WNK4-mediated regulation of renal ion transport proteins

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Peng J-B, Warnock DG. WNK4-mediated regulation of renal ion transport proteins. Am J Physiol Renal Physiol 293: F961–F973, 2007. First published July 18, 2007; doi:10.1152/ajprenal.00192.2007.—Point mutations in WNK4 [for With No K (lysine)], a serine-threonine kinase that is expressed in the distal nephron of the kidney, are linked to familial hyperkalemic hypertension (FHH). The imbalanced electrolyte homeostasis in FHH has led to studies toward an understanding of WNK4-mediated regulation of ion transport proteins in the kidney. A growing number of ion transport proteins for Na⁺, K⁺, Ca²⁺, and Cl⁻, including ion channels and transporters in the transcellular pathway and claudins in the paracellular pathway, are shown to be regulated by WNK4 from studies using models ranging from *Xenopus laevis* oocytes to transgenic and knockin mice. WNK4 regulates these transport proteins in different directions and by different cellular mechanisms. The common theme of WNK4-mediated regulation is to alter the abundance of ion transport proteins at the plasma membrane, with the exception of claudins, which are phosphorylated in the presence of WNK4. The regulation of WNK4 can be blocked by the full-length WNK1, whose action is in turn antagonized by a kidney-specific WNK1 variant lacking the kinase domain. In addition, WNK4 also activates stress-related serine-threonine kinases to regulate members of the SLC12 family members of cation-chloride cotransporters. In many cases, the FHH-causing mutants of WNK4 exhibit differences from wild-type WNK4 in regulating ion transport proteins. These regulations well explain the clinical features of FHH and provide insights into the multilayered regulation of ion transport processes in the distal nephron.

WNK1; kidney; hypertension; ion channel; transporter

THE DISTAL PORTION OF THE nephron, including the distal convoluted tubule (DCT), the connecting tubule (CNT), and the cortical collecting duct (CCT), is important for maintaining an electrolyte balance and, in turn, a normal blood pressure. The roles of these ion transport proteins in maintaining a normal blood pressure have been demonstrated in genetic disorders (59, 103). An array of ion transport proteins located in this portion of the nephron plays important roles in maintaining an electrolyte balance and, in turn, a normal blood pressure. The roles of these ion transport proteins in maintaining a normal blood pressure have been demonstrated in genetic disorders (59, 103). For example, gain-of-function in the amiloride-sensitive epithelial Na⁺ channel (ENaC) causes hyperkalemia in Liddle’s syndrome (32, 90); loss-of-function in the thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC; SLC12A3; also known as TSC or NCCT) results in hypotension in Gitelman syndrome (91).

The linkage of familial hyperkalemic hypertension (FHH; also known as pseudohypoaldosteronism type II, PHAII, or Gordon’s syndrome) (30), an autosomal dominant form of hypertension, to two members of the WNK [With No K (lysine)] family of protein serine-threonine kinases has powered efforts to understand the roles of WNK kinases in regulating ion transporters in the distal tubule (104). The WNK family consists of four members; they all lack an invariant lysine residue in the catalytic site in subdomain II, which is important for binding ATP (36, 99, 104, 109). Mutation in either the WNK1 or WNK4 gene causes FHH (104). The FHH-causing gene mutations in WNK4 are missense mutations clustered in a short “acidic motif” rich in negatively charged amino acids downstream of the kinase domain (29, 104), with the exception of R1185C, which is close to the COOH terminus of the kinase (104). FHH is also caused by overexpression of WNK1, another member of the WNK family, due to deletions in the first intron of the WNK1 gene (104). The clinical features of FHH, including hyperkalemia, hypertension, and metabolic acidosis, are common among patients carrying WNK1 and WNK4 mutations. Abnormal Ca²⁺ homeostasis has been reported in some FHH patients (84, 89, 92). Interestingly, hypercalcuiara was observed in patients carrying that WNK4Q565E mutation (64), but not in patients carrying the WNK1 gene mutation (1). The association of the two WNK kinases with hypertension due to imbalanced electrolyte homeostasis has stimulated studies on their regulation of renal ion transport proteins. While these studies aimed at connections between WNK kinases and the molecular pathogenesis of FHH, insights obtained through these studies are very helpful in understanding the regulation of renal handling of electrolytes. The roles of WNK kinases in regulation of ion transport proteins and in the pathogenesis of FHH have been subject of excellent reviews (9, 22, 26, 30, 31, 42, 43, 45, 79, 95, 108, 110). Due to the limited scope of this review, regulations mediated by WNK1 (2, 7, 10, 53, 68, 73, 101, 102, 111, 112, 123) and WNK3 (13, 41, 56, 81) will not be covered. Instead, we will focus on the WNK4-mediated regulation of ion transport proteins.
Distribution of WNK4 Along the Nephron

Early Northern blot analysis suggests that WNK4 is exclusively expressed in the kidney among 12 organs examined (104). Later reports indicate that WNK4 is also expressed in extrarenal tissues such as colon, brain, testis, heart, liver, prostate, and lung of mice (39) or humans (99). Within the kidney, the strongest WNK4 transcript signal was detected in the DCT and CNT by in situ hybridization approach (71). Moderate levels of WNK4 transcript in the macula densa, medullary thick ascending limb, and collecting duct were also detected (71). This is broader than the immunohistochemical staining results showing WNK4 in the DCT and collecting duct (CD) (104). Interestingly, renal WNK4 is upregulated by high dietary K⁺ intake and is downregulated by low Na⁺ intake, whereas aldosterone treatment does not significantly increase the WNK4 mRNA level (71). This suggests that WNK4 is a factor that mediates the body’s response to alterations in K⁺ and Na⁺ balance, in parallel to but distinct from aldosterone.

WNK4 is fairly restricted to epithelial cells, such as tubular epithelial cells in the kidney (104), cuboidal epithelial cells in the pancreatic and the biliary ducts, pseudostratified columnar epithelial cells of epididymis, and colonic crypt epithelial cells (39). WNK4 is also weakly detected in endothelial cells, such as those comprising the blood-brain barrier (39). At the subcellular level, WNK4 is usually distributed in the cellular junction and often extends to the lateral membrane. In the kidney, WNK4 colocalizes with the tight junction marker ZO-1 in the DCT; in the CD, however, cytoplasm distribution of WNK4 was also observed (104). When transfected into Madin-Darby canine kidney (MDCK) cells, WNK4 localizes to the intercellular junction as well as to the cytosol (40, 114). This pattern of cellular distribution allows WNK4 to readily regulate proteins at the intercellular junction, e.g., the claudins (114). However, it is somewhat puzzling how WNK4 regulates ion transporters and channels in apical or basolateral membranes. In a transgenic mouse line carrying FHH-causing WNK4<sup>D564A</sup>, the mutated WNK4 localizes to the apical membranes in the thick ascending limb of Henle’s loop and to cytoplasm in the distal tubule (115).

