The thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ cotransporter: molecular biology, functional properties, and regulation by WNKs

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Gamba G. The thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ cotransporter: molecular biology, functional properties, and regulation by WNKs. Am J Physiol Renal Physiol 297: F838–F848, 2009. First published May 27, 2009; doi:10.1152/ajprenal.00159.2009.—The thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ cotransporter is the major salt reabsorption pathway in the distal convoluted tubule, which is located just after the macula densa at the beginning of the aldosterone-sensitive nephron. This cotransporter was identified at the molecular level in the early 1990s by the pioneering work of Steven C. Hebert and coworkers, opening the molecular area not only for the $\text{Na}^+\text{-Cl}^-$ cotransporter but also for the family of electroneutral cation-coupled chloride cotransporters that includes the loop diuretic-sensitive $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter of the thick ascending limb of Henle’s loop. This work honoring the memory of Steve Hebert presents a brief review of our current knowledge about salt and water homeostasis generated as a consequence of cloning the cotransporter, with particular emphasis on the molecular biology, physiological properties, human disease due to decreased or increased activity of the cotransporter, and regulation of the cotransporter by a family of serine/threonine kinases known as WNK. Thus one of the legacies of Steve Hebert is a better understanding of salt and water homeostasis.

distal tubule; ion transport; hypertension; diuretics

THIAZIDE-TYPE DIURETICS were developed in the middle of the twentieth century and quickly became popular, since they were the first active agents with proven beneficial effects for lowering blood pressure in patients with arterial hypertension (47, 75). Fifty years later, thiazides are still recommended as the first line of pharmacological therapy for the treatment of hypertension (13). Pioneering studies by Kunau et al. (57) were the first to suggest that thiazide-type diuretics inhibited chloride reabsorption in the distal nephron. Later, studies by Renfro (85, 86) demonstrated that the urinary bladder of the teleost winter flounder (Pseudopleuronectes americanus) exhibited a salt transport mechanism in the apical membrane that featured an interdependence between $\text{Na}^+$ and $\text{Cl}^-$ transport, suggesting the existence of an electroneutral $\text{Na}^+\text{-Cl}^-$ cotransporter. A few years later, Stokes et al. (103) observed that this transport mechanism in flounder urinary bladder was specifically inhibited by thiazide diuretics in a dose-dependent manner. This was followed by the work of Costanzo (15) and Ellison et al. (31), who established that in mammalian kidney the apical membrane of the distal convoluted tubule (DCT) possesses a similar thiazide inhibitable $\text{Na}^+\text{-Cl}^-$ transport pathway, and thus DCT is the site of action for thiazide type diuretics. In the next few years, the $\text{Na}^+\text{-Cl}^-$ cotransporter was studied as the “thiazide receptor” by assessing the binding of the tracer $[\text{H}]\text{metolazone}$ to plasma membranes from the renal cortex (6, 32, 107). However, the molecular area for the renal $\text{Na}^+\text{-Cl}^-$ cotransporter, and the rest of the SLC12 family of electroneutral cation chloride cotransporters, began with the pioneering work of Steven Hebert and coworkers, who isolated a cDNA clone encoding the thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ cotransporter (NCC) from the winter flounder urinary bladder, following an expression cloning strategy in Xenopus laevis oocytes (35).2

After several months of unsuccessful experiments that were designed to clone the rat outer medullary bumetanide-sensitive $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter, based on the observations of Stokes et al. (103) and probably inspired by the remarkable book of Homer Smith From Fish to Philosopher (102), Steven Hebert suggested that we should pursue the expression cloning of the thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ cotransporter from the winter flounder urinary bladder, with the idea that cloning this fish cDNA would probably be the best place to start to identify, not only the mammalian ortholog, but also the bumetanide-sensitive $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporters because, he said, “they probably are related.” He was right. Cloning of the flounder cDNA cotransporter was quickly followed by the molecular identification of rat cDNAs encoding the thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ cotransporter, two isoforms of the bumetanide-sensitive $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporters (24, 34), and four isoforms of the $\text{K}^+\text{-Cl}^-$ cotransporters (40, 73, 81). This is one of the many visionary projects that Steve Hebert often generated.

