Regulation of Renal Electrolyte Transport by WNK and SPAK-OSR1 Kinases

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Abstract

The discovery of four genes responsible for pseudohypoaldosteronism type II, or familial hyperkalemic hypertension, which features arterial hypertension with hyperkalemia and metabolic acidosis, unmasked a complex multiprotein system that regulates electrolyte transport in the distal nephron. Two of these genes encode the serine-threonine kinases WNK1 and WNK4. The other two genes [kelch-like 3 (*KLHL3*) and cullin 3 (*CUL3*)] form a RING-type E3-ubiquitin ligase complex that modulates WNK1 and WNK4 abundance. WNKs regulate the activity of the Na^+:Cl^- cotransporter (NCC), the epithelial sodium channel (ENaC), the renal outer medullary potassium channel (ROMK), and other transport pathways. Interestingly, the modulation of NCC occurs via the phosphorylation by WNKs of other serine-threonine kinases known as SPAK-OSR1. In contrast, the process of regulating the channels is independent of SPAK-OSR1. We present a review of the remarkable advances in this area in the past 10 years.

INTRODUCTION

With no lysine (K) kinases (WNK kinases or WNKs): these kinases owe their name to the absence of catalytic lysine in serine-threonine kinases in subdomain II

Arterial hypertension is the number one risk factor for death worldwide (1), with a prevalence of between 30% and 40% in the adult population. Hypertension is a complex disease, resulting from the combination of an inherited susceptibility and the exposure to environmental factors, such as excessive salt intake and obesity. A combination of physiological, genetic, and clinical studies has suggested that a key component in the development of hypertension is a failure of the kidney at normal blood pressure to properly excrete the salt ingested in a typical Westernized diet. Supporting this, all monogenic diseases associated with increased or decreased arterial blood pressure are due to mutations in genes encoding renal ion transporters or their regulators. One such disease is familial hyperkalemic hypertension (FHHt), or pseudohypoaldosteronism type II, an autosomal dominant or recessive disease featuring arterial hypertension, hyperkalemia, hyperchloremic metabolic acidosis, and hypercalciuria. Strikingly, both the hypertension and the metabolic disorders are corrected by a low dose of thiazide-type diuretics (2). In addition, FHHt is the mirror image of Gitelman's syndrome, which is characterized by hypotension, hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria. In the vast majority of cases, this syndrome is due to inactivating mutations of the renal thiazide-sensitive Na^+:Cl^- cotransporter, NCC. Taken together, these data have suggested that FHHt is mainly due to an increase in the activity of NCC. However, in FHHt patients, no genetic association or mutation has been found in the *SCL12A3* locus, encoding NCC.

The identification of the genes causing FHHt triggered a remarkable amount of work aimed at understanding how the product of these genes modulates ion transport in the distal nephron and, thus, blood pressure. There are four FHHt genes (**Table 1**). Two of them, *WNK1* and *WNK4*, encode serine-threonine kinases of the with no lysine (K) (WNK) family. The other two, *KLHL3* (kelch-like 3) and *CUL3* (cullin 3), form a RING-type E3-ubiquitin ligase complex. Mutations in *KLHL3* produce a dominant or recessive form of FHHt (3, 4), and mutations in *CUL3* cause a severe form of FHHt (3). This complex recruits the WNK kinases for ubiquitination to promote their proteasomal degradation. Mutations in *KLHL3* disrupt the interaction with the substrates or cullin 3, thus preventing the degradation of WNKs. Mutations in *CUL3* also abrogate WNK ubiquitination (WNK1, WNK4, and probably WNK3), but the effect is indirect, via increased

Table 1 Proposed effects of familial hyperkalemic hypertension (FHHt) mutations on the affected genes

Gene	Mechanism	Consequence	Putative effect[a]	Reference(s)
WNK1	Deletion of intron 1	Increased expression of WNK1	↑L-WNK1	34
WNK4	Missense mutations in the acidic domain	Increased expression of WNK4 due to disruption of the kelch 3 recognition site	↑WNK4	3, 5, 34, 118, 119
WNK4	R1185C mutations in the carboxyl-terminal domain	Unknown; potential effect on calmodulin binding site and SGK1 phosphorylation site	Unknown	7, 120
KLHL3	Missense mutations in the BTB or BACK domain	Disruption of the CUL3-KLHL3 interaction	↑WNK1, ↑WNK4 ↑WNK3	3–5, 118, 121
KLHL3	Missense mutation in the kelch propeller blades	Disruption of the substrate binding site	↑WNK1 ↑WNK4 ↑WNK3	3–5, 118, 121
CUL3	Skipping of exon 9	Increased KLHL3 ubiquitylation and degradation	↓KLHL3 ↑WNK1 ↑WNK4 ↑WNK3	3, 5, 6

[a] ↑ indicates increase; ↓ indicates decrease.

ubiquitination and degradation of KLHL3 (5, 6). The mutations in *WNK1* and *WNK4* also lead to increased expression of the corresponding protein. The only reported mutations in *WNK1* are large deletions of the first intron that result in increased expression of an otherwise normal WNK1 protein (7, 8). In the case of WNK4, several missense mutations have been identified, and all except one are located in a small motif called the acidic domain, which is highly conserved among WNKs. This domain is required for the recruitment of WNKs by KLHL3 (5), which is precluded by the FHHt missense mutations, thereby increasing WNK4 protein abundance.

The FHHt phenotype results from increased expression of WNKs that leads to increased salt reabsorption and hypertension, and decreased potassium secretion with hyperkalemia and metabolic acidosis, due to the altered effects of WNKs in distal nephron ion-transport mechanisms. Thus, we review the current understanding of how WNKs regulate ion-transport pathways in the distal nephron.

Sterile20-related proline-alanine-rich kinase and oxidative stress response-1 (SPAK and OSR1): serine-threonine kinases of the Sterile (Ste)-20 superfamily

Cation-coupled chloride cotransporters: Na^+:K^+:2Cl^- cotransporters NKCC1 and NKCC2, Na^+:Cl^- cotransporter NCC, and K^+:Cl^- KCC1 to KCC4 of the SLC12 family

THE WNK/SPAK COMPLEX OF SERINE-THREONINE KINASES

SPAK and OSR1 Interact with and Phosphorylate the Cation-Chloride Cotransporters

The Sterile20-related proline-alanine-rich kinase (SPAK) and oxidative stress response kinase 1 (OSR1) were identified through the search for the kinase responsible for coordinated phosphorylation of the K^+:Cl^- cotransporters and Na^+:K^+:2Cl^- cotransporters (9). It is well known that the influx of Cl^- is mediated by the Na^+:K^+:2Cl^- cotransporter NKCC1, and the K^+:Cl^- cotransporter KCC (KCC1–4) is responsible for Cl^- efflux in many epithelial cells (10). Cell shrinkage or a decrease in intracellular chloride concentration $([Cl^-]_i)$, or both, triggers the phosphorylation of NKCC1 and KCCs, promoting, respectively, their activation and inactivation. Conversely, cell swelling or an increase in $[Cl^-]_i$, or both, promotes the net dephosphorylation of both branches of the family, thereby inhibiting NKCC and activating KCC. These observations suggested that a common Cl^--sensitive kinase is involved in these processes. In order to identify this kinase, a yeast two-hybrid screening was performed. It identified SPAK and OSR1 as proteins that interact with the K^+:Cl^- cotransporter KCC3 (9). It was then shown that they also interact with NKCC1 and NKCC2, but not with KCC1 and KCC4 (9).