WNK4 Regulates Ion Transporters and Channels in the Transcellular Pathway and Claudins in the Paracellular Pathway

The distribution of WNK4 allows it to regulate a variety of ion transport proteins along the distal tubule as tested in experimental systems including <i>Xenopus laevis</i> oocytes and mammalian cells. The regulations of ion transport proteins by WNK4 are summarized in Table 1 and Fig. 1. These include apical or basolateral ion transporters, ion channels in the

Table 1. WNK4-mediated regulation of ion transport proteins

<table>
<thead>
<tr>
<th>Ion Transport Protein</th>
<th>Role</th>
<th>Renal Distribution</th>
<th>Effect of WNK4</th>
<th>Effect of Kinase-Dead Mutant</th>
<th>Effect of FHH Mutants</th>
<th>Mechanism of Regulation</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCC</td>
<td>Na⁺-Cl⁻ cotransport</td>
<td>DCT, apical</td>
<td>Inhibition</td>
<td>Not effective</td>
<td>Less effective</td>
<td>Block of forward transport from Golgi; increased lysosomal degradation</td>
<td>5, 28, 29, 52, 96, 105, 116, 118</td>
</tr>
<tr>
<td>ROMK</td>
<td>K⁺ secretion</td>
<td>CNT, CCD, apical</td>
<td>Inhibition</td>
<td>Effective</td>
<td>More effective</td>
<td>Increased clathrin-mediated endocytosis</td>
<td>33, 44</td>
</tr>
<tr>
<td>ENaC</td>
<td>Na⁺ reabsorption</td>
<td>CNT, CCD, apical</td>
<td>Inhibition</td>
<td>Inhibition or not effective</td>
<td>Less effective with NCC present</td>
<td>Increased degradation mediated by Nedd4-2</td>
<td>21, 82</td>
</tr>
<tr>
<td>TRPV5</td>
<td>Ca²⁺ reabsorption</td>
<td>DCT, CNT, apical</td>
<td>Enhancing</td>
<td>Inhibition or not effective</td>
<td>Less effective</td>
<td>Likely increase in secretory pathway</td>
<td>38</td>
</tr>
<tr>
<td>TRPV4</td>
<td>Osmolarity sensing</td>
<td>TAL, DCT, CNT, CD, basolateral</td>
<td>Inhibition</td>
<td>Not effective</td>
<td>Less effective</td>
<td>Decreased surface expression</td>
<td>23</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cl⁻ secretion</td>
<td>Renal tubules and extrarenal</td>
<td>Inhibition</td>
<td>Not effective</td>
<td>More effective</td>
<td>Decreased surface expression</td>
<td>117</td>
</tr>
<tr>
<td>CFEX</td>
<td>Cl⁻/anion exchange</td>
<td>PT, apical extrarenal</td>
<td>Inhibition</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NKCC1</td>
<td>Na⁺, K⁺, 2Cl⁻ cotransport</td>
<td>Extrarenal</td>
<td>Inhibition</td>
<td>ND</td>
<td>ND</td>
<td>Decrease surface expression</td>
<td>39</td>
</tr>
<tr>
<td>KCC1</td>
<td>K⁺, Cl⁻ cotransport</td>
<td>Renal; extrarenal</td>
<td>Inhibition</td>
<td>Not effective</td>
<td>Same as WT WNK4</td>
<td>ND</td>
<td>27</td>
</tr>
<tr>
<td>KCC3</td>
<td>K⁺, Cl⁻ cotransport</td>
<td>PT; basolateral; extrarenal</td>
<td>Inhibition</td>
<td>Not effective</td>
<td>Same as WT WNK4</td>
<td>ND</td>
<td>27</td>
</tr>
<tr>
<td>KCC4</td>
<td>K⁺, Cl⁻ cotransport</td>
<td>PT; TAL, DCT, CD, basolateral; extrarenal</td>
<td>Inhibition</td>
<td>Not effective</td>
<td>Same as WT WNK4</td>
<td>ND</td>
<td>27</td>
</tr>
<tr>
<td>Claudins</td>
<td>Paracellular permeability</td>
<td>Claudin 4 in CCD, tight junction</td>
<td>Enhancing</td>
<td>ND</td>
<td>More effective</td>
<td>Phosphorylation of Claudins</td>
<td>40, 114</td>
</tr>
</tbody>
</table>

WNK4, With No K (lysine); NCC, Na⁺-Cl⁻ cotransporter; ROMK, renal outer medullary K⁺ channel; ENaC, epithelial Na⁺ channel; TRPV4 and TRPV5, transient receptor potential vanilloid subfamily member 4 and member 5, respectively; CFEX, Cl⁻/formate exchanger; NKCC1, Na⁺-K⁺-2Cl⁻ cotransporter 1; KCC, K⁺-Cl⁻ cotransporter; CFTR, cystic fibrosis transmembrane conductance regulator; PT, proximal tubule; TAL, thick ascending limb; DCT, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct; CD, collecting duct; WT, wild-type; ND, not determined.

AJP-Renal Physiol • VOL 293 • OCTOBER 2007 • www.ajprenal.org
transcellular pathway, and claudins in the paracellular pathway, which will be summarized below.

Thiazide-sensitive Na\(^{+}\)/H\(^{+}\)-Cl\(^{-}\)/H\(^{+}\) cotransporter NCC. NCC is the target of thiazide diuretics and is localized to the DCT (25). Loss-of-function of NCC results in Gitelman syndrome, featuring hypokalemia, hypotension, and hypocalciuria (91). The clinical features of FHH, such as hyperkalemia, hypertension, metabolic acidosis, and hypercalciuria, are opposite those of Gitelman syndrome (64). Because FHH patients are responsive to thiazide administration (20) and WNK4 colocalizes with NCC in the DCT (104), two groups independently studied the regulation of NCC by WNK4 (105, 116). Both groups found that WNK4 inhibits NCC by decreasing its level at the plasma membrane in the \(X. laevis\) system (105, 116). Yang et al. (116) found that while WNK1 alone has no effect on NCC, it blocks the inhibitory effect of WNK4. Wilson et al. (105) found that WNK4 interacts with NCC and the kinase activity of WNK4 is required for the regulation. Both groups found the FHH-causing WNK4Q562E mutant (mouse WNK4, equivalent to Q565E in FHH/PHAII patients), denoted as Tg(Wnk4 WT) mice exhibit hypertension, hyperkalemia, and hypercalciuria similarly to the patients carrying WNK4Q565E (63, 64); whereas the Tg(Wnk4 WT) mice have opposite manifestations (52). Immunostaining using antibodies against tubular markers reveals a marked reduction of NCC luminal surface area in Tg(Wnk4 PHAII) mice and a significant increase in NCC-positive nephron segments in the Tg(Wnk4 PHAII) mice. On the other hand, renal outer medullary K\(^{+}\)/H\(^{+}\) channel (ROMK) and ENaC staining are not altered (52). Targeted deletion of NCC in mice results in a significant reduction of DCT structures (87). Therefore, the hyperplasia of DCT is likely caused by the increased expression of NCC in the Tg(Wnk4 PHAII) mice due to the impaired inhibition of NCC by Wnk4 Q562E, although the mechanism by which NCC controls the morphology of DCT is unknown. Mice lacking NCC exhibit a phenotype similar to Gitelman syndrome (67, 87), which is opposite that of FHH. Thus increased NCC is likely the cause of the FHH-like phenotype in the Tg(Wnk4 PHAII) mice. This is confirmed by