As shown in Fig. 1, the DCT mediates reabsorption of 5–10% of the glomerular filtrate and is the first segment of the

1 This is to my knowledge the first study that demonstrated the existence of an electrically silent Na-coupled chloride cotransport mechanism.

2 The thiazide-sensitive Na-CI cotransporter was the first member of the SLC12A family that was identified at the molecular level.
aldosterone-sensitive distal nephron. It is divided into early and late segments, also known as DCT1 and DCT2, respectively. The major salt reabsorption pathway in the DCT is the thiazide-sensitive NCC (15, 31, 57, 82, 109). In DCT1, salt transport is driven exclusively by NCC, whereas in DCT2, the epithelial sodium channel (ENaC) also participates (10, 31, 62, 63, 78, 84). The sodium gradient that drives transport from the lumen to the interstitium is generated and maintained by the Na\(^+\)-K\(^+\)-ATPase that is polarized to the basolateral membrane (28). Part of the potassium entering the cell at the basolateral membrane is secreted at the luminal membrane via K\(^+\)/H\(^+\) channels (120) and via an apical K\(^+\)/H\(^+\)-Cl\(^-\) cotransporter (3). Thus the rate of Na\(^+\)-Cl\(^-\) reabsorption determines, in part, the rate of K\(^+\) secretion. In addition, NCC modulates magnesium reabsorption in parallel with sodium reabsorption, and it regulates calcium reabsorption inversely with sodium reabsorption. The higher the sodium reabsorption, the lower the calcium reabsorption and vice versa (15). Because of its anatomical localization just after the macular densa cells, the DCT is the first part of the nephron in which the salt reabsorption rate is not subjected to compensation by the tubuloglomerular feedback mechanism, and thus it affects the final concentration of salt in urine. Therefore, salt reabsorption in the DCT is expected to affect extracellular fluid balance and arterial blood pressure.

**Molecular Biology of NCC**

The NCC belongs to solute carrier family 12 (SLC12; Human Genome Organization), known as the electrically silent, cation-coupled chloride cotransporter family (36, 46). The SLC12 family of membrane transporters translocate chloride together with Na\(^+\) and/or K\(^+\) observing a stoichiometry of 1:1 (chloride:cation), and their activity is modulated by cell volume. SLC12A, the gene encoding the thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter is located on chromosome 16q13 in humans (65, 101), 19p12–14 in rat (105), and 8 in mouse (80). In humans, SLC12A3 is a 55-kb gene containing 26 exons (101). The NCC has been identified at the molecular level in human (65, 101) rat (34), mouse (58), rabbit (110), and eel kidney (17), as well as in flounder urinary bladder (35). The NCC is a membrane protein of 1,002 to 1,028 amino acid residues with a proposed topology featuring a central hydrophobic domain made up of 12 putative transmembrane (TM) spanning regions. A hydrophilic loop connects TMs 7 and 8 and contains two glycosylation sites. The hydrophobic domain is flanked by a short amino-terminal domain and a long carboxy-terminal domain that are located within the cell. Soon after the molecular identification of NCC, anti-NCC polyclonal antibodies were generated and used to demonstrate that this cotransporter is specifically expressed in the apical membrane of the DCT of the kidney (4, 82). The degree of identity among mammalian NCCs is \(\sim 90\%\) and that of any mammalian NCC with the flounder NCC is \(\sim 60\%\). Two putative NCC genes from eel have been reported, and interestingly, the degree of identity shared with the mammalian or flounder NCCs is \(\sim 50\%\) for both (17). Two alternatively spliced isoforms differing in the length of the 3'-untranslated regions were discovered in rat (34), but no alternative splicing isoform affecting the primary structure of NCC has been found in mammals. Rabbit and human NCCs are longer than rat and mouse orthologs due to the presence of 17–26 amino acid residues in the carboxy-terminal domain. These extra residues in humans are encoded by a separate exon (exon 20) that is not present in mouse or rat. Interestingly, this exon contains a putative PKA site that is not present in mouse and rat NCCs.

