WNK3 is a Putative Chloride-sensing Kinase

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Key Words

WNK • Salt transport • Hypertension • Epilepsy • Phosphatase

Abstract

The with-no-lysine kinase 3 (WNK3) is a serine/threonine kinase that modulates the activity of the electroneutral cation-coupled chloride cotransporters (CCC). Using the *Xenopus laevis* oocyte heterologous expression system, it has been shown that WNK3 activates the Na⁺-coupled chloride cotransporters NKCC1, NKCC2, and NCC and inhibits the K⁺-coupled chloride cotransporters KCC1 through KCC4. Interestingly, the effect of catalytically inactive WNK3 is opposite to that of wild type WNK3: inactive WNK3 inhibits NKCCs and activates KCCs. In doing so, wild type and catalytically inactive WNK3 bypass the tonicity requirement for activation/inhibition of the cotransporter. Thus, WNK3 modulation of the electroneutral cotransporters promotes Cl⁻ influx and prevents Cl⁻ efflux, thus fitting the profile for a putative “Cl⁻-sensing kinase”. Other kinases that potentially have these properties are the Ste20-type kinases, SPAK/OSR1, which become phosphorylated in response to reductions in

intracellular chloride concentration and regulate the activity of NKCC1. It has been demonstrated that WNKs lie upstream of SPAK/OSR1 and that the activity of these kinases is activated by phosphorylation of threonines in the T-loop by WNKs. It is possible that a protein phosphatase is also involved in the WNK3 effects on its associated cotransporters because activation of KCCs and inhibition of NKCCs by inactive WNK3 can be prevented by known inhibitors of protein phosphatases, such as calyculin A and cyclosporine, suggesting that a protein phosphatase is also involved in the protein complex.

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Introduction

The with-no-lysine kinase (WNK) is a family of serine/threonine kinases discovered a decade ago during a cloning project to identify novel members of the MAP/extracellular-signal regulated protein kinase (ERK) kinase

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(MEK) family. This new family of kinases was named WNK because family members lack the classical catalytic lysine residue of subdomain II found in all other serine/threonine kinases. WNK1 is a serine/threonine kinase consisting of 2382 amino acid residues. It has a short amino terminal domain, followed by the kinase domain and then by a long carboxyl terminal domain containing at least two coil-coil domains that are indicative of potential protein interaction points [1]. In that study, it was observed that elimination of specific residues (D368A or K233M) rendered WNK1 catalytically inactive. It was also observed that WNK1 is capable of autophosphorylation. From the many different potential stimuli, including epidermal growth factor, nocodazole, anisomycin, and salt, the only one that induced and increased autophosphorylation of the kinase was increasing the salt concentration in the extracellular medium. A subsequent study demonstrated that the catalytic lysine is located in subdomain I [2] and that there are four different members of the WNK family [3].

Interest in WNKs increased greatly because it was soon demonstrated that mutations in two members of the family, WNK1 and WNK4, are associated with the development of an inherited disease known as Gordon Syndrome, Familial Hyperkalemic Hypertension (FHH) or Pseudohypoaldosteronism type II (PHAII) [4]. This autosomal dominant inherited disease is characterized by arterial hypertension with hyperkalemic, metabolic acidosis, hypercalciuria, and decreased bone mineral density [5, 6]. These symptoms are corrected by administering low doses of hydrochlorothiazide, a thiazide-type diuretic that specifically inhibits the activity of the renal Na⁺-Cl⁻ cotransporter (NCC). The clinical picture of PHAII is the exact mirror image of Gitelman's syndrome; an autosomal recessive disease characterized by arterial hypotension with hypokalemic metabolic alkalosis, accompanied by hypocalciuria and increased bone mineral density that is due to inactivating mutations of NCC. Thus, mutations of a single gene, WNK1 or WNK4, are capable of producing arterial hypertension and electrolyte abnormalities in adults, presumably by increasing the activity of NCC, resulting in salt-sensitive hypertension.

The mutations in WNK1 that are associated with PHAII are deletions of 22 to 40 kb of intron 1, which results in increased expression of an otherwise normal WNK1 protein [4]. Thus, increased expression/activity of WNK1 produces arterial hypertension. Consistent with this, several single nucleotide polymorphisms in the WNK1 gene have been shown to be associated with increased

blood pressure in different populations [7, 8]. In addition, heterozygous WNK1 null mice are hypotensive. Mice with complete knockout of WNK1 die during embryonic development due to abnormalities in development of the cardiovascular system [9, 10]. Mutations in WNK4 that are associated with PHAII do not involve intronic deletions but are instead missense mutations occurring within a highly conserved acidic region in the carboxyl terminal domain of WNKs, just beyond the first coil-coil domain [4]. In this region, a charged residue is substituted by another residue of the opposite charge in patients with PHAII. One exception is a family with a mutation in the proline 1173 residue at the very end of the carboxyl terminal domain [11].