![Diagram](image_url)
the observation that the removal of NCC in Tg(Wnk4PHAI) background corrects all the abnormalities observed in the Tg(Wnk4PHAI) mice (52). It appears that all the features of FHH could be explained by the impaired regulation of NCC by WNK4. Nevertheless, altered regulations of other ion transport proteins by WNK4PHAI also explain the clinical features of FHH.

The most recent results obtained from the Wnk4D561A/+ knockin mice confirmed the importance of NCC in the pathogenesis of FHH (119). The Wnk4D561A/+ knockin mouse is an excellent model for FHH. In this model, mice develop hyperkalemia, hypertension, and metabolic acidosis at 3.5 mo of age, and the abnormalities could be corrected by hydrochlorothiazide (119). In contrast to the lack of difference between wild-type WNK4 and FHH-causing D561A (corresponding to human D564A), a mutant of WNK4 as tested in X. laevis oocytes (116) and MDCK cells (120), the NCC protein level in the total lysates and the membrane fractions of the kidney is elevated in the Wnk4D561A/+ mice (119). Consistent with the observation in Tg(Wnk4PHAI) mice, the luminal surface area of some NCC-positive segments appears to be increased. In addition, the phosphorylation of NCC at serine 71 (Ser71) is increased (119). Because the phosphorylation of NCC increases its activity (74), it is likely both the surface level of NCC and the activity of individual NCC are increased in the Wnk4D561A/+ mice. Although other changes were observed in the Wnk4D561A/+ mice, such as elevated levels of ENaC, maxi-K, NCC is the only one that remains elevated in Wnk4D561A/+ mice after chronic thiazide treatment, suggesting that alterations in NCC play a primary role in the pathogenesis of FHH caused by the WNK4 gene mutation (119).

ROMK. ROMK is a K+ channel in the aldosterone-responsive collecting duct that mediates K+ secretion. The driving force for K+ secretion is created by the Na+ influx via ENaC. Thus a net Na+ uptake and K+ secretion in the collecting duct are mediated by the concerted actions of ROMK and ENaC. FHH features hypertension with hyperkalemia, suggesting a possible involvement of dysregulation of ROMK-mediated K+ secretion. In the X. laevis oocyte system, Kahle et al. (44) found that ROMK-mediated K+ current is drastically inhibited by WNK4 due to the reduction of the ROMK protein level at the plasma membrane. In contrast to the WNK4-mediated regulation of NCC, the action of WNK4 on ROMK is independent of kinase activity. The FHH-causing mutants of WNK4 tested, including Q562E and E559K (44) and D564H (29), further inhibited ROMK. This gain-of-function effect on WNK4PHAI mutants, which is very different from the loss-of-function effect on NCC (105), could explain the hyperkalemia in FHH. However, the inhibitory effects of FHH-causing WNK4 mutants were not observed in two transgenic mouse models, carrying WNK4D564A (115) or WNK4D562E (52), suggesting that the change in surface expression of ROMK is not a primary regulatory event. In Wnk4D561A/+ knockin mice, the abundance of maxi-K(α) protein level is significantly elevated in contrast to the unaltered ROMK level (119). Because the elevation of maxi-K(α) is not evident after chronic thiazide treatment, the increase in maxi-K is likely a compensatory mechanism for the need to increase K+ secretion in the Wnk4D561A/+ mice (119).

ENaC. ENaC mediates electrogenic, amiloride-sensitive Na+ influx in the aldosterone sensitive segment of the distal nephron (6). A luminal negative potential created by ENaC-mediated Na+ influx is necessary for K+ secretion and paracellular Cl− flux. By measuring the nasal potential difference in FHH patients carrying WNK4D566E, Farfel and colleagues (21) provided evidence that WNK4D566E upregulates amiloride-sensitive Na+ transport likely mediated by ENaC in the airway. By coexpression of ENaC subunits with WNK4 in X. laevis oocytes, Ring and colleagues (82) found that wild-type WNK4 inhibits ENaC activity. The kinase-dead WNK4 is capable of inhibiting ENaC to the extent of wild-type WNK4, but the FHH-causing WNK4D566E fails to inhibit ENaC. The amiloride-sensitive Na+ transport in the distal colon in the Tg(Wnk4PHAI) mice is elevated compared with that in wild-type mice, indicating that ENaC is regulated by WNK4 in vivo (82). The increased ENaC activity could be due to increased ENaC protein in the apical membrane, although no change in the ENaC protein level in Tg(Wnk4PHAI) mice has been reported so far. In the Wnk4D561A/+ knockin mice, the protein levels of ENaC subunits are elevated and the luminal surface area of the ENaC-positive segment appears to be increased (119). Consistent with these observations, the amiloride-sensitive Na+ permeability is also increased in Wnk4D561A/+ mice as determined by CCD microperfusion (119). However, in contrast to NCC, the difference in ENaC expression between wild-type and the Wnk4D561A/+ mice is not evident after chronic thiazide treatment, suggesting the increase in ENaC could be a secondary effect (119). Nevertheless, the regulation of ENaC by WNK4 and the loss-of-function behavior of the FHH-causing mutants of WNK4 are consistent with the hypersecretion manifestation in FHH, because the increased Na+ reabsorption by ENaC in patients carrying the Q566E mutant will cause volume expansion and hypertension.