Tissue distribution analysis by Northern blot in the rat revealed renal-specific expression of NCC (34). However,
recent studies have clearly demonstrated NCC protein expression in intestine (5) and bone (30) tissues. Although NCC has been postulated to exist in brain (25), blood vessels (14), pancreas (7), peripheral blood mononuclear cells (2), gallbladder (16), and heart (29), confirmation of its presence in these tissues at the molecular level has been unsuccessful. The role of NCC in the intestine is not clear, but it is probably related to salt and calcium absorption. It has been shown in many clinical studies that the use of thiazide diuretics in elderly subjects promotes an increase in bone mineral density and helps to prevent pathological fractures (49, 98). Consistent with this beneficial effect of thiazides, NCC is expressed in rat and human bones. Addition of thiazides to osteoblasts in culture increases the formation of mineralized nodules, an effect that was no longer present after decreasing NCC expression by transfecting cells with a NCC antisense plasmid (30).

The NCC is able to form dimers, and it is likely that it functions as a dimer (19). The NCC is glycosylated at two sites (N404 and N424) located in the long extracellular loop (48). Elimination of each site reduced the rat NCC activity by 50%, and elimination of both sites reduced it by 95%. Decreased expression in the plasma membrane accounts for most of the reduction in NCC activity. Thus the glycosylated loop between TM 7 and 8 must be oriented toward the extracellular space, and glycosylation of NCC plays a key role in trafficking the protein to the cell surface. Interestingly, absence of glycosylation in the rat NCC (48), but not in the flounder NCC (70), is associated with increased affinity for thiazides, suggesting that in rat sugar moieties on NCC probably prevent thiazide from reaching its binding site on the cotransporter.

### Functional Properties of NCC

Robust and reproducible expression of the flounder, rat, mouse, and human NCCs has been achieved by several groups using the heterologous expression system of X. laevis oocytes (18, 34, 35, 42, 58, 69, 93). Only two groups (20, 88) have reported some degree of functional NCC expression by transfecting human NCC cDNA into MDCK or HEK-293 cells, respectively. These systems turned out to be better tools for studying NCC at the biochemical level rather than at the protein level, thus increasing the natriuretic response to the loop diuretic. Finally, little is known about residues or domains defining specificity for ion transport or thiazide inhibition. Chimeric constructs between NCC and the apical bumetanide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter NKCC2 suggest that the key residues must be within the central hydrophobic domain because the amino and carboxy terminals play no role in this issue (106).

### NCC and Human Disease

Inactivating mutations of the SLC12A3 gene encoding NCC are the cause of Gitelman’s disease, an inherited autosomal recessive disease featuring hypokalemic metabolic alkalosis, arterial hypotension, hypocalciuria, and hypomagnesemia that are not usually recognized until the second decade of life. More than 70 families have been studied and more than 100 different mutations of NCC have been reported (http://archive.uwcm.ac.uk/uwcm/mg; Ref. 36). Functional and biochemical analysis of human or rat NCCs containing some of the reported point mutations revealed two major groups: one in which NCC

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<th>Table 1. Functional properties of rat, mouse, and flounder NCC</th>
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<td>Na(^+) K(_{m}), mM</td>
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<td>Cl(^-) K(_{m}), mM</td>
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Data are means ± SE. Table uses information from Refs. 69, 70, 93, 108. NCC, thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter.
The discovery that PHAII is due to mutations in WNK1 and WNK4 (116) suggested that WNKs are potential regulators of ion transport in the kidney, particularly through the regulation of NCC. First, PHAII patients feature a clinical condition that mirrors Gitelman’s syndrome in which NCC is not functional (101). Second, clinical studies (44, 66) have shown that PHAII patients are particularly sensitive to very low doses of thiazide diuretics, as 20% of the typical “thiazide” dose is sufficient to improve hypertension and the other clinical features. Third, the expression of both WNK1 and WNK4 proteins were specifically localized to the DCT (116). Thus using *X. laevis* oocytes, we learned that wild-type WNK4 reduces the activity of NCC, whereas WNK4 mutants lacking catalytic activity (obtained by introducing a D318A mutation in kinase domain of WNK4) or harboring the PHAII-type mutation E562K no longer inhibit NCC (117). The reduction in NCC activity by WNK4 was at least in part due to a decrease in the number of NCCs present at the cell surface. These observations were corroborated by other groups also using *X. laevis* oocytes (42, 122) or epithelial cells (9) in which NCC expression at the cell surface was assessed by confocal image techniques. Thus the proposed pathophysiological mechanism of PHAII is that WNK4 is a natural inhibitor of NCC and that this effect is lost through PHAII-type mutations in WNK4, increasing the activity of NCC, resulting in hypertension, hyperkalemia, and metabolic acidosis.