It was soon demonstrated that WNK4 is a powerful regulator of NCC, and this is affected by the PHAII type mutations [12, 13]. In *Xenopus laevis* oocytes, WNK4 inhibits the activity of NCC, and this effect is prevented by eliminating WNK4 catalytic activity or by introducing one of the PHAII type mutations into WNK4. Eliminating WNK4 catalytic activity or introducing PHAII type mutations is thought to reduce the amount of NCC present in the plasma membrane [12-15], probably due to trafficking, as well as increased degradation of NCC via lysosomal granules [16]. WNK1 has not been shown to directly modulate NCC. However, it has been proposed that WNK1 modulates the effect of WNK4 on NCC [17]. Additionally, both WNK1 and WNK4 have been shown to modulate other ion transport pathways in the distal nephron, such as the epithelial sodium channel ENaC [18, 19], rat outer medulla potassium channel ROMK [20], transient receptor potential cation channel 5 [21], and paracellular protein claudins [22, 23]. More detailed explanations of what is currently known about WNK1 and WNK4 modulation of ion transport pathways in the distal nephron, leading to regulation of blood pressure and thus, arterial hypertension when the kinases are mutated, is beyond the scope of this work. Interested readers are referred to excellent work on the subject [24-27].

The WNK family

The WNK kinase family is composed of four members [3]. WNK1 consists of 2382 amino acid residues, and it is encoded by the *PRKWINK1* gene located on chromosome 12. This kinase is expressed in many tissues and is thought to be a housekeeping gene. Additionally, a single exon of WNK1 is expressed as an

exon-less gene exclusively in the central nervous system (CNS), and when mutated, this gene gives rise to another disease known as Hereditary Sensory Neuropathy (HSN) [28]. WNK2 is composed of 2217 amino acid residues and is encoded by the *PRKWINK2* gene located on chromosome 9q22.31. The expression of WNK2 is restricted mostly to the CNS, in which it is expressed as early as P10.5 of embryonic life and throughout adulthood [29]. WNK3 is composed of 1800 amino acid residues. The human *PRKWINK3* gene encodes WNK3, which is located on chromosome Xp11.22. This gene is made up of 24 exons [30] in a region of 705 kb that exhibits conserved synteny with the segment of chromosome 9 in which the WNK2 gene is located. WNK4 is the smallest of the WNK family, as it is made up of only 1243 amino acid residues. It is encoded by the *PRKWINK4* gene located on chromosome 17 [4].

The general structure of all four WNKs is similar. However, while the degree of homology at the protein level is greater than 80 % for the serine/threonine domain, it is lower than 15 % for the amino terminal and carboxyl terminal domains [31]. The biochemical properties of WNK1 have been extensively studied. WNK1 contains a 50-residue stretch (residues 515-569) that makes up an autoinhibitory sequence that is just C-terminal to the catalytic domain conserved in WNKs across species; this conserved stretch contains two phenylalanine residues that play key roles in autoinhibition [32]. WNKs form tetramers due to the coiled-coil domain downstream to the autoinhibitory domain. The autoinhibitory domain of one WNK1 molecule may also inhibit the kinase activity of another WNK1 molecule within the tetramer. It seems likely that the formation of tetramers may enhance autoactivation of WNKs [32].

WNK1 senses decreases as well as increases in osmotic strength. It is known that autophosphorylation of WNK1 increases about 5-fold in response to hypertonic stress induced by sorbitol, NaCl and KCl in kidney epithelial cells, breast and colon cancer cell lines. Interestingly, however, a modest increase was also observed in WNK1 activity in hypotonic stress conditions [33]. Thus, the precise stimuli for WNKs activation are not clear at the moment. WNK1 activation induced by hypertonic conditions is related to autophosphorylation at multiple residues, specifically at the T-loop Ser 382 residue, which has been suggested to occur by transautophosphorylation [33, 34]. Conservation of Ser 382 in all species of WNK isoforms is consistent with the notion that phosphorylation of this residue plays a crucial role in controlling the activity of all WNKs. Phosphorylation

between WNKs has also been reported, and there is evidence that WNK1 phosphorylates both WNK2 and WNK4. Furthermore, cell biological studies have suggested a functional interaction between WNK1 and WNK4 [27, 34, 35].

WNK1 has also been shown to interact with a protein kinase of the STE20 family, termed STE20/SPS1-related proline/alanine-rich kinase (SPAK), as well as a closely related kinase known as oxidative stress response kinase-1 (OSR1). Phosphorylation by WNK1 and WNK4 at an equivalent [36] residue located within the T-loop of the catalytic domain (Thr 233 in SPAK, Thr 185 in OSR1) markedly increases SPAK and OSR1 activity. Thus, WNKs lie upstream of SPAK and OSR1 kinases [37-40].