Epithelial Ca2+ channel TRPV5. TRPV5 (for transient receptor potential cation channel, vanilloid subfamily, member 5) is a Ca2+-selective channel that is expressed in the apical membrane of DCT and CNT (34, 61, 76). TRPV5 plays an important role in regulating urinary Ca2+ excretion. Mice lacking TRPV5 exhibit a sixfold increase in Ca2+ excretion (35). Because FHH patients carrying WNK4D566E exhibit hypercalciuria (64), the possible regulation of TRPV5 by WNK4 was investigated (38). In contrast to the inhibitory effects of WNK4 on other ion transporter proteins in the tubular pathway, WNK4 increases TRPV5-mediated Ca2+ uptake by approximately twofold in X. laevis oocytes. The regulation appears to have a certain level of specificity. On the one hand, WNK1 has no effect on TRPV5; on the other, WNK4 has no significant effect on TRPV6 (75, 77), which also participates in renal reabsorption of Ca2+ in addition to its major role in intestinal Ca2+ absorption (3). WNK4 increases the level of mature TRPV5 at the plasma membrane without significantly affecting the channel property. WNK4-mediated regulation of TRPV5 likely involves the kinase activity of WNK4, because the kinase-dead mutant of WNK4 significantly inhibits TRPV5 at 1 day after injection of cRNA instead of upregulating it. This is likely due to a dominate negative effect of the kinase-dead WNK4 on endogenous WNK4 (38). All the FHH-causing mutants tested, including E562K, D564A, Q566E, and R1185C, behave very much like wild-type WNK4 in enhancing TRPV5 (38).

In the distal tubule, Ca2+ reabsorption is inversely related to Na+ reabsorption (11). The opposite effect of WNK4 on

AJP-Renal Physiol. • VOL 293 • OCTOBER 2007 • www.ajprenal.org
WNK4-mediated regulation of ion transport proteins

TRPV5 and NCC further suggests that WNK4 might be involved in the inverse relation of Na\(^+\) and Ca\(^{2+}\) transport. Interestingly, the effect of WNK4 on TRPV5 is abrogated by NCC in a dose-dependent fashion (38). In the presence of NCC, WNK4\(^{Q565E}\) fails to significantly increase TRPV5-mediated Ca\(^{2+}\) uptake, whereas wild-type WNK4 is capable of enhancing TRPV5 significantly (38). This suggests that the effect of WNK4 on TRPV5 depends on the protein level of NCC in the later portion of the DCT, where the three proteins are coexpressed. In the case of the Q565E mutation, the level of NCC is increased due to the loss-of-function effect of WNK4 on NCC. The increased NCC will further block the enhancing effect of WNK4\(^{Q565E}\) on TRPV5, resulting in increased Na\(^+\) reabsorption and decreased Ca\(^{2+}\) reabsorption. Thus it is likely that this mechanism may contribute to the increased urinary Ca\(^{2+}\) excretion in FHH patients carrying WNK4\(^{Q565E}\).

Osmolarity-sensitive cation channel TRPV4. TRPV4 (transient receptor potential cation channel, vanilloid subfamily, member 4) is a nonselective cation channel that could be activated by reduction of osmolarity in the extracellular solution (16, 58, 93, 107). It is expressed in the basolateral membrane of the water-impermeant nephron segments from the thin ascending limb of Henle’s loop with the exception of the macula densa (97). By coexpressing WNK4, WNK1, or WNK4 and WNK1 with TRPV4 stably expressed in human embryonic kidney (HEK) cells, Fu et al. (23) demonstrated that both WNK4 and WNK1 decrease TRPV4 activity by reducing the cell surface level of TRPV4. FHH-causing mutants E559K and Q562E are less effective in blocking TRPV4. The effect is likely kinase dependent as the kinase-dead mutant of WNK4 is not effective in blocking TRPV4. Intriguingly, WNK4 constructs without portions in the NH\(_2\)-terminal region, which contains the kinase domain, are effective in blocking TRPV4. However, without the extreme COOH-terminal region of WNK4, the blocking effect of WNK4 on TRPV4 is abolished. These complicated structural requirements for the blocking effect of WNK4 on TRPV4 suggest that a certain conformation of the WNK4 protein is necessary for the inhibition of TRPV4 to take place. Since the physiological role of TRPV4 is less clear in the kidney (8), how much this WNK4-mediated regulation of TRPV4 is involved in the pathogenesis of FHH is yet to be investigated.

Other members of the SLC12 family of cation-coupled Cl\(^-\) cotransporters. Besides NCC, WNK4 also inhibits some other members of the SLC12 family. NKCC1, a Na\(^+\)\(-\)K\(^+\)\(-\)2Cl\(^-\) cotransporter in the basolateral membrane of Cl\(^-\)secreting epithelia, is inhibited by WNK4 through the reduction of expression at the plasma membrane (39). Three K\(^+\)\(-\)Cl\(^-\) cotransporters, KCC1, KCC3 and KCC4, are also negatively regulated by WNK4 (27). Coexpression of WNK4 with the three KCCs reduces their activity in swollen oocytes, which is necessary for the activation of the KCCs. The effect of WNK4 on these KCCs requires the kinase activity as the kinase-dead WNK4 has a reduced effect on KCC1 and has no effect on KCC3 and KCC4. Three FHH-causing mutants tested, including E559K, D561A, and Q562E, inhibit KCC4 to a similar extent. Among the three KCCs, KCC4 is known to be expressed in the α-intercalated cells of the CD and KCC4 null mice exhibit metabolic acidosis (4). Nonetheless, due to the absence of any alterations of the FHH-causing mutants on KCC4, it is unlikely that the metabolic acidosis in FHH is caused by the WNK4\(^{Q565E}\) mediated regulation of KCC4.

CFTR. Mutations of the CFTR gene cause cystic fibrosis (CF), a common hereditary disease with pathophysiological abnormalities in the lung and other epithelial tissues (88). WNK4 was shown to be well colocalized with CFTR in the colon (39). More recently, Yang et al. (117) reported that CFTR is inhibited by both WNK1 and WNK4. The effect of WNK4 on CFTR is similar to that on ROMK (117). The inhibition of CFTR by WNK4 is likely due to the reduction in surface expression, and the inhibition is independent of WNK4 kinase activity. The FHH-causing WNK4\(^{Q562E}\) exhibits a gain-of-function effect and further reduces CFTR activity than does wild-type WNK4 (117). In contrast to the moderate level of CFTR in the lung, CFTR is abundantly expressed in the kidney (12), in tubular cells including those in the CCD (18, 98). However, CF patients have no significant abnormality in renal function (106). CFTR functionally regulates ENaC (57, 94) and ROMK (51, 62, 85), which are also regulated by WNK4. How much the enhanced inhibition of CFTR by WNK4\(^{FHH}\) affects ENaC and ROMK, and to what extent the overall effect contributes to the pathogenesis of FHH, are questions to be addressed in future studies.

Transport proteins outside the kidney. Since WNK4 is rather broadly expressed in many epithelial cells, its regulatory effects are not restricted to the ion transporters within the kidney. In fact, WNK4 regulates amiloride-sensitive Na\(^+\) transport in the airway (21) and the colon (82). In addition, the chloride-formate exchanger (CFEX) is inhibited by WNK4 (39). Since WNK4 has not been detected in the proximal tubule, where CFEX is expressed in the kidney (49), the regulation of CFEX by WNK4 is likely significant outside the kidney, in many Cl\(^-\)-secreting epithelia where CFEX is expressed.