SPAK and OSR1 belong to the germinal center kinase group VI of the Ste20-related kinases superfamily. The degree of homology between these two kinases is approximately 67% and increases to 89% when only the kinase domain is considered (11). One major difference between SPAK and OSR1 is the presence of a proline- and alanine-rich region (PAPA box) in the N-terminal domain of SPAK, upstream of the catalytic domain (12). SPAK was originally identified as a new kinase that specifically activates the p38 pathway in pancreatic β cells, suggesting that it may act as a novel mediator of stress-activated signals (13). OSR1 was originally identified through a search for genes involved in tumor suppression (14).

The *STK39* gene, which encodes SPAK, gives rise to three isoforms. The first one is the full-length isoform (FL-SPAK), which is expressed ubiquitously, with higher expression in the brain, heart, and testis (15, 16). The second, SPAK2, is also expressed ubiquitously (15–17), but lacks the N-terminal PAPA box and part of the kinase domain. The third one, also devoid of kinase activity, is expressed predominantly in the kidney, hence its name KS-SPAK (kidney-specific SPAK). In the kidney, the expression of SPAK (or FL-SPAK) is lower than that of the two other isoforms (17). FL-SPAK is expressed only in the thick ascending limb of Henle's loop (TAL) and the distal convoluted tubule (DCT) (16, 17). Its expression in the DCT is higher than in TAL. Conversely, SPAK2 and KS-SPAK are more abundant in TAL than in the DCT (17). Interestingly, SPAK is mostly apical in TAL, whereas it is concentrated in dense puncta in the DCT, with only a weak

expression at the apical membrane (18). No SPAK isoforms have been detected in the other nephron segments (16, 17). Shorter forms of SPAK are also generated by proteolytic cleavage by the aspartyl aminopeptidase Dnpep at sites located within the PAPA box and the catalytic domain (19).

WNK1 variants: in the kidney 70% of WNK1 copies represent the Δ11 variant, 20% the Δ11,12 variant, and 10% the full L-WNK1 protein

Like SPAK, OSR1 is expressed ubiquitously (9, 20). In the kidney, OSR1 is found at the apical membrane of TAL and DCT cells (18, 21). OSR1 is not present in the SPAK-containing puncta in the DCT of wild-type animals. However, such a distribution has been observed in the DCT of *SPAK*$^{-/-}$ animals (18, 21). Two isoforms have been detected by Western blot, but they have not been characterized in detail. The short isoform could be generated by the use of an alternative translational start site (9, 15). No proteolytic cleavage has been observed for OSR1 (19).

After the discovery of SPAK and OSR1 as partners of NKCCs and KCCs, several groups studied the regulation of the cotransporters by kinases. Surprisingly, in in vitro models, the overexpression of SPAK alone is not sufficient to activate NKCC1, suggesting that SPAK requires an upstream regulator (9–15).

WNK Kinases Lie Upstream of SPAK-OSR1

A yeast two-hybrid screen identified WNK1 and WNK4 as partners of SPAK (9, 15, 22). Biochemical studies then demonstrated that WNKs are the upstream kinases that activate SPAK-OSR1 (22–24). WNKs phosphorylate SPAK and OSR1, respectively, on residues Thr243 and Thr185 of the T-loop, and Ser373 and Ser325 of the S-motif (25). However, only the phosphorylation of the T-loop is absolutely required to activate SPAK and OSR1.

The WNK family comprises four members, WNK1 to WNK4 (26). Their name derives from the fact that WNKs lack the invariant catalytic lysine in subdomain II of protein kinases that is crucial for binding to ATP (adenosine triphosphate). This lysine is displaced in the first subdomain (27). Despite these changes, WNKs exert kinase activity. WNK1 was originally identified through the search for new members of the mitogen-activated protein (MAP)–extracellular signal-regulated protein kinase (ERK) (MEK) family in the rat brain (27), and was later found to be differentially expressed in various colorectal cell lines (26). Therefore, similar to SPAK, there was no evidence that WNK1 had a role in regulating renal ion transport and blood pressure prior to the discovery of *WNK1* and *WNK4* mutations in FHHt patients. A short amino-terminal domain and a long carboxyl-terminal domain flank the kinase domain of WNKs. In addition to the aforementioned acidic domain, the carboxyl terminal domain contains the well-described autoinhibitory domain of kinase activity and two coiled-coil domains. It also contains the so-called HQ domain near the end of the protein, required for WNK–WNK interaction and, thus, activity (28, 29).

WNK1, WNK3, and WNK4, but not WNK2, are expressed in the kidney. The *WNK1* gene is a complex gene giving rise to two isoforms and several variants (26, 30–32). A proximal promoter, located upstream of the first exon, drives the expression of L-WNK1 (long-WNK1), containing the entire kinase domain, and expressed ubiquitously. A second promoter, located in intron 4, allows for the expression of a shorter isoform called KS-WNK1 (kidney-specific WNK1), which is expressed only in the distal nephron and is devoid of kinase activity. In addition, the alternative splicing of 6 exons (8b, HSN2, 11, 12, 26a, and 26b) generates several WNK1 variants expressed in a tissue-specific manner. In particular, the variant containing HSN2 is expressed only in the peripheral nervous system. Mutations located in the HSN2 exon are responsible for another inherited disease, known as hereditary sensory neuropathy type 2 (33). There is little information about the WNK1 protein. Wilson and collaborators (34) reported that the WNK1 protein is found only in the cytoplasm of cells in the DCT and the cortical collecting duct (CCD).

WNK3 encodes a 1,800-residue protein and gives rise to alternatively spliced isoforms due to the presence or absence of exons 18a, 18b, and 22, with specific tissue expression (35).

WNK3 is extensively expressed in the central nervous system and the kidney (36). In the latter, reverse transcriptase–polymerase chain reaction (RT-PCR) showed that WNK3 is expressed in all nephron segments, but the relative expression level in each segment has not been measured (37).

WNK4 encodes a 1,243-residue protein expressed in several tissues, including the distal nephron. WNK4 transcripts have been detected by in situ hybridization in TAL (including in the macula densa), the DCT, and the CCD and outer-medullary collecting duct (OMCD) (38). Expression is the highest in the DCT. This pattern differs somewhat at the protein level (39). Immunofluorescence microscopy has confirmed expression in the cortical TAL, DCT, connecting tubule (CNT) and CCD (principal and intercalated cells), and OMCD. It also revealed that WNK4 is present in podocytes and the inner medullary collecting ducts (39). There was no signal in the medullary TAL.

REGULATION OF NCC BY THE WNK/SPAK COMPLEX

As discussed in the Introduction, FHHt is probably the consequence of activation of the Na^+:Cl^- cotransporter NCC, which is the target of thiazides. In addition, a common pathway in FHHt pathophysiology seems to be the increased expression of WNK1 or WNK4, or both, in the distal nephron, regardless of the mutated gene. Thus, a remarkable amount of effort has been dedicated to trying to understand the role of WNKs and SPAK-OSR1 in the modulation of the expression and activity of NCC. Although the essential role of SPAK in the phosphorylation and, thus, activity of NCC was quickly established, both in vitro and in vivo, the regulation of NCC by WNK1 and WNK4 has been a matter of controversy for many years.

