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

Gerardo Gamba

Molecular Physiology Unit, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, and Instituto Nacional de Cardiología Ignacio Chávez, Mexico City, Mexico

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Gamba G. The thiazide-sensitive Na\textsuperscript{+}-Cl\textsuperscript{−} 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 Na\textsuperscript{+}-Cl\textsuperscript{−} 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, who isolated a cDNA clone encoding the thiazide-sensitive Na\textsuperscript{+}-Cl\textsuperscript{−} cotransporter (NCC) from the winter flounder urinary bladder, following an expression cloning strategy in Xenopus laevis oocytes (35).

After several months of unsuccessful experiments that were designed to clone the rat outer medullary bumetanide-sensitive Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} 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 Na\textsuperscript{+}-Cl\textsuperscript{−} 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 Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} 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 Na\textsuperscript{+}-Cl\textsuperscript{−} cotransporter, two isoforms of the bumetanide-sensitive Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporters (24, 34), and four isoforms of the K\textsuperscript{+}-Cl\textsuperscript{−} 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 Na\textsuperscript{+}-Cl\textsuperscript{−} 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 Na\textsuperscript{+}-Cl\textsuperscript{−} cotransporter (NCC) from the winter flounder urinary bladder, following an expression cloning strategy in Xenopus laevis oocytes (35).

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 fish possesses a similar thiazide inhibitable Na\textsuperscript{+}-Cl\textsuperscript{−} transport mechanism in the apical membrane that featured salt transport mechanism in the apical membrane that featured an interdependence between Na\textsuperscript{+} and Cl\textsuperscript{−} transport, suggesting the existence of an electroneutral Na\textsuperscript{+}-Cl\textsuperscript{−} 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 Ellisson et al. (31), who established that in mammalian kidney the apical membrane of the distal convoluted tubule (DCT) possesses a similar thiazide inhibitable Na\textsuperscript{+}-Cl\textsuperscript{−} transport pathway, and thus DCT is the site of action for thiazide type diuretics. In the next few years, the Na\textsuperscript{+}-Cl\textsuperscript{−} cotransporter was studied as the “thiazide receptor” by assessing the binding of the tracer [3H]metolazone to plasma membranes from the renal cortex (6, 32, 107). However, the molecular area for the renal Na\textsuperscript{+}-Cl\textsuperscript{−} 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 Na\textsuperscript{+}-Cl\textsuperscript{−} cotransporter (NCC) from the winter flounder urinary bladder, following an expression cloning strategy in Xenopus laevis oocytes (35).

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-Cl cotransporter was the first member of the SLC12A family that was identified at the molecular level.

Address for reprint requests and other correspondence: G. Gamba, Vasco de Quiroga No. 15, Tlalpan 14000 Mexico City, Mexico (e-mail: gamba@biomedicas.unam.mx or gamba@quetzal.innsz.mx).
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\(^{+}\)-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 an 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 \(\approx 90\%\) and that of any mammalian NCC with the flounder NCC is \(\approx 60\%\). Two putative NCC genes from eel have been reported, and interestingly, the degree of identity shared with the mammalian or flounder NCCs is \(\approx 50\%\) 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 functional level because robust expression of NCC was observed in MDCK or HEK-293 cells. As shown in Table 1, a number of interesting differences in functional properties between the fish and mammalian NCCs have been observed. The apparent $K_m$ values for Na$^+$ and Cl$^-$ in rat (69) or mouse NCC proteins (93) are significantly lower than the $K_m$ values observed in the flounder NCC (108). NCC activity is specifically inhibited by thiazide type diuretics, with the following potency profile: polythiazide > metolazone > bendroflumethiazide > trichloromethiazide > chlorothalidone. In all cases, the flounder NCC exhibited lower affinity for each thiazide. While a 100-μM concentration of the less potent thiazides trichloromethiazide and chlorothalidone inhibited the rat NCC by >95% (69), the flounder NCC activity was reduced by only 68 and 46%, respectively (108).