Claudins in the paracellular pathway. In addition to the transporters in the transcellular pathways of ion transport, WNK4 is capable of regulating the paracellular pathway of Cl\(^-\) transport likely via claudins. Increased reabsorption ability for Cl\(^-\) in the distal nephron, known as “chloride shunt,” which results in a decreased driving force for K\(^+\) and H\(^+\) secretion later in the tubule, has been postulated by Schambelan et al. (86) as the cause of FHH. It is not clear whether the increased Cl\(^-\) permeability is via a transcellular pathway or paracellular pathway. Since the majority of WNK4 proteins are in the tight junction of the distal tubule (104), it is possible that the paracellular pathway is affected. Yamauchi et al. (114) developed MDCK cell lines stably expressing wild-type WNK4 or the FHH-causing WNK4\(^{Q564A}\) in a tetracycline-regulated manner. The paracellular Cl\(^-\) permeability of the MDCK monolayer is increased on the induction of WNK4 and is further increased by the FHH-causing WNK4\(^{D564A}\). On induction of WNK4, protein levels and cellular distributions of occludin and claudins 1-4 are not altered. However, the phosphorylation of claudins 1-4 but not occludin is increased. A further increase in phosphorylation of claudins 1-4 was observed by the overexpression of WNK4\(^{D564A}\). WNK4 binds to claudins via a YV motif in the COOH-terminus of claudins, and the phosphorylation of claudins occurs at the COOH-terminal region as well (114).

Because claudins 1 and 2 are not expressed in the distal nephron where WNK4 is expressed, the regulation of these two claudins by WNK4 may not be of physiological importance in...
the kidney. Claudins 3 and 4 are distributed in the distal nephron (48), and a recent study suggests that claudin 4 is involved in Cl− permeability (37). Therefore, the regulation of claudin 4 by WNK4 might be of pathophysiological significance.

Using a similar experimental system, Kahle et al. (40) also demonstrated that wild-type WNK4 (but not the kinase-dead D318A mutant) decreases the transepithelial resistance of MDCK cells by increasing Cl− permeability without altering the tight junction structure. The FHH-causing mutants Q562E and E559K further increased Cl− permeability. Thus both studies indicate a gain-of-function effect of WNK4FH in regulating paracellular Cl− permeability. Thus both studies indicate a gain-of-function effect of WNK4FH in regulating paracellular Cl− permeability. Interestingly, WNK1 overexpression in MDCK cells also causes the same effect as WNK4 mutants, including increased paracellular Cl− permeability and increased phosphorylation of claudin 4 (73). Coexpression of WNK4 could not further increase the WNK1-induced increase in Cl− permeability, suggesting a common pathway of the WNK4- and WNK1-mediated increase in the paracellular route of Cl− transport (73).

Although an increase in paracellular Cl− permeability by FHH-causing mutations of WNK4 is evident in MDCK cell models (40, 114), the involvement of this mechanism in the pathogenesis of FHH is not supported by results from the Wnk4D561A/+ mice (119). A significant difference in the Cl−/Na+ permeability ratio (Pcl/Pna) of the CCD was observed between Wnk4D561A/+ and wild-type mice; this difference was eliminated in the presence of the ENaC blocker amiloride (119). This indicates that the Na+ permeability mediated by ENaC, rather than paracellular Cl− permeability, is increased in the Wnk4D561A/+ mice (119).

Downstream Effectors of WNK4: WNK4 Activates STE20 Kinases to Regulate Members of the SLC12 Family

WNK4 is able to activate the STE20-like kinases, which are stress-related serine-threonine kinases, such as SPAK and OSR1 (17), and in turn regulates members of the SLC12 family. SPAK and OSR1 interact with the cation-chloride cotransporters, including NKCC1, NKCC2, and KCC3 (78). Under both isosmotic (basal) and hyperosmotic (stimulated) conditions, neither SPAK nor WNK4 alone regulates KCCL1; in contrast, the coexpression of both kinases significantly increased NKCC1 activity in isotonic conditions, and no further effect was observed when the oocytes were exposed to hypertonicity (24). The regulation requires kinase activity of both SPAK and WNK4. WNK4 interacts with SPAK through an interacting motif, RFQV, in the COOH-terminal region of WNK4 (24). Thus WNK4 likely activates SPAK, which in turn enhances NKCC1. Indeed, Vitari and colleagues (100) demonstrated that both WNK1 and WNK4 interact with and phosphorylate SPAK and OSR1 at specific sites and markedly increase their kinase activity (100). In addition, a robust phosphorylation of the NH2-terminal region of NKCC1 was observed only when WNK1 or WNK4 together with either SPAK or OSR1 (100). Moriguchi et al. (66) also observed the direct phosphorylation of NH2-terminal-regulatory regions of NKCC1, NKCC2, and NCC by SPAK and OSR1. WNK4 could be phosphorylated by WNK1, which is activated by hypertonic stress, and to a lesser extent by hypotonic stress in kidney epithelial cells as well as in breast and colon cancer cells (55). Thus WNK1 and WNK4 regulate ion flux mediated by cation-chloride cotransporters via the STE20 kinases to restore cell volume in response to hypertonic or hypotonic stress.

The significance of WNK4-mediated regulation of SPAK/OSR1 in the pathogenesis of FHH is highlighted by the study using Wnk4D561A/+ mice (119). While the protein levels of OSR1 and SPAK are not altered, their phosphorylated forms (OSR1 at Ser325 or SPAK at Ser380) are increased in the kidney of Wnk4D561A/+ mice (119). Phosphorylation of key residues activates OSR1/SPAK (100), which in turn phosphorylates NCC (66) and increases its transport activity (74). Thus the WNK4-OSR1/SPAK-NCC signaling pathway plays an important role in blood pressure control and is involved in the pathogenesis of FHH (119).

Upstream Modulators of WNK4

WNK4 interacts with long and kidney-specific WNK1. In one of the two initial reports showing NCC is inhibited by WNK4, Yang et al. (116) found that the effect of WNK4 is abrogated by WNK1, which has no effect on NCC alone. The effect of WNK1 on WNK4 involves a physical association between WNK1 and WNK4 via their kinase domains, and the intact kinase activity of WNK1 as well as the entire protein of WNK1 (118).