SPAK Is Essential for the Phosphorylation and Activity of NCC

The minimum SPAK binding motif, defined as RFx(V/I) by alanine screening (9), is present in the N-terminal tail of NCC. SPAK and OSR1 phosphorylate NCC on three residues: Thr46, Thr55, and Thr60 (40). NCC can also be phosphorylated on one additional site during Cl^- depletion (Ser91) by a yet unidentified kinase (40). Although SPAK and OSR1 are equally able to phosphorylate NCC in vitro, SPAK is essential for NCC phosphorylation and activity in vivo. Two mouse models have been generated to study SPAK function (**Table 2**). The first one is a global knockout, inactivating both the full-length and truncated isoforms (17, 21, 41). The second one ($SPAK^{T243A}$) consists of the introduction of an inactivating missense mutation in the T-loop of SPAK, which precludes WNKs from activating SPAK (16). In both models, NCC phosphorylation is reduced dramatically. Consequently, the length and area of the early part of the DCT (DCT1) is decreased (21) and is compensated for by an increase in the length and activity of the CNT (42), as observed in NCC knock-out mice (43). NCC inhibition in $SPAK^{T243A}$ mice is associated with all the features of Gitelman's syndrome.

The genetic inactivation of *OSR1* results in embryonic lethality due to defects in angiogenesis and cardiovascular development (**Table 2**) (16, 44, 45). The targeted inactivation of OSR1 in the distal nephron results in a Bartter-like syndrome, with mild volume depletion and hypokalemia, caused by reduced expression, phosphorylation, and activity of NKCC2 (44). NCC expression is increased in these mice, probably to compensate for the decreased activity of NKCC2 (44). The expression and phosphorylation of NKCC2 are reduced in the $SPAK^{T243A}$ model, but they are increased in total SPAK knockout mice. This difference is due to the inhibitory effect exerted by shorter isoforms of SPAK on NKCC2 (17, 19), which are still present in $SPAK^{T243A}$ mice but not in the knockout mice. Thus, SPAK is an activator of NCC and its absence cannot be fully compensated for by OSR1. This could be due to the displacement of OSR1 from the apical

Table 2 Mouse models in which WNK or SPAK-OSR1 kinases have been genetically altered (ordered by manipulated gene and chronology)

Gene	Modification	Blood pressure	NCC expression and activity[a]	Phenotype	Reference
Wnk1	*Wnk1*$^{+/-}$	Decreased	Normal	None	49, 128
	Wnk1$^{-/-}$	NA	NA	Embryonic lethal (cardiovascular development defects)	48, 49
	Wnk1$^{+/FHHt}$	↑	↑↑	FHHt	8
KS-Wnk1	*KS-Wnk1*$^{-/-}$	Normal	↑↑	Decreased aldosterone secretion	91
	KS-Wnk1$^{-/-}$	↑	↑↑	Salt-sensitive hypertension	122
	Transgenic mice overexpressing fragment 1–253 of KS-Wnk1	↓	↓	Decreased abundance of NKCC2	122
Wnk4	Overexpression of Wnk4	↓	↓	Gitelman-like syndrome	76
	Overexpression of Wnk4-Q562E	↑	↑↑	FHHt	76
	Knock-in of FHHt D561A mutation *Wnk4*$^{D561A/+}$	↑	↑↑	FHHt	102
	Wnk4 hypomorphic mice (deletion of exons 7–8)	↓	↓	Impaired ability to conserve Na during Na depletion	123
	Bacterial artificial chromosome *Wnk4*^{TG} Low copy	↑	↑	FHHt	118
	Bacterial artificial chromosome *Wnk4*^{TG} High copy	↑	↑↑	FHHt	
	Wnk4$^{-/-}$	Normal	↓↓↓	Hypokalemia and metabolic alkalosis	58, 124
Wnk3	*Wnk3*$^{-/-}$	Normal	Normal	Hypotension during Na depletion	37
	Wnk3$^{-/-}$	Normal	Normal	Upregulation of WNK1 mRNA	54
SPAK	*SPAK*$^{T243A/T243A}$	↓	↓↓	Gitelman's syndrome	16
	SPAK$^{-/-}$	↓	↓↓	Gitelman's syndrome	21, 41
	SPAK$^{+/-}$	↓	↓		41
	SPAK$^{-/-}$	↓ when fed a Na^+-depleted diet	↓↓	Hypokalemia when fed a K^+-depleted diet	17
	SPAK$^{-/-}$	ND	↓↓	Vasopressin-induced phosphorylation of NCC, but not NKCC2, is blunted	18
	SPAK$^{-/-}$	ND	ND	NKCC2-mediated Na^+ reabsorption is decreased in these mice	126

(*Continued*)

Table 2 (*Continued*)

Gene	Modification	Blood pressure	NCC expression and activity[a]	Phenotype	Reference
OSR1	Kidney-specific inactivation (*KSP-OSR1*$^{-/-}$)	Normal	↑↑	Bartter's syndrome	44
	OSR1$^{+/-}$	↓	↑↑	Bartter's syndrome	44
	OSR1$^{-/-}$	NA	NA	Embryonically lethal	45
	OSR1$^{-/-}$ *hOSR1*^{ca} (transgenic expression of a constitutively active OSR1)	ND	ND	Rescues embryonic lethality	
	WNK1$^{-/-}$ *hOSR1*^{ca}	ND	ND	Rescues embryonic lethality	
Wnk4 SPAK-OSR1	*Wnk4*$^{D561A/+}$ *SPAK*$^{T243A/+}$	Partial correction	↑	Partial correction	127
	Wnk4$^{+/+}$ *OSR1*$^{T185A/+}$	Partial correction	ND	Partial correction	
	Wnk4$^{+/+}$ *SPAK*$^{T243A/+}$ *OSR1*$^{T185A/+}$	Normal	Normal	Correction of FHHt phenotype	
	Wnk4$^{D561A/+}$ *OSR1*$^{T185A/+}$ *SPAK*$^{T243A/T243A}$	↓	↓↓	Hypokalemia	
Wnk1 Wnk4	*Wnk1*$^{+/-}$	Normal	Normal		128
	Wnk4$^{D561A/+}$ *Wnk1*$^{+/+}$	↑	↑↑	FHHt	
	WNK4$^{D561A/+}$ *WNK1*$^{+/-}$	↑	↑↑	FHHt	
	WNK1$^{+/FHHt}$ *WNK4*$^{-/-}$	↑	↑↑	FHHt	28
Wnk4 SPAK OSR1	*Wnk4*$^{D561/+}$ *KSP-OSR1*$^{-/-}$	↑	↑↑	FHHt	129
	Wnk4$^{D561/+}$ *SPAK*$^{-/-}$	Normal	Normal	None	

[a] ↑ indicates increase; ↓ indicates decrease. The number of up or down arrows denotes the relative magnitude of increase or decrease.
Abbreviations: FHHt, familial hyperkalemic hypertension; NA, not applicable; ND, not determined.

membrane to the dense punctate, which has been observed within the cytoplasm of the DCT in SPAK knockout animals and which would prevent the interaction of OSR1 with NCC (21). Conversely, OSR1 is required for NKCC2 phosphorylation and activity.