The hypothesis described above has been confirmed in vivo by two groups using different strategies in transgenic animals. Yang et al. (126) produced a knockin WNK4 D561A/+ that imitates the genetics of PHAII because this mouse model has one normal and one mutated WNK4 allele. The mice developed the PHAII phenotype that was corrected by thiazide administration and exhibited increased expression of phosphorylated NCC at Ser-71, which together with threonines 53 and 58 were previously shown to be required for full activation of NCC by intracellular chloride depletion (79), and phosphorylation of the STE-20 serine/threonine kinases SPAK/OSR1 that have been proposed to lie downstream of WNK1 and WNK4 (38, 72, 111). Additionally, it was demonstrated that activation of NCC by intracellular chloride depletion requires NCC-SPAK/OSR1 interaction and that in this condition SPAK/OSR1 induces phosphorylation of human NCC at threonine residues 45, 55, and 60 and serine 91 (87, 88), corresponding to threonines 43, 53, and 58, and serine 89 in rat NCC (79).

Lalioti et al. (59) produced BAC transgenic mice with four alleles of wild-type WNK4 (WNK4+/+/+/+) and PHAII-type mutant mice with two alleles of wild-type WNK4 and two of mutant WNK4 (WNK4 PHAII). Thus in addition of the two normal alleles of WNK4, these mice exhibit two extra alleles of wild-type or mutant WNK4. WNK4+/+/+/+ mice developed a Gitelman’s-like condition, with hypoplasia and hypotrophy of the DCT, indicating that, as suggested by in vitro studies (117, 122), wild-type WNK4 indeed inhibits NCC. In contrast, WNK4 PHAII mice developed a clear PHAII-like phenotype, with arterial hypertension and hyperkalemia and hyperplasia of the DCT. When exposed to a high potassium diet, WNK4 PHAII mice actually died due to hyperkalemia. A very intriguing observation was that crossing WNK4 PHAII mice with NCC null mice (99) fully prevented the phenotype, including the hyperkalemia during high potassium diet, unmasking the importance of WNK4 in the aldosterone-sensitive nephron (76, 116).

Gordon’s disease, pseudohypoaldosteronism type II (PHAII), or familial hypertension with hyperkalemia are the terms that have been assigned over the years for a rare inherited illness featuring arterial hypertension with hyperkalemia and metabolic acidosis, despite a normal glomerular filtration rate (43, 66). Three loci for PHAII have been observed in humans in the following regions: 1q31-42 (64), 12p13 (26), and 17p11-q21 (64). Linkage for these loci has been excluded in at least two unrelated kindreds with autosomal dominant PHAII, indicating there is a fourth locus involved (27). The gene responsible for this condition in families with Gordon’s disease linked to chromosome 1 remains a mystery, but in chromosomes 12 and 17, PHAII cosegregates with mutations in two serine/threonine kinases known as WNK1 and WNK4, respectively (116). Intronic deletions in the first intron of WNK1 lead to an increased expression of a normal WNK1 protein, whereas missense mutations in a conserved acidic region of WNK4 are responsible for the disease. Most of the pathophysiology of PHAII seems to be explained by the effects of WNKs upon distal nephron ion transport proteins, particularly the NCC. Thus the rest of the present work is devoted to an analysis of the regulation of NCC (and other distal nephron transport proteins) by WNKs and the implication of this new knowledge in our understanding of distal nephron physiology.

**Effects of WNK4 on NCC**

The WNK family of serine/threonine kinases is composed of four genes known as WNK1 to WNK4, which are located on human chromosomes 12, 9, X, and 17, respectively (53). These kinases exhibit a conserved serine/threonine kinase domain that lacks the typical catalytic lysine in subdomain II (hence, the name WNK; with no lysine (K); Ref. 118) flanked by an unconserved short amino-terminal domain and a long carboxy-terminal domain. The carboxy-terminal domain varies in size and contains an autoinhibitory domain, two coiled-coil domains, and a highly conserved acidic region of 10 residues in which most of the WNK4-PHAII-type missense mutations occur. WNK1, WNK3, and WNK4 are expressed in several tissues, including chloride absorbing or secreting epithelia (22, 50, 52, 89, 116, 118). In the nephron, WNK1 transcripts and WNK3 proteins have been shown to be expressed in all nephron segments (76, 89), whereas WNK4 is present mainly in the aldosterone-sensitive nephron (76, 116).