Full-length WNK1 (noted as L-WNK1) is rather broadly expressed in multiple tissues (109). A shorter splice variant without the kinase domain is specifically expressed in the kidney (designated as KS-WNK1) (15, 72, 113). L-WNK1 is ubiquitously expressed throughout the kidney, whereas KS-WNK1 is most abundant in the DCT (15, 71). KS-WNK1 but not L-WNK1 is induced by aldosterone in a mouse cortical collecting duct cell line (68). L-WNK1 is increased whereas KS-WNK1 is decreased by dietary K+ restriction in the rat kidney (53). KS-WNK1 is significantly upregulated by a high-K+ diet, high-Na+ diet, and aldosterone in mice as well (71). KS-WNK1 was reported to stimulate ENaC (68). Thus KS-WNK1 appears to be an important regulatory factor responsive to K+ and Na+ balance and aldosterone.

In a follow-up study on the effect of WNK1 on WNK4-mediated regulation of NCC, Subramanyam et al. (96) found that KS-WNK1 alleviates L-WNK1-mediated suppression of WNK4 dose dependently. KS-WNK1 interacts with L-WNK1 and attenuates its kinase activity as demonstrated with in vitro kinase assay (96). Similarly, KS-WNK1 also acts as an antagonist of the L-WNK1-mediated regulation on ROMK (53, 102). In response to high K+ intake or aldosterone release, the KS-WNK1 level is increased, and the increased KS-WNK1 blocks NCC-mediated Na+−Cl− reabsorption (96). This will increase the availability of Na+ and Cl− in the CCD and promote luminal negative potential due to increased Na+ influx mediated by ENaC and consequently result in increased K+ secretion. Thus this additional layer of regulation of NCC and ROMK by KS-WNK4 modulates K+ secretion in response to aldosterone and K+ intake.

SGK1 phosphorylates WNK4 to deliver the action of aldosterone. Na+ reabsorption and K+ secretion in the distal tubule are modulated by aldosterone, which is produced in response to either a reduction in intravascular volume or a high K+ level in the circulation. Aldosterone regulates Na+ and K+ transport in...
part by rapid induction of a serum- and glucocorticoid-inducible kinase (SGK1). SGK1 phosphorylates Nedd4-2, an ubiquitin ligase which interacts with the PY domain in the β- and γ-subunits of ENaC and mediates their retrieval from the plasma membrane (47). The phosphorylated Nedd4-2 does not bind to ENaC and therefore makes ENaC stay in the plasma membrane for a longer time period (14). ROMK is also positively regulated by SGK1 by increasing its abundance at the surface (121, 122). Thus SGK1 is an important mediator of aldosterone action. In a recent study, Ring and colleagues (83) showed that WNK4 can be phosphorylated by SGK1, and a SGK1 phosphorylation site (Serine 1169) was identified close to the COOH-terminal region of WNK4. The S1169D WNK4 mutant mimics the phosphorylated state of WNK4 and alleviates the inhibition of WNK4 on both ENaC and ROMK (83). Increased ENaC activity will promote the luminal negative potential for ROMK-mediated K+ secretion. Thus the phosphorylation of WNK4 by SGK1 promotes Na+ reabsorption and K+ secretion to restore intravascular volume and to reduce the serum K+ level, respectively. Therefore, WNK4 is likely a mediator of aldosterone action in response to volume depletion and hyperkalemia (see Fig. 3).

Aldosterone promotes ENaC-mediated Na+ reabsorption and ROMK-mediated K+ secretion through at least four layers of regulation. 1) Aldosterone induces SGK1 expression, which increases the activity of ENaC and ROMK, respectively, through phosphorylation of Nedd4-2 (14) and ROMK channel protein itself (121). 2) Aldosterone decreases NCC-mediated Na+-Cl− reabsorption through upregulating KS-WNK1, which promotes WNK4-mediated inhibition on NCC by abrogating the blocking effect of L-WNK1 on WNK4 (96). This allows Na+ to reach a more distal portion of the nephron to be reabsorbed by ENaC. The resulted luminal negative potential due to Na+ influx via ENaC will promote ROMK-mediated K+ secretion. 3) In addition, elevated KS-WNK1 in response to aldosterone also increases ROMK-mediated K+ secretion by abrogating L-WNK1-mediated ROMK inhibition (53, 102). 4) The phosphorylation of the SGK1 site in WNK4 by SGK1 abrogates the WNK4-mediated inhibition of both ENaC and ROMK, resulting in a net increase in Na+ reabsorption and K+ secretion (83). Because L-WNK1 activates SGK1 (111), WNK4 is a target of SGK1 (83); in addition, WNK1, WNK4, and SGK1 interact with each other (83, 111, 118), and it is likely that the three kinases form a protein complex at some points of the process to deliver the effect of aldosterone on Na+ reabsorption and K+ secretion.

**Cellular Mechanisms of WNK4-Mediated Regulation**

Many studies, such as WNK4-mediated regulation on NCC (28, 29, 105, 116), ROMK (44), TRPV5 (38), ENaC (82), KCCs (27), and CFTR (117), were carried out using the X. laevis oocyte system; however, the mechanisms underlying these regulations differ. The common feature of WNK4-mediated regulation on ion transporters or channels in the transcellular pathway is that WNK4 alters the abundance of a transport protein at the plasma membrane but does not significantly alter its transport function. However, the regulation differs in three aspects (Table 1). First, the direction of WNK4-mediated regulation differs. TRPV5 is upregulated by WNK4 (38), whereas the rest of the ion transport proteins tested are down-regulated. Second, the regulation differs in the dependence of the WNK4 kinase activity, as tested with the kinase-dead mutant. The effects of WNK4 on NCC (28, 105), TRPV5 (38), and KCCs (27) are kinase dependent, whereas those on ROMK (44), ENaC (82), and CFTR (117) are kinase independent. Third, the effects of WNK4FHH on ion transport proteins are different. The effects of WNK4FHH are indistinguishable to wild-type WNK4 on TRPV5 (in the absence of NCC) (38) and KCCs (27); loss-of-function effects were observed on NCC (5, 29, 105, 116), TRPV4 (23), and ENaC (82); and gain-of-function effects were observed for ROMK (33, 44) and CFTR (117), and perhaps claudins (114). Thus WNK4 is capable of regulating ion transport proteins through different mechanisms, which are not yet fully understood. Some details of the WNK4-mediated regulations of ROMK (33, 44), ENaC (82), NCC (5, 28), and TRPV5 (38) are illustrated in Fig. 2 and will be discussed below.