WNKs Act Upstream of SPAK to Activate NCC

Two pathways were initially proposed for the regulation of NCC by WNK1. A first group of studies showed that the overexpression of L-WNK1 in *Xenopus* oocytes or cell lines did not affect NCC expression or activity, or both, directly but precluded WNK4 from inhibiting NCC (see below) (46, 47). However, and as mentioned above, L-WNK1 can activate SPAK by

phosphorylation, suggesting that L-WNK1 could activate NCC in a SPAK-dependent manner (22, 40). It is impossible to test the in vivo relevance of these pathways using a simple L-WNK1 knockout model because it results in developmental defects in the cardiovascular system, similar to those observed in OSR1-deficient embryos (**Table 2**) (45, 48, 49). Therefore, we took advantage of a mouse model of FHHt caused by *WNK1* mutations (8). These mutations are large deletions of the first intron of the gene (34). The deletion of the whole first intron in mice fully recapitulates the FHHt phenotype (**Table 2**) (8). *WNK1*$^{+/FHHt}$ mice exhibit only an increased expression of L-WNK1, specifically in the DCT and CNT; the expression of KS-WNK1 is not modified. In agreement with the initial hypothesis, NCC expression and phosphorylation are increased. In addition to an increased level of SPAK phosphorylation on the T-loop and the S-motif in the DCT of WNK1-FHHt mice, we also observed that SPAK is more abundant at the apical membrane of the DCT in mutant mice compared with wild-type mice. Finally, the FHHt phenotype is maintained even when WNK4 is absent (**Table 2**) (28). Taken together, these studies favor the L-WNK1/SPAK pathway for NCC activation. Because SPAK is expressed in *Xenopus* oocytes, it was difficult to understand why the overexpression of L-WNK1 did not stimulate NCC activity in this system.

In the kidney, the predominant variant of WNK1 is L-WNK1-Δ11, lacking exon 11 (32). The variant used in the *Xenopus* studies was a rat L-WNK1-Δ11–12 cDNA (complementary DNA), which represents approximately 20% of L-WNK1 in the nephron but is predominant in the brain, from which it has been cloned (27). We tested the activity of the kidney variant in oocytes (28). In this system and in HEK293 cells, the coexpression of the human L-WNK1-Δ11 with NCC results in a significant threefold activation of NCC (28). The effect of L-WNK1-Δ11 requires kinase activity and depends on interaction with SPAK (28). It also requires WNK–WNK interaction because missense mutations in the C-terminal HQ domain preclude L-WNK1 from activating NCC. Surprisingly, human L-WNK1-Δ11–12 also stimulates NCC, although with less potency. We identified a mutation in the C-terminal tail of the rat L-WNK1-Δ11–12 cDNA (p.Gly2120Ser). Corrected rat cDNA is then able to activate NCC, similarly to human cDNA. Thus, L-WNK1 is a powerful activator of NCC (**Figure 1**). When reviewing the literature, it is important to keep in mind that a significant number of studies of L-WNK1 or KS-WNK1 used the mutated WNK1-Δ11–12 construct.

Similar to L-WNK1, WNK3 is a potent activator of NCC by a kinase- and SPAK- dependent mechanism (50, 51) (**Figure 1**). In the absence of kinase activity, WNK3 no longer activates NCC. In contrast, the kinase-dead WNK3 mutant becomes a powerful inhibitor of the cotransporter, showing that in the absence of activation, WNKs can promote the opposite effect on their target protein (51–53). WNK3 knockout mice show a very mild phenotype, with only a slight decrease in blood pressure during NaCl depletion (**Table 2**) (37, 54). In these models, L-WNK1 expression is increased in the kidney, where it probably compensates for the absence of WNK3, thereby allowing the maintenance of NCC phosphorylation.

WNK4 has been the most difficult kinase to understand. There is evidence in vitro, as well as in vivo, that WNK4 can behave as an inhibitor or an activator of NCC (55). Almost all in vitro studies have suggested that WNK4 inhibits NCC activity by preventing the effect of WNK1 or WNK3 on NCC (56) (**Figure 1**). Apparently, in certain circumstances, inactive WNK4 is able to interact with WNK1 or WNK3, or both, thus precluding the effect of these kinases on the SPAK/NCC complex. This effect is prevented by angiotensin II (angII) (57). However, WNK4 can phosphorylate SPAK in vitro (24). Moreover, evidence in vivo has shown that WNK4 is required in mice for basal NCC phosphorylation and activity through its interaction with SPAK (**Table 2**). The inactivation of WNK4 (58) results in a significant reduction in NCC expression and activity that is associated with hypokalemia and hypochloremic metabolic alkalosis. Interestingly, the absence of WNK4 precludes angII from stimulating the phosphorylation of SPAK

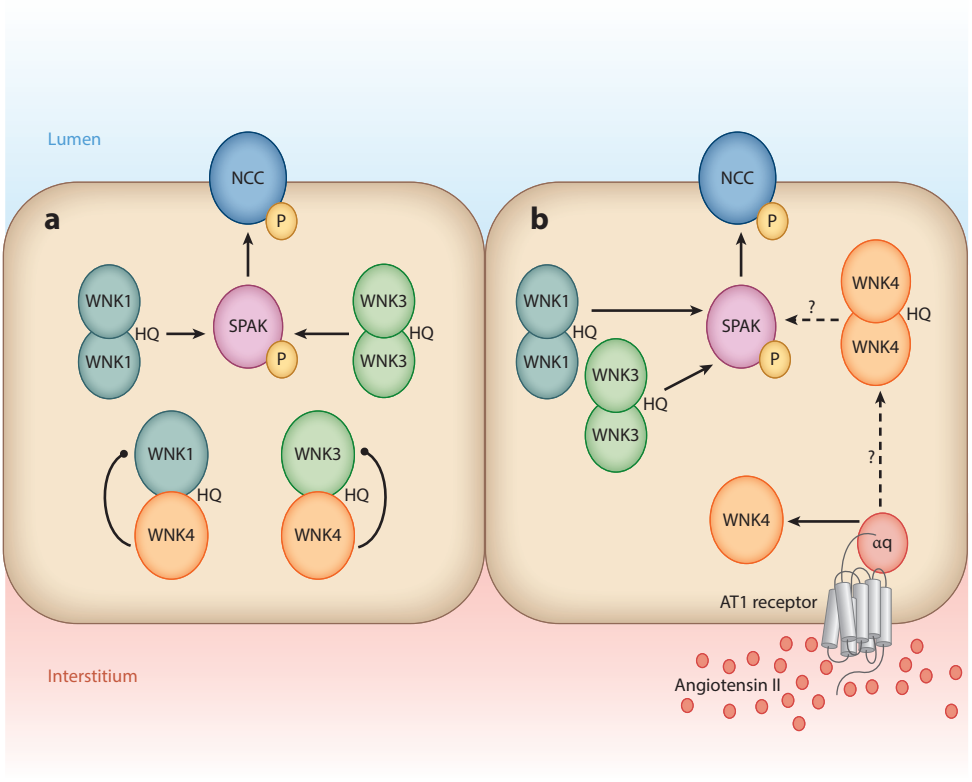

Figure 1

(*a*) Effect of WNKs on SPAK and NCC phosphorylation and activity observed using in vitro systems. WNK1 and WNK3 activate NCC by a SPAK-dependent mechanism (28, 50). SPAK is phosphorylated by these kinases and, in turn, phosphorylates NCC. Interaction between WNKs through the HQ motif is required (28, 29). WNK4 remains inactive toward SPAK and NCC in most cells tested. Through the HQ domain, inactive WNK4 is capable of interacting with WNK1 or WNK3, or both, thus decreasing the availability of these kinases to activate SPAK and NCC. (*b*) When angiotensin II interacts with its membrane receptor, it abrogates the inhibitory effect of WNK4 on NCC (57). One potential mechanism of this action may be that angiotensin II prevents WNK4 from interacting with WNK1 or WNK3, thus liberating these kinases to activate SPAK and NCC. Another possibility is that angiotensin II promotes the activation of free inactive WNK4, which, in turn, can activate the SPAK/NCC complex. Abbreviations: AT1 receptor, angiotensin II receptor type 1; DCT, distal convoluted tubule. Adapted from Reference 55.

and NCC (58). Additionally, the effect of the aldosterone-serum/glucocorticoid kinase-1 (SGK1) pathway seems to be translated, at least in part, by SGK1-induced WNK4 phosphorylation (59).