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**Fig. 2. Structure-function relationship model for NCC** [from Moreno et al. (69)].

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of NCC activity, not only in blood pressure regulation but also in potassium and hydrogen regulation in the collecting duct.

The mechanism by which WNK4 modulates the activity of NCC is not fully understood. Wild-type WNK4 induces a decrease in NCC expression in the plasma membrane in both oocytes (42, 117, 122) and WNK4-NCC-transfected Cos-7 cells (9). In these studies, decreased expression of NCC in plasma membrane was not observed with a WNK4-harboring PHAII-type mutant. In addition, in transgenic mice mimicking PHAII, mutant WNK4 is associated with increased expression of NCC in DCT apical membrane (59, 126). The effect of wild-type WNK4 is not prevented by wild-type or mutant dynamin, suggesting that clathrin-induced vesicle internalization is not involved. Treatment of Cos-7 cells with bafilomycin A1, an inhibitor of the vacuolar-type H^+ -ATPase, partially reduced the WNK4-induced decrease in NCC expression, suggesting that at least part of the reduction of NCC expression is due to increased degradation of the cotransporter in lysosomal compartment (9).

Modulation of Distal Nephron Ion Transport Systems by WNK4

The clinical features of PHAII suggested that, in addition to NCC, other renal ion transport systems of the distal nephron could be regulated by WNKs. Thus effects of WNK4 on these transport systems have been extensively studied over the last few years (for review see Refs. 37, 54, 94). The results obtained (Table 2) using wild-type WNK4, PHAII-type WNK4, and a constitutively phosphorylated WNK4 in a canonical site for the serum glucocorticoid kinase (SGK; WNK4-S1169D) positioned WNK4 as a potential switch that can work non catalytically. Similar to their effects on ROMK, PHAII-type mutations in WNK4 are associated with further increases in Cl^- permeability and phosphorylation of claudin-4. The effect of WNK4-S1169D on paracellular chloride permeability or claudin phosphorylation has not been addressed. Finally, wild-type WNK4 also inhibits the activity of the K^+-Cl^- cotransporters by a mechanism that requires catalytic activity (39). KCs claudin-4. The effect of WNK4 catalytic phosphorylation on WNK4 are that ENaC and ROMK activities. The combination of these effects maximizes potassium secretion without increasing salt reabsorption. In contrast, as explained in the interactive Supplemental Fig. 1, the consequences of WNK4-S1169D resemble what would be expected to occur during hyperkalemia when aldosterone is increases (supplemental data for this article are available online at the Am J Physiol Renal Physiol website). The consequences of S1169 phosphorylation on WNK4 are that ENaC and ROMK activities are increased (91). It is known, for example, that these channel activities are modulated by SGK (11, 127). In contrast, NCC activity remains suppressed because WNK4-S1169D inhibits NCC similarly to wild-type WNK4 (95). Thus salt escapes reabsorption in DCT1, and sodium delivered to DCT2 and CNT is uncharged with potassium due to increased ENaC and ROMK activities. The combination of these effects maximizes potassium secretion without increasing sodium reabsorption. In contrast, as explained in the interactive Supplemental Fig. 2, the consequence of PHAII-type mutations in WNK4 resembles what would be expected to occur during intravascular volume depletion. The activities of NCC, ENaC, and paracellular chloride transport are increased by WNK4-PHAII, whereas the activity of ROMK is further reduced. Thus DCT1

Table 2. Effect of wild-type and mutant WNK4 or wild-type WNK1 on distal nephron transport systems