WNK4 enhances the endocytosis of both ROMK (33, 44) and ENaC (82), however, in different ways. The removal of ROMK from the plasma membrane is mediated by clathrin-dependent endocytosis, and this process involves a NPXY internalization motif in the COOH terminus of ROMK (44, 124). Removal or mutation of this NPXY motif abolished WNK4-mediated inhibition on ROMK (44). Furthermore, disruption of clathrin-dependent endocytosis by a dominant-negative mutant of dynamin (K44R) prevents the action of WNK4 on ROMK. Thus the WNK4-mediated regulation on ROMK is to facilitate the removal of ROMK through the clathrin-mediated endocytosis (44). The molecular mechanism of the regulation of ROMK by WNK4 involves the interaction between WNK4 and ROMK and intersectin (ITSN) (33). ITSN is a linker protein that is involved in the clathrin-dependent endocytosis (70). Three PXXP motifs adjacent to the FHH-causing mutations in WNK4 interact with the SH3 domains of ITSN, and FHH-causing WNK4E559K increases this interaction (33). Furthermore, the interaction of ROMK with WNK4 is increased by the FHH-causing mutations. Thus the increased interactions between WNK4FHH and ROMK and ITSN coincide with the strengthened inhibition of ROMK by WNK4FHH (33).

**Fig. 2.** Cellular mechanisms of WNK4-mediated regulation. **Left:** WNK4 likely blocks the forward trafficking of NCC and enhances that of TRPV5 (for transient receptor potential cation channel, vanilloid subfamily, member 5). The direction of regulation is indicated by plus and minus signs. PM, plasma membrane; TGN, trans-Golgi network; ER, endoplasmic reticulum.
The mechanism of WNK4-mediated regulation of ENaC involves its COOH-terminal PY motif, which is responsible for the interaction of ENaC with E3 ubiquitin ligase Nedd4-2 (82). Each ENaC unit possesses a PY motif that interacts with WW domains 3 and 4 in Nedd4-2 (46). Ubiquitylation by Nedd4-2 facilitates the removal of ENaC from the plasma membrane for degradation. A truncating mutation that removes the PY motif in the β- or γ-unit of ENaC causes Liddle’s syndrome, a genetic form of hypertension due to the increased ENaC activity (32, 90). WNK4 no longer inhibits ENaC mutants of Liddle’s syndrome, indicating that WNK4 facilitates the removal of ENaC through the Nedd4-2-mediated retrieval and degradation of ENaC (82).

With regard to the cellular mechanism by which WNK4 regulates NCC, a novel mechanism involving enhanced degradation of NCC via a lysosomal pathway by WNK4 was reported by Cai and colleagues (5) using mammalian cell models. They found that WNK4-mediated inhibition on NCC surface expression is independent of the clathrin-mediated endocytosis, as indicated by the lack of effect of dominant-negative dynamin mutant K44A. In contrast, bafilomycin, a vacuolar proton pump inhibitor which disrupts the acidification of lysosomes, dose dependently abrogates the inhibitory effect of WNK4 on the steady-state level of NCC (5). This indicates that the inhibitory effect of WNK4 on NCC surface expression results from enhancing degradation of NCC via a lysosomal pathway, suggesting that WNK4 interferes with the forward trafficking of NCC to the plasma membrane. Golbang and colleagues (28) subsequently reported the similar result confirming the findings of Cai and colleagues (5) and further support the notion that WNK4 appears to block the forward trafficking of NCC. When the forward trafficking from the Golgi apparatus is blocked by brefeldin-A, the rate of NCC endocytosis is no longer affected by WNK4 (28). In the WnkD561A+ knockin mice, the NCC protein level in the kidney lysate and that in the membrane fractions are increased, indicating either the synthesis of NCC is increased or the degradation of NCC is decreased by the mutant WNK4 (119), which is consistent with finding of Cai and colleagues (5). In addition, the phosphorylation of Ser71, one of the potential phosphorylation sites for SPAK/OSR1 in NCC (66), is increased in WnkD561A+ mice (119). Because of the concomitant increase in phosphorylated SPAK/OSR1 and NCC, it is likely WNK4D561A+ increases the phosphorylation of SPAK/OSR1, which in turn phosphorylates NCC. The phosphorylation of NCC at Ser71 and other sites increases its transport activity (74). Therefore, both the protein abundance and transport activity of NCC are likely increased in FHH.

In contrast to the action on NCC, WNK4 appears to facilitate the forward trafficking of TRPV5 because the complex glycosylated mature form of TRPV5 is increased by WNK4 (38). Abolishing the N-linked glycosylation of TRPV5 by site-directed mutation abrogates the effect of WNK4 on TRPV5 (unpublished observations). The complex glycosylation in the Golgi apparatus is a step in the secretory pathways of protein delivery to the plasma membrane. TRPV6 is not well complex glycosylated in X. laevis oocytes and likely goes to the plasma membrane via an alternative route because the majority of TRPV6 in the plasma membrane is in core-glycosylated form (unpublished observations). Since WNK4 selectively acts on TRPV5, WNK4 likely enhances a certain step(s) of the secretory pathway via which mature TRPV5 reaches the plasma membrane.

WNK4-mediated regulation involves protein-protein interaction. WNK4 associates with its downstream targets, including NCC (5, 105, 118), ROMK (33, 44), ITSN (33), claudins (114), and SPAK/OSR1 (100) and upstream modulators, including WNK1 (118) and SGK1 (83). WNK4 interacts with NCC and ROMK via the cytoplasmic COOH termini of the channels (33, 44, 105, 118). While the role of the interaction between WNK4 and NCC in the regulation of NCC by WNK4 is unclear, the interaction between WNK4 and ROMK alone appears to be insufficient to mediate the inhibition of ROMK by WNK4. A segment of WNK4 (amino acid residues 1–444) interacts with ROMK, but this segment alone is incapable of inhibiting ROMK (33). Rather, the interaction between WNK4 and ITSN through a stretch of WNK4 (amino acids 473–584) containing a proline-rich motif is necessary in mediating the inhibition of ROMK via a clathrin-dependent endocytosis pathway (33). The interaction of WNK4 with the conserved COOH-terminal YV motif of claudins is likely involved in the WNK4-mediated phosphorylation of the COOH terminal of claudins (114). Similarly, the interaction between WNK4 and SPAK via the interacting motif RFQV in the COOH-terminal region of WNK4 (24) could be involved in the phosphorylation of SPAK by WNK4 (100). The association of WNK4 with WNK1 via their kinase domain is necessary for the modulation of WNK4 by WNK1 (118). The association of WNK4 with SGK1 could be involved in the phosphorylation on Ser1169 of mouse WNK4, which abrogates the inhibitory effects of WNK4 on ENaC and ROMK (83). Because WNK1, WNK4, and SGK1 associate with each other (83, 111, 112, 118), it is possible that they may coexist in a protein complex that interacts with their targets such as SPAK and OSR1 and relevant ion transport proteins they regulate. It should be noted that not all ion transport proteins regulated by WNK4 directly interact with it. TRPV4 does not coimmunoprecipitate with WNK4 (23). There is no evidence that a direct interaction is involved in the regulation of TRPV5 by WNK4.