REGULATION OF THE WNK/SPAK CASCADE BY EXTRA- AND INTRACELLULAR OSMOLARITY

The Activity of WNK Kinases Is Modulated by Intracellular Chloride Concentration

Several lines of evidence support the theory that the activity of WNKs could be modulated by the extra- and intracellular environment, in particular $[Cl^-]_i$. First, wild-type WNKs induce the activation of NKCC1, NKCC2, and NCC. In contrast, mutant, inactive WNKs (caused by

elimination of the catalytic activity or the WNK–WNK interaction) are powerful inhibitors of these cotransporters (28, 36, 51, 60–63). Second, NKCC1, NKCC2, and NCC are clearly activated or inhibited by, respectively, a decrease or increase of $[Cl^-]_i$, and the activation is associated with increased phosphorylation of N-terminal key residues by SPAK and OSR1 (50, 60, 64–66). Thus, the effect of $[Cl^-]_i$ on these transporters is translated through SPAK but sensed by an upstream, intracellular, soluble kinase. It is known that WNKs lie upstream of SPAK (24, 67), which makes WNKs good candidates for acting as the chloride sensor of the cell (68).

Indeed, recent studies have shown that $[Cl^-]_i$ modulates the kinase activity and autophosphorylation and activation of WNK1 and WNK4. Piala et al. (69) demonstrated that WNK1 has a chloride-binding pocket formed by Leu369 and Leu371 in the DLG motif. The binding of chloride precludes the autophosphorylation of WNK1. Thus, the higher the chloride concentration is, the lower the autophosphorylation and activity of WNK1. Similarly, as shown in **Figure 2**, the effect of WNK4 on NCC is modulated by $[Cl^-]_i$. In *Xenopus* oocytes, the effect of WNK4 on NCC is switched from inhibitory in control conditions to activating when cells are exposed to low-chloride hypotonic stress, which promotes chloride efflux from the cell and decreases $[Cl^-]_i$ (62). The activation of NCC by WNK4 requires SPAK. The phosphorylation of WNK4 is undetectable in control conditions and is stimulated by low-chloride hypotonic stress. Finally, the elimination of chloride binding to WNK4 by mutating Leu322 (which corresponds to Leu369 in L-WNK1) results in the constitutive phosphorylation and activation of WNK4 and, thus, stimulation of NCC by WNK4 in control conditions (62). Thus, the inhibitory effect versus the activating effect of WNKs on NCC is modulated by $[Cl^-]_i$. Although the chloride-binding pocket is conserved in all WNKs, WNK4 is the WNK kinase with the highest sensitivity for $[Cl^-]_i$ (**Figure 2**) (70).

The Effect of Extracellular Potassium on NCC Activity Is Translated by the Chloride-Sensitive WNK/SPAK Pathway

The sensitivity of the WNK/SPAK pathway to $[Cl^-]_i$ could explain how NCC is regulated by potassium intake. NCC expression is indeed increased during potassium depletion and decreased during potassium load (71–73). During the latter, the reduction of NCC activity in the DCT results in increased delivery of NaCl to the CNT and CCD. This in turn promotes Na^+/K^+ exchange via the ENaC [epithelial sodium (Na) channel] and ROMK (renal outer medullary potassium) channels; thus, sodium reabsorption switches from predominantly electroneutral (NCC) to predominantly electrogenic (ENaC and ROMK), favoring potassium secretion. Aldosterone secretion is also stimulated by potassium intake, thereby promoting potassium secretion rather than sodium reabsorption, probably because aldosterone has little effect on NCC. In addition, acute potassium intake does not increase aldosterone production but promotes potassium secretion by inhibiting NCC activity (73, 74). In contrast, when angII and aldosterone are both increased, as occurs during Na^+ depletion or hypovolemia, NCC is activated by angII, and electroneutral salt reabsorption increases in the DCT. This is called the aldosterone paradox (75). Thus, NCC activation seems to have a critical role when potassium secretion must be minimized. Accordingly, potassium retention in FHHt is largely the consequence of NCC activation. In fact, crossing WNK4-FHHt mice with NCC null mice completely corrects the hyperkalemia (76).

Several studies have shown that NCC expression and activity in rodents are increased or decreased when they are fed a diet that is, respectively, low or high in KCl (71, 72, 77–79). Interestingly, however, when rats are fed a high–K acetate diet, NCC expression and activity are increased (71). In both cases, the secretion of aldosterone is increased. This suggests that chloride is essential for potassium modulation of NCC. This was demonstrated in recent work

