosphorylation and also by reducing total expression levels, thereby preventing WNK degradation via the ubiquitin proteasome system (UPS) and increasing WNK4 (and likely WNK1) levels. Secondly, hypokalemia reduces intracellular Cl^- levels (shown as $[Cl^-]$) by promoting Cl^- efflux; this results in reduced Cl^- binding to WNK1/4 and increases the kinase activity.

pressure through both vasoconstrictive and salt-retaining effects. Accumulating data indicate that the latter effect is mediated by NCC activity. Interestingly, the CNI tacrolimus (FK506) induced NCC phosphorylation in mice kidney (Hoom *et al.* 2011) and caused salt-sensitive hypertension in wild-type mice but not in NCC knockout mice. Consistently, kidney transplant recipients treated with tacrolimus showed greater response to thiazide diuretics than those without. In the kidney, calcineurin colocalizes with NCC, and CNI administration associated with activation of the WNK/SPAK pathway, suggesting that WNK and SPAK mediate the NCC activation caused by CNIs. A recent study also showed that the 12-kDa FK506 binding protein (FKBP12) is required for CNI-induced hypertension, since FKBP12 deletion in the renal nephron abolished NCC phosphorylation and blood pressure elevation in the kidney of mice treated with tacrolimus (Lazelle *et al.* 2016). How calcineurin interacts with SPAK and WNK remains to be determined.

In summary, the evidence to date indicates that two distinct ubiquitin ligases (i.e., Nedd4-2 and KLHL3/CUL3) modulate NCC function, with two mechanisms converging on the regulation of WNK. However, the former is inactivated by aldosterone via Sgk1, whereas the latter is regulated by AngII and plasma K^+ levels. Whether KLHL3/CUL3 is regulated by aldosterone remains unknown.

NaCl reabsorption and K^+ secretion in the distal nephron: integration of MR-dependent and -independent effects

A key function of the renal distal nephron is to regulate NaCl reabsorption and K^+ secretion, both of which are stimulated by aldosterone. In the context of volume depletion, aldosterone increases NaCl reabsorption without increasing K^+ secretion, whereas in hyperkalemia, aldosterone maximizes K^+ secretion without altering NaCl reabsorption. These distinct effects of aldosterone are exerted by the coordinate actions of DCT cells, principal cells and intercalated cells.

Characterizing the pathways that regulate the activity of channels and transporters in the distal nephron has provided insights into the mechanisms that maintain the balance between NaCl reabsorption and K^+ secretion in high-aldosterone states. In the case of volume depletion or hyperkalemia, aldosterone production induces ENaC activation through MR and its downstream effectors, as described above. However, in the former instance, AngII promotes renal intercalated cell MR dephosphorylation, thereby promoting Cl^- influx through these cells (Shibata *et al.* 2013a). In addition, AngII stimulates NCC by increasing the kinase activity of WNKs and inhibiting KLHL3/CUL3-based ubiquitin ligase, resulting in increased NaCl reabsorption in DCTs. These mechanisms

act in concert to eliminate the lumen negativity driving K^+ secretion in the collecting duct, thereby suppressing urinary K^+ excretion. Conversely, hyperkalemia inhibits NCC and also blocks Cl^- reabsorption in intercalated cells by increasing MR^{S843-P} , even when aldosterone levels are elevated. These changes favor K^+ secretion in exchange for electrogenic Na^+ reabsorption through ENaC.

Besides regulating fluid volume, several lines of evidence support the importance of DCT and intercalated cells in renal K^+ handling. In PHAI, both high blood pressure and hyperkalemia can be corrected by thiazide treatment. Hyperkalemia in a mouse model of PHAI is also normalized by mating with NCC knockout mice (Laloti *et al.* 2006). These data demonstrate that NCC activation contributes to the conservation of K^+ . In addition, in individuals with Pendred syndrome, thiazide treatment induces severe hypokalemia, highlighting the compensatory actions of pendrin and NCC in K^+ homeostasis. Furthermore, mathematical models of electrolyte handling in the collecting duct have demonstrated that the activation of intercalated cells along with principal cells results in maximal NaCl reabsorption while conserving K^+ (Weinstein 2002, Wall & Weinstein 2013).

These mechanisms have implications for pathological conditions. For example, it is well known that the reduced K^+ intake increases blood pressure (Mente *et al.* 2014). Studies summarized in this article suggest that this inverse association is attributable to the regulation of NCC by hypokalemia/hyperkalemia via KLHL3/CUL3 ubiquitin ligase and WNK/SPAK signaling. In primary aldosteronism, high levels of circulating aldosterone are associated with decreased plasma K^+ concentration. The accompanying hypokalemia may aggravate high blood pressure by increasing NCC activity (Terker *et al.* 2015a) and also by modulating MR^{S843-P} and pendrin expression in renal intercalated cells (Xu *et al.* 2017). Although these represent adaptive changes to prevent renal K^+ loss in hypokalemia, they result in increased NaCl reabsorption and hypertension.

Conclusion

This review summarized the current state of knowledge regarding mechanisms modulating the function of MR and its downstream targets in the distal nephron. Principal, intercalated and DCT cells are differentially regulated by the interplay of aldosterone, AngII and plasma K^+ , which controls the balance between NaCl reabsorption and K^+ secretion.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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