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<tr>
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<th>WNK4</th>
<th>S1169D WNK4</th>
<th>PHAII WNK4</th>
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ENaC, epithelial sodium channel. NKCC, apical bumetanide-sensitive Na^+-K^+-2Cl^- cotransporter. PHAII, pseudohypoaldosteronism type II.
salt reabsorption is increased with the consequent reduction in salt delivery to DCT2 or the CNT, even though the salt that reaches the CNT is reabsorbed due to increased ENaC and claudin activities. Because transepithelial sodium reabsorption is accompanied by paracellular chloride transport, the luminal negative potential that is required for potassium secretion is not generated, and additionally, ROMK is further inhibited by WNK4-PHAII. Thus salt reabsorption is maximized without increasing potassium secretion. These observations suggest that the PHAII mutations mimic a natural state resulting from volume depletion. The next question to pursue addresses what the upstream regulatory signal for this natural state might be. Since the hallmark of intravascular volume depletion is the increase in the peptide hormone ANG II circulating levels, which are not increased during hyperkalemia, signaling of ANG II through its G-protein-coupled receptor AT$_1$R is an attractive candidate to be the physiological counterpart of the functional state generated by PHAII mutations in WNK4. Supporting this hypothesis, reconstitution experiments in Xeno- nopus oocytes have recently demonstrated that ANG II signaling acts through WNK4 and the kinase SPAK to increase NCC activity (95). ANG II increases NCC activity only in the presence of WNK4, and this effect is completely prevented by the AT$_1$R blocker losartan. In addition, WNK4-PHAII that no longer inhibits NCC also exhibited no response to ANG II, providing evidence that the ANG II effect can be substituted in full by PHAII mutations in WNK4. Conversely, ANG II signaling did not reverse WNK4 inhibition of ROMK. Interestingly, it was also observed that ANG II signaling increased phosphorylation at key regulatory sites in both SPAK and NCC in mammalian cells. Several studies (33, 72, 83, 87, 88, 111) have suggested that WNKs lie upstream of SPAK, which probably phosphorylates the cotransporters. These findings place WNK4-SPA in the signaling pathway between ANG II and NCC and reveal a key role for the ANG II-WNK4-SPA-NCC pathway in the renal response to intravascular volume depletion. Supporting these observations, experiments in vivo have shown that ANG II promotes the trafficking of NCC to the apical membrane of DCT cells (97) and has an inhibitory effect on ROMK activity (115). Additionally, a recent genomic wide association study analysis in the Amish population showed a strong association signal between blood pressure levels and common variants within the SPAK gene (114). Taking all this information together, our current understanding of the WNK4 modulation of NCC activity is as follows (Fig. 3). During normovolemia (Fig. 3A), in which the renin-ANG system is suppressed, WNK4 behaves as a natural inhibitor of NCC, reducing its activity by decreasing the number of NCCs present in the plasma membrane (9, 97, 117). Although a protein-protein interaction between WNK4 and NCC has been observed in oocytes (117) and in mice (59), at this point it is not clear whether the negative effect of WNK4 on NCC is direct. During hypovolemia (Fig. 3B), when the renin-ANG system is activated, ANG II interacts with its AT$_1$R, turning WNK4 into an activator of NCC by increasing NCC trafficking to the plasma membrane, and this is likely mediated through SPAK (88, 95). Supporting this proposal, eating a low-salt diet is associated with phosphorylation of SPAK and NCC (12) in similar phosphoacceptor sites to those phosphorylated after exposing oocytes to ANG II (95). The model shown in Fig. 3 depicts the location of AT$_1$R at the basolateral membrane. However, renin and ANG-converting enzymes have been localized within the distal nephron (56, 92), and apical AT$_1$R was shown to be present in the collecting duct (115), introducing the possibility that ANG II present in the distal tubular fluid may have an effect on distal tubule transport. According to our observations, patients with PHAII (Fig. 3C) have one allele with mutations that imitate the effect of ANG II. Thus there is a constitutive activation of SPAK and NCCs, abnormally
increasing salt reabsorption and decreasing potassium secretion, consistent with the proposal, the PHAII-type knockin mouse exhibits increased phosphorylation of SPAK and NCC in the kidney (126). These proposals imply that PHAII is a “gain of function” type of disease, which agrees with its dominant mode of inheritance. The gain of function is to make WNK4-SPAK-NCC behave as if there was a continuous activation of AT1R in the DCT.