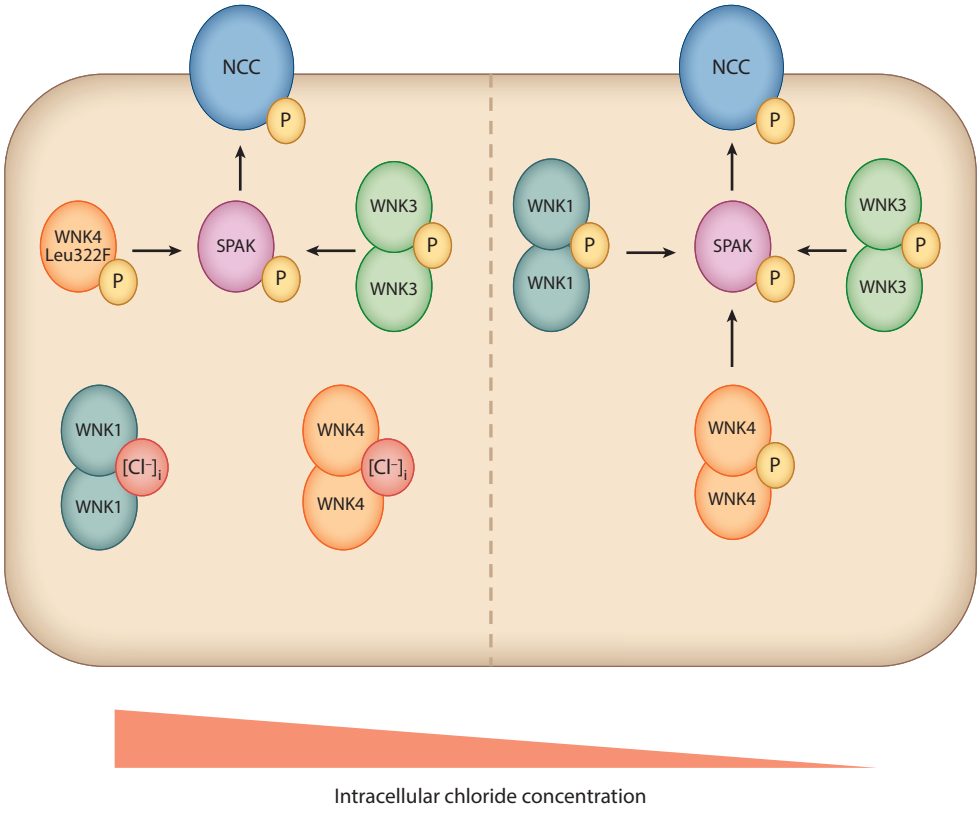

Figure 2

The activity of WNK1 and WNK4 toward SPAK/NCC is modulated by $[Cl^-]_i$. In an environment with high $[Cl^-]_i$, WNK4 remains unphosphorylated and, thus, cannot activate SPAK/NCC. In these circumstances, WNK4 interacts with WNK1 and WNK3, precluding their effects on NCC, thus behaving as an inhibitor. The absence of the critical leucine for chloride binding in the mutant WNK4-L322F allows WNK4 to activate SPAK and NCC (62). WNK1 sensitivity to chloride is lower than WNK4 sensitivity because WNK1 can partially activate SPAK-NCC at high $[Cl^-]_i$. In contrast, WNK3 seems to be less sensitive to chloride because it can fully activate SPAK-NCC at high $[Cl^-]_i$ (55). When $[Cl^-]_i$ is reduced, WNK4 becomes phosphorylated and, thus, active toward SPAK, promoting NCC activity (62). WNK4 is the most sensitive WNK kinase for $[Cl^-]_i$, making it a key regulator of NCC in the distal convoluted tubule (70). Adapted from Reference 55.

by Terker et al. (78). They showed that the regulation of NCC by extracellular concentrations of potassium is due to a potassium-dependent modulation of the membrane voltage in DCT cells, which in turn modulates $[Cl^-]_i$ (**Figure 3**). A decrease in extracellular potassium results in DCT hyperpolarization with a consequent chloride efflux, decreasing the $[Cl^-]_i$ and activating NCC in a WNK-dependent manner. These results are supported by the characterization of the mechanisms by which inactivating mutations in the basolateral K^+ channel, KCNJ10 (Kir4.1), result in NCC inhibition (80). The inactivation of KCNJ10 increases $[Cl^-]_i$ because it prevents the generation of the driving force for chloride secretion by the CLCKb chloride channel. This would then inhibit the WNK1–4/SPAK pathway. Accordingly, SPAK expression and phosphorylation are decreased in *KCNJ10*$^{-/-}$ mice (81).

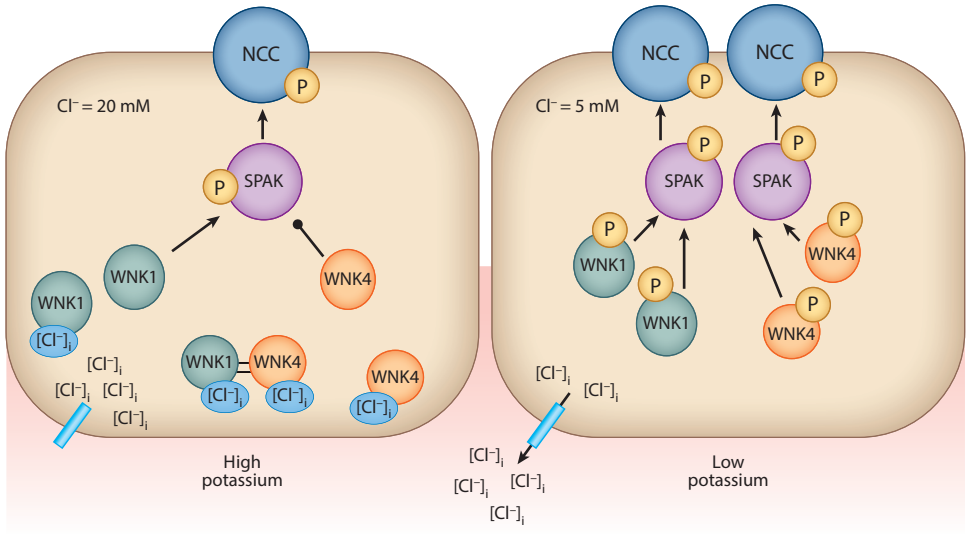

Figure 3

Proposed mechanism for the modulation of NCC activity by extracellular potassium. The basal $[Cl^-]_i$ in distal convoluted tubule cells when extracellular potassium is at normal-to-high concentrations is about 20 mM (130, 131). In this environment, WNK4 is inhibited mostly by chloride, and WNK1 is partially active due to chloride binding to the kinase, which precludes autophosphorylation. In addition, inactive WNK4 is able to interact with WNK1, thus preventing its activity. A decrease in extracellular potassium depolarizes the cell and also stimulates chloride efflux from the cell, thus decreasing $[Cl^-]_i$ (70). Consequently, WNK4 and WNK1 are no longer inhibited by chloride, and become phosphorylated and active, thus activating more SPAK units, which results in increased phosphorylation and activation of NCC.

REGULATION OF ION TRANSPORT BY WNK1 AND WNK4 INDEPENDENTLY OF SPAK OR OSR1

Within the distal nephron, WNK1 and WNK4 are expressed not only in the cortical TAL and DCT but also in the CNT and CCD, where they regulate several transporters and channels. However, the expression of SPAK and OSR1 is barely detectable in these two segments, suggesting that WNKs act independently of their downstream kinases.

Regulation of Potassium Secretion in the Connecting Tubule and Cortical Collecting Duct by WNK1 and WNK4

Two potassium channels are mainly responsible for K^+ excretion in the CNT and CCD. In principal cells, the ROMK channel acts in concert with the ENaC channel to secrete potassium. The second potassium channel is the large-conductance calcium-activated potassium channel BKCa, also known as BK, which is responsible for the flow-induced K^+ secretion in the CNT and CCD. WNK1 and WNK4 are both expressed in the principal cells and intercalated cells of the CNT and CCD (39). Several studies, mostly in vitro, have shown that WNK1 and WNK4 may inhibit these channels.

In contrast to the phosphorylation-dependent regulation of NCC, all studies have shown that the kinase activity of WNK1 is not required for the regulation of ROMK. The N-terminal proline-rich domain seems to be crucial for this process (82, 83). L-WNK1 inhibits membrane

expression of ROMK by stimulating its clathrin-mediated endocytosis in transfected HEK293 or COS cells and *Xenopus* oocytes (82, 84). The N-terminal 1–491 fragment of L-WNK1, containing both the first proline-rich region and the kinase domain, is sufficient to inhibit ROMK surface expression (8, 83, 85). The same fragment bearing three mutated proline residues at positions 94, 103, and 114 fails to inhibit ROMK (86). The regulation of ROMK by L-WNK1 is not direct, and two potential partners have been identified. The first one is the multimodular endocytic scaffold protein intersectin (84), which is required for L-WNK1 to inhibit ROMK. Mutation of the proline residues of the N-terminal proline-rich domain disrupts this interaction (84). The second one is ARH (autosomal recessive hypercholesterolemia), a clathrin adaptor molecule that recruits ROMK to clathrin-coated pits for endocytosis (85). ARH is coexpressed with ROMK predominantly in the DCT, CNT, and CCD, and to a lesser extent in TAL. Moreover, its expression is downregulated in the mouse kidney cortex by a potassium load (85). Overexpression of L-WNK1 enhances ARH-mediated endocytosis in COS cells by phosphorylating ARH and inhibiting its proteasomal degradation (87). This last result contradicts the aforementioned studies, which showed that the kinase activity of L-WNK1 is not required to inhibit ROMK. Therefore, two pathways may coexist for the regulation of ROMK by L-WNK1: a kinase-independent regulation of ROMK by intersectin and a kinase-dependent regulation of ROMK by ARH. The characterization of their respective physiological relevance using an in vivo integrated model is required to understand when and where they regulate ROMK. Interestingly, we observed a decreased abundance of ROMK at the apical membrane, as well as diffuse cytoplasmic ROMK staining in the late DCT (DCT2) and CNT of *WNK1*$^{+/FHHt}$ mice, suggesting that ROMK endocytosis is increased (8). Of note, these modifications are observed only in the DCT2 and CNT, where L-WNK1 expression is increased, and not in the CCD, where L-WNK1 expression is not modified. The decreased apical abundance of ROMK could contribute to the hyperkalemia observed in *WNK1*$^{+/FHHt}$ mice, but this remains to be formally demonstrated. If regulation of ROMK by L-WNK1 is observed in this pathological situation, there is so far no evidence for a role of L-WNK1 in the regulation of ROMK expression in physiological conditions. The level of expression of L-WNK1 messenger RNA is indeed not modified by K^+ load or depletion (38, 88; J. Hadchouel et al., unpublished data). Because the inactivation of L-WNK1 results in embryonic death (49), the consequences of the absence of L-WNK1 on potassium balance in adult mice have not been characterized.