An interesting question is how WNK4 manages to regulate a variety of ion transport proteins using different mechanisms, such as those observed for NCC, ENaC, ROMK, and TRPV5? It is likely that WNK4 affects multiple pathways via its kinase activity and/or specific domains, and the specificity of the regulation lies in the ion transporter side. For instance, plasma membrane retrieval and degradation of ENaC depend on the interaction with the E3 ubiquitin ligase Nedd4-2 through the PY motif in ENaC β- and γ-subunits (82). On the other hand, the endocytosis of ROMK is through a clathrin-dependent pathway, which is dependent on a NPXY internalization motif in the COOH terminal of ROMK (124). Although WNK4 may affect both pathways of endocytosis, the effect is observed only when the channel protein is affected by one of the pathways. If the protein is no longer going through the pathway, e.g., ENaC without the PY motif or ROMK without NPXY motif, the regulatory effect of WNK4 will be abolished. Similarly, the differential effects of WNK4 on TRPV5 and TRPV6 also depend on the mechanisms by which they are delivered to the plasma membrane as discussed earlier.

It is still unclear why WNK4 affects NCC and TRPV5 in different directions in the same experimental system and WNK4 appears to prevent the forward trafficking of NCC (28).
WNK4-MEDIATED REGULATION OF ION TRANSPORT PROTEINS

Invited Review

but to promote that of TRPV5 (38). What causes this difference? In addition to the difference in channel proteins between NCC and TRPV5, the ions that NCC and TRPV5 transport are different. Expression of TRPV5 leads to an increase in intracellular Ca\(^{2+}\), an important intracellular messenger which is involved in the interaction of WNK1 and synaptotagmin 2 (54). Thus Ca\(^{2+}\) ions may have a role in switching the action mode of WNK4, as WNK4 is proposed as a molecular switch in regulating diverse pathways of ion transport (45). However, the inhibitory effect of WNK4 on TRPV4 (23), which is a Ca\(^{2+}\)-permeable cation channel, argues against the role of Ca\(^{2+}\) in determining the regulation direction of WNK4.

Summary

WNK4 is a protein kinase with the exceptional ability to regulate an array of ion transport proteins in the kidney. The majority of the information was obtained in the *X. laevis* system; thus the question arises as to what extent these in vitro results reflect the in vivo situation. First, the results obtained are consistent with the clinical features of FHH. In most cases, the in vitro results well explain the clinical manifestations of FHH (Fig. 1). The hypertension in FHH could be caused by the impaired inhibitory effects of WNK4\(^{FHH}\) on NCC and ENaC, leading to increased Na\(^{+}\) reabsorption and in turn intravascular volume expansion. Hyperkalemia in FHH could be explained by the decreased K\(^{+}\) secretion due to three effects of WNK4\(^{FHH}\) action: 1) the strengthened inhibition of ROMK by WNK4\(^{FHH}\) reduces the secretion of K\(^{+}\); 2) the increased Cl\(^{-}\) flux likely mediated by claudin 4 in the CCD by WNK4\(^{FHH}\) diminishes the luminal negative potential for K\(^{+}\) secretion; and 3) the increased Na\(^{+}\) reabsorption by NCC limits Na\(^{+}\) delivery to the CCD for ENaC-mediated Na\(^{+}\) reabsorption and in turn reduces the secretion of K\(^{+}\) and H\(^{+}\) capabilities in the CCD. The NCC and ENaC activity and the surface area expressing NCC and ENaC are increased in the mouse models of FHH (52, 82, 119), supporting the third mechanism. Metabolic acidosis in FHH is likely secondary to the decreased K\(^{+}\) secretion. Hypercalciuria of FHH could be caused by the diminished enhancing effect of WNK4 on TRPV5 through the increased level of NCC. Alternatively, due to the inverse relationship of Ca\(^{2+}\) and Na\(^{+}\) reabsorption in the distal tubule (11), an increased Na\(^{+}\) reabsorption by NCC and ENaC may reduce the reabsorption of Ca\(^{2+}\), likely through enhancing passive Ca\(^{2+}\) transport in the proximal tubule (69). In addition, a direct effect of WNK4 on the Na\(^{+}/Ca\(^{2+}\) exchanger and/or the Ca\(^{2+}\) pump in the basolateral membrane could not be ruled out. Second, the regulation of NCC by WNK4 observed in transgenic mice is consistent with the in vitro results obtained using oocytes (52). Although the effect of increased DCT mass in Tg(WNK4\(^{FHH}\)) mice could not be demonstrated in the oocyte system, it is a logical result of increased NCC due to the alleviated inhibition by WNK4\(^{FHH}\), given the fact that the removal of NCC results in diminished DCT structure (87). Third, the increased ENaC-mediated Na\(^{+}\) transport as demonstrated in oocyte and transgenic mice is also evident in human subjects carrying the WNK4\(^{Q256S}\) mutation (21). Thus it is reasonable to assume that the effects observed in the in vitro system largely reflect the reality in vivo, which is expected to be illustrated in future studies.

Investigations of WNK4-mediated regulation are progressing very rapidly. The list of ion transport proteins regulated by WNK4 will likely continue to grow in the next few years. A clearer understanding of the WNK4-mediated regulation will likely be achieved at the cellular and molecular levels. Identifying cellular processes regulated by WNK, and proteins that are either targets or modulators of WNK4 action, will certainly help in decoding the regulatory processes. In addition, a target of WNK4 may act as a modulator of the regulations of WNK4 on other ion transport proteins, e.g., NCC in WNK4-mediated regulation of TRPV5 (38). The cross talk between WNK4 and its targets adds more layers in WNK4-mediated regulation.

Compared with all the progress made in the actions of WNK4 on its downstream targets, very little is known on how WNK4 responds to physiological cues. WNK4 responds to aldosterone indirectly through the action of SGK1, which phosphorylates WNK4 and in turn abrogates the regulatory effect of WNK4 on ENaC and ROMK (Fig. 3) (83). Lalioti et al. (52) suggest that the renin-angiotensin system likely provide upstream signals to shape the action of WNK4. Investigations in these areas are likely to provide further insight into how WNK4 reacts in different pathophysiological conditions.

The role of WNK4 in the molecular pathogenesis of FHH suggests that the genetic variations in the WNK4 gene are possibly involved in the common forms of hypertension. Indeed, associations of single-nucleotide polymorphisms in the WNK4 gene with the risk of essential hypertension in general populations have been reported (19, 50), although further studies are needed to verify these preliminary results and to establish the role of the WNK4 gene in essential hypertension. WNK4 could become a novel drug target for diseases related to imbalanced ion homeostasis, such as hypertension. If specific
delivery of drugs to the distal nephron becomes achievable, drugs may be developed to temporally lock WNK4 in a certain mode, acting on a specific ion transport protein. Understanding the mechanisms of the WNK4-mediated regulation of ion transport processes is an essential step in this direction.

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