**Modulation of NCC Activity by Other WNKs**

The effect of WNK1 upon NCC and upon the rest of the ion transport systems in the aldosterone-sensitive distal nephron is complex and still unclear. Two isoforms of WNK1 are expressed in the kidney (23, 77): the complete long WNK1 (L-WNK1) and a shorter transcript that results from alternative splicing of exons 1-4 (KS-WNK1 that lacks the first 437 amino acid residues, including the entire kinase domain). The L-WNK1 transcript isoform is expressed along the entire nephron, whereas KS-WNK1 transcripts are only present in the DCT and the CNT (76). WNK1 seems to be a key regulator of other WNKs, either by phosphorylation processes (61) or by direct interactions at the protein level (123, 125). For instance, there is no evidence so far that WNK1 has a direct effect on NCC. Nevertheless, since NCC expression in mammalian cells has not been properly achieved, it is not known if the absence of WNK1 effects on NCC using oocytes as the expression system can be extended to mammalian cells. In this regard, Yang et al. (122) observed in oocytes that WNK1 prevents the WNK4-induced inhibition of NCC. That is, in the presence of WNK1, WNK4 no longer inhibits NCC. This interaction is modulated by the alternatively spliced KS-WNK1 isoform because KS-WNK1, by interacting with L-WNK1 in a dominant-negative fashion, eliminates the L-WNK1-induced inhibition of WNK4 (104). Thus it has been proposed that in normal subjects KS-WNK1, by high-jacking L-WNK1, prevents its inhibitory effect upon WNK4, allowing WNK4 to inhibit NCC. In contrast, in PHAII patients, the KS-WNK1-to-L-WNK1 ratio is reduced due to intronic deletions of the WNK1 gene that increase the expression of L-WNK1. As a consequence, L-WNK1 is able to inhibit WNK4 and thus NCC activity is increased, augmenting salt reabsorption in the DCT and thus arterial pressure. Consistent with these observations, KS-WNK1 and L-WNK1 also interact with each other to regulate ROMK activity. L-WNK1 decreases ROMK activity, an effect that requires catalytic activity and that is prevented by KS-WNK1 (60, 113). L-WNK1 is also a modulator of the ENaC via SGK (119). WNK1 induces the phosphorylation of SGK, which in turn phosphorylates and inhibits a protein named Nedd4 that is known to reduce ENaC activity by promoting its endocytosis via a clathrin-dependent mechanism (1). In addition, in mouse cortical collecting duct cells in culture aldosterone seems to modulate the L-WNK1-to-KS-WNK1 ratio of expression (74). Therefore, ENaC activity is enhanced. The increased expression of L-WNK1 in the DCT of a mouse model carrying a deletion of WNK1 intron 1 has been confirmed (22). However, increased expression of KS-WNK1 in DCT was also observed in the same study. Thus the molecular mechanism for WNK1-induced NCC activity in PHAII patients with WNK1 intron 1 deletion remains unknown.

WNK3, a member of the WNK family not associated with PHAII, is a powerful regulator of all the members of the SLC12 family and is expressed along the entire nephron, as well as in several epithelial and nonepithelial cells outside the kidney (53, 94). WNK3 is an activator of the Na+-K+-ATPase, what WNK3 seems to promote is the net chloride movement into the cells. Activation by WNK3 is associated with phosphorylation of two amino-terminal domain threonines in NKCC1 and NKCC2 (52, 89). Because threonines that are conserved in both NCC and the NKCC1/2 are phosphorylated by similar stimuli, such as intracellular chloride depletion (72, 79, 88), it is highly likely that these threonines in NCC also become phosphorylated by WNK3. WNK3 activation of NCC is associated with increased numbers of NCC on the cell surface, suggesting that WNK3 promotes the insertion of NCC vesicles into the plasma membrane (89). The WNK3-induced activation of NKCC1/2s or inhibition of KCCs occurs in oocytes even during cell swelling, which is a well-known inhibitor of NKCC1/2 and NCC activities (34, 41, 69). Interestingly, elimination of WNK3 catalytic activity by the D294A substitution in the kinase domain (WNK3-D294A) not only prevented the WNK3-induced activation of NKCC1/2 and NCC and its inhibition of KCCs but also turned WNK3 into a powerful inhibitor of NKCC1/2 and NCC and an activator of KCCs, even in isotonic or hypertonic conditions, inducing cell shrinkage, which is known to activate NKCC1/2 and inhibit KCCs. For example, microinjection of cRNA encoding the K+-Cl– cotransporters KC1, KC3, or KC4 induces expression of these cotransporters that can be demonstrated by assessing the Cl– dependent 86Rb+ influx but only when oocytes are exposed to a hypotonic medium to induce cell swelling. When incubated in isotonic medium, the cotransporters are completely inactive (67, 68). In contrast, when coinjected with WNK3-D294A cRNA, KCCs are fully active when incubated in isotonic medium (21). Thus inactive WNK3 promotes net chloride movement out of the cells. Since the activation of KCCs by WNK3-D294A can be prevented with calyculin A and/or cyclosporine A, it is likely due to a WNK3-D294A activation of protein phosphatases 1 and 2B (21). Thus WNK3 modulates the activity not only of NCC but also of all members of the SLC12 family and, in doing so, bypasses the changes in cell volume and/or intracellular chloride concentration that are usually required for their activation or inhibition. For these reasons, it has been proposed that WNK3 could be the intracellular kinase sensitive to cell volume and/or intracellular chloride concentration (53).