The kidney-specific isoform of WNK1, KS-WNK1, precludes L-WNK1 from inhibiting ROMK in HEK293 cells and *Xenopus* oocytes. Thus, KS-WNK1 should favor K^+ secretion. This is consistent with expression studies, which have shown that the level of expression of KS-WNK1 increases during K^+ load and decreases during K^+ depletion (38, 83, 88). A first set of in vivo studies confirmed the in vitro data. Overexpression of the N-terminal 1–253 fragment of KS-WNK1 in the distal nephron of transgenic mice decreased plasma K^+ and increased ROMK apical abundance (89). Conversely, the inactivation of KS-WNK1 in mice reduced K^+ secretion by ROMK but not flow-induced K^+ secretion by BKCa in the CCD (90). However, we obtained contradictory results. Indeed, we reported that the apical abundance of ROMK and the expression of BKCa are increased in the DCT2 and CNT of mice bearing an inactivation of KS-WNK1 (91), suggesting that ROMK expression could be inhibited rather than stimulated by KS-WNK1 in vivo. The reasons for this discrepancy have not been determined.

Similarly to L-WNK1, WNK4 inhibits ROMK by stimulating the clathrin-dependent endocytosis of the channel in a kinase-independent manner (92). The inhibition of ROMK by WNK4 is reversed by SGK1, which phosphorylates WNK4 on the residues Ser1169 and Ser1196 (59, 93). It has been postulated that this mechanism could contribute to the increased apical abundance of ROMK in the distal nephron of animals fed a high potassium diet, which stimulates aldosterone secretion and, thus, SGK1 activity. Conversely, the protein tyrosine kinase c-Src could be involved

in preserving the correct K^+ balance during Na^+ depletion, during which aldosterone secretion and SGK1 activity are also stimulated. In vitro, c-Src prevents the phosphorylation of WNK4 by SGK1 (94) through the phosphorylation of residues Tyr1092, Tyr1094, and Tyr1143 (95). The protein tyrosine kinase c-Src may act downstream of angII, whose production is increased by Na^+ depletion and which has been shown to inhibit ROMK activity. AngII increases c-Src phosphorylation, and the inhibition of protein tyrosine kinase in perfused rat CCDs blocks the effect of angII on ROMK (94). Taken together, these data suggest that the angII/c-Src pathway prevents an increase in ROMK apical abundance and, thus, K^+ secretion triggered by increased SGK1 during Na^+ depletion.

However, it is important to note that the stimulation of ROMK endocytosis by WNK4 has not been reported in vivo. Additionally, an opposite effect has been observed in mice in which WNK4 is overexpressed. These mice exhibit a significant twofold increase in the level of ROMK transcripts and slightly increased ROMK apical abundance in the distal nephron (76). The same authors reported that ROMK expression is not modified in their FHHt model. More surprisingly, O'Reilly and collaborators (38) showed that a K^+ load increases the level of WNK4 transcripts in the kidney.

WNK1 and WNK4 may also regulate K^+ balance by modulating the expression and activity of BKCa, but in this case by acting in opposite ways. One study recently showed that L-WNK1 activates BKCa via the inhibition of ERK1 and ERK2 (96). Conversely, WNK4 inhibits the membrane expression of BKCa (97–99). Two mechanisms have been described. The first involves activation of the ERK1–ERK2 and p38 pathways with enhanced lysosomal degradation (97, 99). The second relies on the ubiquitination and subsequent degradation of the channel (98).

Regulation of ENaC by WNKs

Several studies aimed to define the potential part played by WNK1 and WNK4 in regulating the expression and activity of ENaC (**Figure 4**). This channel is expressed in a wide range of tissues (such as the kidney, lung, colon, heart, and arteries). In the kidney, it is expressed in the principal cells of the DCT2, the CNT, and the CCD, where it is responsible for electrogenic Na^+ reabsorption, providing the driving force for K^+ secretion by ROMK. A functional ENaC channel is composed of three subunits (in the kidney, α, β, and γ). The importance of ENaC in maintaining the correct Na^+ balance is highlighted by the fact that mutations in the genes encoding its subunits result in salt-losing or hypertensive diseases. Loss-of-function mutations in any of the three subunits lead to pseudohypoaldosteronism type I, an autosomal recessive syndrome associated with salt-wasting and hyperkalemia. Conversely, gain-of-function mutations in the genes encoding the β and γ subunits are responsible for Liddle's syndrome, an autosomal dominant disease characterized by early-onset hypertension and hypokalemia.

WNK4 inhibits ENaC in *Xenopus* oocytes (100). As for ROMK, the kinase activity of WNK4 is not required to regulate ENaC. The same authors showed that the C-terminal domain of ENaC is necessary because WNK4 cannot inhibit channels containing a truncated β or γ subunit. In patients with Liddle's syndrome, this domain is either mutated or deleted. It contains a PY motif that binds to the ubiquitin ligase Nedd4-2. The interaction between ENaC and Nedd4-2 results in ubiquitination, internalization, and proteasomal degradation of the channel. The requirement of the C-terminal domain for WNK4-mediated regulation of ENaC suggests that WNK4 may be involved in the interaction between Nedd4-2 and ENaC or in the modulation of this interaction. Accordingly, overexpression of WNK4 in the A6 distal nephron cell line decreases both the apical and total abundances of all three subunits of ENaC, without affecting open-channel probability (101). This is not caused by inhibiting the trafficking of the channel to the membrane,