Differences in functional properties between the rat and flounder NCCs have been exploited to design and analyze chimeric and site-directed mutagenesis approaches to gain insight into NCC structure-function relationships (70). Observations in this study revealed that the functional characteristics of NCC are defined by the central hydrophobic domain, and it was possible to define different segments within the central domain defining Cl$^-$ or thiazide affinity (Fig. 2). Chimeras in which the TM segment 1–7 was interchanged between flounder and rat demonstrated that affinity-defining residues for Cl$^-$ are located within this segment. In contrast, the affinity-defining residues for thiazide inhibition are located within TM segments 8–12. This information argued against a previous proposal that thiazide diuretics and chloride compete for the same binding site (107). The observation that Cl$^-$ affinity is defined by residues located within TM segments 1–7 is supported by another study from Moreno et al. (71) in which the functional consequences of a single nucleotide polymorphism (SNP) that change a single residue within NCC were analyzed. It was observed that a highly conserved glycine within TM 4 plays a critical role in defining the level of cotransporter activity and the affinity for Cl$^-$. Accordingly, a glycine to alanine SNP at position 264 resulted in a 50% decrease in cotransporter activity associated with an increased affinity for Cl$^-$ of one order of magnitude. This observation has been translated to pharmacogenomics because it was later observed that people harboring the G264A SNP exhibit a stronger diuretic response to the loop diuretic furosemide (112). Because of the decreased activity of NCC harboring the G264A polymorphism, it is possible that the DCT cannot increase salt reabsorption in response to increased salt delivery that follows furosemide administration, 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).

### Table 1. Functional properties of rat, mouse, and flounder NCC

<table>
<thead>
<tr>
<th></th>
<th>Rat NCC</th>
<th>Mouse NCC</th>
<th>Flounder NCC</th>
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<tbody>
<tr>
<td>Na$^+$ $K_m$, mM</td>
<td>5.5±1.0</td>
<td>7.2±0.4</td>
<td>30.0±6.0</td>
</tr>
<tr>
<td>Cl$^-$ $K_m$, mM</td>
<td>2.6±0.6</td>
<td>5.6±0.5</td>
<td>15.0±2.0</td>
</tr>
<tr>
<td>Polythiazide IC$_{50}$, μM</td>
<td>3×10$^{-7}$</td>
<td>4×10$^{-7}$</td>
<td>7×10$^{-6}$</td>
</tr>
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Data are means ± SE. Table uses information fromRefs. 69, 70, 93, 108. NCC, thiazide-sensitive Na$^+$/Cl$^-$ cotransporter.

**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, hypocalcuria, 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/g; 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
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 WNK4D561A/+ 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 (WNK4PHAI). 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, WNK4PHAI mice developed a clear PHAII-like phenotype, with arterial hypertension and hypertrophy and hyperplasia of the DCT. When exposed to a high potassium diet, WNK4PHAI mice actually died due to hyperkalemia. A very intriguing observation was that crossing WNK4PHAI mice with NCC null mice (99) fully prevented the phenotype, including the hyperkalemia during high potassium diet, unmasking the importance of WNKs in the regulation of NCC.

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-WHAI-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).
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 as a non-canonical site for the serum glucocorticoid kinase (SGK; WNK4-PHAII). WNK4, and a constitutively phosphorylated WNK4 in a calcium-activated state (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).

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

<table>
<thead>
<tr>
<th></th>
<th>WNK4</th>
<th>S1169D WNK4</th>
<th>PHAII WNK4</th>
<th>WNK1</th>
</tr>
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<tbody>
<tr>
<td>NCC</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>ND</td>
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<tr>
<td>ROMK</td>
<td>↓</td>
<td>↑</td>
<td>↓↓</td>
<td>↓</td>
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<tr>
<td>ENaC</td>
<td>↓</td>
<td>↑</td>
<td>ND</td>
<td>↑</td>
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<tr>
<td>Claudin 4</td>
<td>↓</td>
<td>ND</td>
<td>↑</td>
<td>ND</td>
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<tr>
<td>NKCC</td>
<td>↑</td>
<td>ND</td>
<td>↑</td>
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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 transcellular 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 AT1R 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 X. 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 AT1R 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-SPAK in the signaling pathway between ANG II and NCC and reveal a key role for the ANG II-WNK4-SPAK-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 AT1R, 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 AT1R at the basolateral membrane.

Fig. 3. Model for ANG II modulation of WNK4-SPAK-NCC interaction in normovolemia (A), in hypovolemia (B), and in patients with PHAII type mutations (C) [from San Cristobal et al. (95)].

However, renin and ANG-converting enzymes have been localized within the distal nephron (56, 92), and apical AT1R 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 that PHAII patients with WNK1 intron 1 deletion remain 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\(^{+}\)-driven cotransporters NKCC1/2 and NCC and is also an inhibitor of the K\(^{+}\)-driven cotransporters KCC1-KCC4 (21, 52, 89). That is, WNK3 activates chloride influx pathways and inactivates chloride efflux pathways. Since the movements of sodium into and potassium out of the cell are quickly resorted by 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 KCC1, KCC3, or KCC4 induces expression of these cotransporters that can be demonstrated by assessing the Cl\(^{-}\} dependence of 48\textsuperscript{RB}\) 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 calycin A and/or cyclosporin 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 carboxyl-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 SPANK/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. Campean. Without his passion for understanding salt transport mechanisms in the kidney, most of what is discussed in the present work would not be possible.

REFERENCES


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