From observations discussed above, it is evident that wild-type WNK3 and WNK4 have opposite effects on NCC. Whereas WNK3 is an activator, WNK4 is an inhibitor. Because both kinases are expressed in the DCT, it is possible that NCC activity at any given time is a result of the algebraic sum of both effects. In this regard, Yang et al. (124) performed a series of experiments in X. laevis oocytes by coinjecting NCC cRNA with different fragments of WNK3 and WNK4 cRNA.
and concluded that these kinases interact and regulate each other by means of the carboxy-terminal domains. For instance, although the effects of WNK3 and WNK4 on NCC require kinase activity when full-length constructs are used (9, 42, 89, 117, 124), it was observed that WNK3 activation or WNK4 inhibition of NCC was reproduced using only the carboxy-terminal domain that lacks the entire kinase domain (124).

These types of interactions were not observed with wild-type full-length proteins or with full-length proteins harboring a single point mutation that eliminates catalytic activity. Unlike what was proposed for L-WNK1/KS-WNK1 regulation of NCC, there is no evidence that such truncated isoforms of WNK3 or WNK4 are expressed in the DCT. In addition, the presence of endogenous WNK3 and WNK4 transcripts in X. laevis oocytes (96) and the known interaction between WNKs (61, 123, 124) make these data difficult to interpret. Thus, at the moment, the physiological relevance of WNK3 and WNK4 carboxy-terminal domain fragments interactions is unclear.

Several differences between WNK3 and WNK4 suggest that each kinase could serve different purposes. The effect of WNK4, but not of WNK3, on NCC can be modulated by ANG II (95), suggesting that NCC activity is regulated by intravascular volume via WNK4, but not via WNK3. Additionally, WNK1, the other WNK member clearly associated with blood pressure regulation, prevents WNK4 but not WNK3 effects on NCC (122, 124). On the other hand, elimination of WNK4 catalytic activity abrogates WNK4 inhibition of NCC, whereas elimination of WNK3 catalytic activity switches WNK3 from an activator to an inhibitor. Thus it is possible that WNK3 and WNK4 respond to different upstream signals for modulation of NCC activity, as it is possible that downstream pathways between WNKs and NCC are also different. In this regard, we have constructed chimeric proteins between WNK3 and WNK4 by swapping the low conserved amino- or carboxy-terminal domains (12–15% identity) that flank the well-conserved kinase domain (>80% identity). Chimeras were functional and possessed catalytic activity. The chimera containing the amino-terminal domain of WNK3 swapped into the kinase and carboxy-terminal domain of WNK4 activated NCC in a kinase-dependent fashion. In contrast, the chimera containing the amino-terminal domain of WNK4 swapped into the kinase and carboxy-terminal domain of WNK3 inhibited NCC in a kinase-dependent fashion. These observations suggest that WNK3 and WNK4 amino-terminal domains contain sequences defining the type of effects that they have on NCC (96).

The understanding of NCC structure-function relationship and its regulation by WNKs and downstream kinases like SPAK/OSR1 have advanced quickly in the past few years, but there is still a lot of exciting discovering waiting for us to get there. Meanwhile, this little stop in our journey was done to recognize the invaluable contribution and legacy of Steve C. Hebert. Without his passion for understanding salt transport mechanisms in the kidney, most of what is discussed in the present work would not be possible.

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TISSAIDE-SENSITIVE Na\(^+\)-Cl\(^-\) COTRANSPORTER