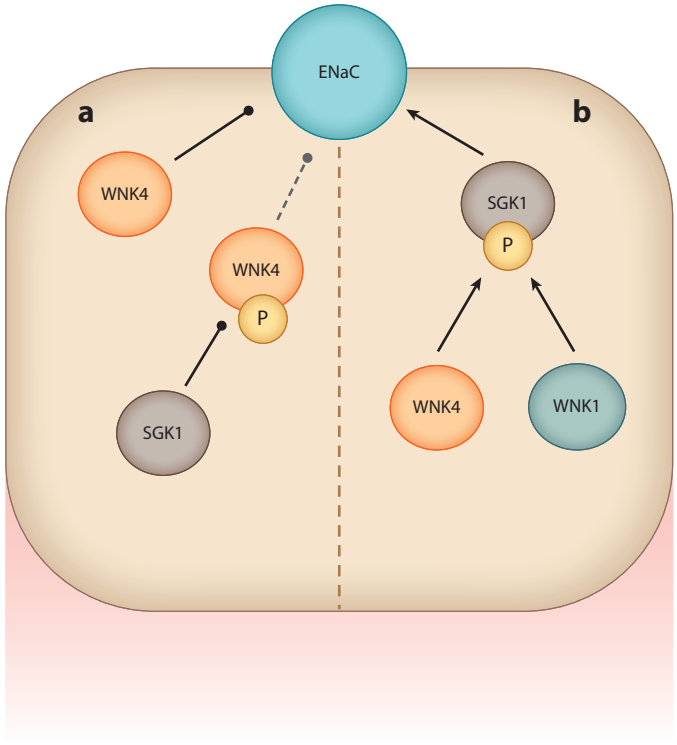

Figure 4

Two different pathways for the regulation of ENaC by WNKs. Two different pathways have been identified by in vitro studies. (*a*) Some in vitro studies have identified a pathway showing that WNK4 inhibits ENaC (93, 100). The phosphorylation of WNK4 by SGK1 precludes the kinase from inhibiting ENaC. (*b*) Other studies have shown that WNK4, and also L-WNK1, can phosphorylate SGK1, thus contributing to the increased apical expression of ENaC (103, 104).

but rather by increasing its internalization and retrograde trafficking (101). However, WNK4 can still decrease the expression of an ENaC β subunit containing one of the mutations causing Liddle's syndrome (101), suggesting that WNK4 regulates the level of expression of ENaC independently of Nedd4-2-mediated ubiquitination. The precise mechanisms by which this is achieved remain to be identified.

One of the WNK4 mutants expressed in FHHt patients (WNK4-Q562E) loses the ability to inhibit ENaC (100). This has been confirmed in vivo in a mouse model overexpressing this mutant WNK4 (76) by measuring the Na^+ flux in the colon in the presence or absence of amiloride, an ENaC-blocker. Amiloride-sensitive Na^+ flux is increased in the colon of these mice compared with wild-type littermates (100). However, the same authors have also reported that ENaC expression is not modified in the kidneys of mice overexpressing the wild-type or mutant WNK4 (76). This contradicts observations from the other WNK4-FHHt model generated by mutation of the endogenous *WNK4* gene. In this model, the level of expression and proteolytic cleavage of the α subunit of the channel is increased. The level of expression of the β subunit is also increased, but only the proteolytic processing of the γ subunit is enhanced (102). In addition, the transepithelial voltage is lower in the isolated, perfused CCD of mutant mice compared with wild-type littermates, and this difference is corrected by the addition of amiloride to the perfusate, suggesting that ENaC is stimulated by the mutation of WNK4. However, it is important to note

that, although this difference is significant, it is very small. In WNK4 knockout mice, amiloride-sensitive Na^+ reabsorption in isolated, perfused CCDs and the natriuretic response to amiloride are greatly enhanced (71). However, this is not due to the direct effect of the inactivation of WNK4 on ENaC, but rather to a compensatory mechanism for the strong decrease in NCC expression and, thus, Na^+ reabsorption by the DCT.

As described above, in vitro studies have shown that SGK1 can phosphorylate WNK4 and, thus, regulate its activity toward ROMK. The same is true for ENaC (**Figure 4**). When the phosphorylation of WNK4 by SGK1 is inhibited, WNK4 can no longer inhibit ENaC in *Xenopus* oocytes (93). However, a few studies have found evidence that WNK1 and WNK4 can activate SGK1. The overexpression of WNK1 in HEK293 cells activates the kinase activity of SGK1, notably the phosphorylation of Nedd4-2, and, thus, increases amiloride-sensitive sodium currents in an SGK1- and Nedd4-2-dependent fashion (103). Interestingly, the kinase activity of WNK1 is not required, but WNK1 needs to be phosphorylated by an Akt/PI3-kinase-related pathway to activate SGK1 (103). The same results have been found when WNK4 is overexpressed in HEK293 cells (104). The contradiction with the aforementioned studies could be due to differences in the experimental systems, notably the extracellular and intracellular ion concentrations that influence the phosphorylation of WNKs. The activation of SGK1 has never been measured in the different FHHt or WNK4 knockout models.

Regulation of Cl^- Transport by WNKs

In addition to NCC, the WNK kinases may regulate several molecules involved in Cl^- transport, such as CFTR (105) and CFEX (106). A few studies have also focused on the regulation of claudins by WNK1 and WNK4. The overexpression of WNK1 in MDCK cells increases Cl^- permeability and claudin-4 phosphorylation (107). Similar results were obtained with WNK4, which is found at the tight junctions of transfected MDCK cells (108). The overexpression of the FHHt-mutant WNK4-D564A significantly increases Cl^- permeability in MDCK cells; the effect of wild-type WNK4 follows the same trend but is not significant. In vitro kinase assays have shown that both proteins phosphorylate claudins 1 to 4, as well as claudin-7 (109), but not occludin (108).

Regulation of Ca^{2+} Reabsorption by WNKs

An additional feature of FHHt is hypercalciuria. The study of a large family with a mutation in *WNK4* showed that this is associated with low bone mineral density (2). Hypercalciuria could be the consequence of increased NCC activity because the pharmacological or genetic inhibition of NCC induces the reverse phenotype—that is, hypocalciuria. It could also be caused by a decrease in the activity of NKCC2, which would reduce the paracellular reabsorption of calcium in Henle's loop. Indeed, the natriuretic response to furosemide is blunted in the knock-in mouse model of FHHt ($WNK4^{+/D564A}$) (110). However, it cannot be excluded that the diminished Na^+ excretion observed after furosemide injection in these mice is not due to increased downstream Na^+ reabsorption by NCC.

A few studies have shown that WNK4 may regulate calcium reabsorption directly but, as is often the case with the WNKs, the results are contradictory. WNK4 has indeed been found to increase (111, 112) or decrease (113) the membrane expression and, thus, the activity of the epithelial calcium channel known as transient receptor potential vanilloid-5 (TRPV5). In vivo studies support the idea of stimulation of TRPV5 by WNK4. In mice, fibroblast growth factor-23 (FGF23) is a positive regulator of apical abundance of TRPV5 (114). The infusion of FGF23 in wild-type mice increases WNK4 phosphorylation by SGK1 and induces a subcellular redistribution of the kinase

from the cytoplasm to the subapical compartment, thereby allowing interaction between WNK4 and TRPV5. This interaction is decreased in mice in which FGF23 is inactivated.

CONCLUSIONS

The discovery that WNK kinases are responsible for an inherited form of salt-sensitive hypertension in humans triggered the interest of many groups of researchers. The remarkable amount of work that has been completed during the past decade is beginning to unravel some of the mechanisms involved in the fine-tuning of ion transport in the distal nephron. WNK kinases modulate the activity of NCC in the DCT through a SPAK-dependent mechanism and ion-transport channels in the DCT2, CNT, and CD by a SPAK-independent mechanism. Thus, the WNK and SPAK protein complex is a potential area for targeting the development of new drugs that could have potent antihypertensive effects (115–117).

The activity of the WNK kinases toward SPAK/NCC is modulated by $[Cl^-]_i$, making WNKs good candidates for acting as the chloride sensor of the cell. Additionally, the stability of WNK kinases is modulated by a RING-type E3-ubiquitin ligase complex formed by KLHL3 and CUL3 proteins, which are also mutated in inherited cases of FHHt. Although our understanding of the effects of WNKs on ion-transport proteins has improved during the past few years, there are several unanswered questions that will require the input and cooperation of many groups of researchers before they are resolved.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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