The SLC12 family of electroneutral cation chloride cotransporters

The SLC12 family is composed of nine genes encoding integral membrane proteins that serve as cation coupled chloride cotransporters [41]. The functional properties of seven members of the family (SLC12A1 to A7) are well known, while functional details regarding the other two have been only proposed. The family is divided into two branches: the Na⁺-coupled branch in which the transport of chloride is coupled with Na⁺ or with potassium, and the K⁺-coupled branch in which the transport of chloride is coupled only to K⁺. Three members belong to the Na⁺-coupled branch. SLC12A1 and SLC12A2 encode for isoforms of the Na⁺-K⁺-2Cl⁻ cotransporters known as NKCC2 and NKCC1, respectively. NKCC2 is exclusively expressed in the thick ascending limb of the loop of Henle in the mammalian kidney, whereas NKCC1 is a housekeeping gene. SLC12A3 encodes the thiazide-sensitive Na⁺-Cl⁻ cotransporter present in the distal convoluted tubule of the mammalian kidney. Four members compose the K⁺-coupled branch. SLC12A4 to SLC12A7 encode isoforms of the K⁺-Cl⁻ cotransporters known as KCC1, KCC2, KCC3, and KCC4, respectively. KCC1 is a housekeeping gene [42]. KCC2 is exclusively expressed in the CNS and retina [43], KCC3 and KCC4 are expressed in many different tissues [44]. One of the characteristics shared by all members of the family is that their activity is modulated by cell volume and intracellular chloride concentration ([Cl⁻]).

As shown in Figure 1, translocation of ions by SLC12 family members follows the gradient generated by the

Fig. 1. General function of the electroneutral cation-coupled chloride cotransporters. Following the gradients established by the $\text{Na}^+:\text{K}^+:\text{ATPase}$, members of the family using Na^+ as the coupled cation promote influx, while those using only K^+ promote efflux of chloride. Because cations are quickly restored by activity of the sodium pump, chloride is the ion that actually changes in concentrations within the cell. Activity of these cotransporters is modulated by changes in cell volume and or intracellular chloride concentration by means of phosphorylation/dephosphorylation processes.

$\text{Na}^+:\text{K}^+:\text{ATPase}$. Thus, due to the Na^+ gradient from outside to inside the cell, NKCC1, NKCC2, and NCC promote the influx of ions. In contrast, following the K^+ gradient, the $\text{K}^+:\text{Cl}^-$ cotransporters promote the efflux of ions from the cell. Movement of ions serves to modulate cell volume. Thus, NKCC1 and NKCC2 are activated during regulatory volume increase. By increasing ions within the cell, these cotransporters help cells regain volume during cell shrinkage. In contrast, by decreasing ions within the cell, the KCCs help to decrease the volume of swollen cells. The activity of these cotransporters is also critical to establish the $[\text{Cl}^-]_i$. The Na^+ and/or K^+ that are cotransported with chloride are rapidly pumped back inside or outside of the cell, respectively, so the ion concentration that actually changes due to the activity of SLC12 cotransporters is chloride. Thus, the Na^+ -coupled cotransporters promote an increase in the $[\text{Cl}^-]_i$, whereas the K^+ -coupled branch reduces it. Because most cells in the body express NKCC1 and KCC1, it is believed that coordinated activity of these cotransporters is critical to modulating cell volume and $[\text{Cl}^-]_i$.

It is clear that the activity of the SLC12 cotransporters are modulated by both cell volume changes and $[\text{Cl}^-]_i$ (Fig. 1). The activity of NKCC1, and to a lesser extent NKCC2 and NCC is increased by exposing cells to hypertonicity and decreased by hypotonicity [41]. NKCC1 is more sensitive at responding to changes in tonicity, and its activity is

completely abolished by hypotonicity [45]. NKCC2 and NCC are also inhibited by hypotonicity, but the degree of their inhibition is less than that of NKCC1 [45, 46]. In contrast, KCCs are completely inhibited, not only in hypertonic conditions but also in isotonic conditions [41]. The exception is KCC2. When expressed in *Xenopus laevis* oocytes, KCC1, KCC3, and KCC4 are completely inactive in isotonic conditions [47, 48], whereas KCC2 shows a level of activity that is approximately 10-20% of that observed in hypotonic conditions [49]. When oocytes are exposed to hypotonic media, all four KCCs are maximally activated. A similar situation occurs with $[\text{Cl}^-]_i$. Intracellular chloride depletion is associated with activation of NKCC1/2 or NCC and inhibition of KCCs, whereas augmenting intracellular chloride activates KCCs and inhibits NKCCs [50, 51]. It has been firmly established that activation/inhibition of the SLC12 family members is associated with phosphorylation/dephosphorylation of the cotransporters [52]. Phosphorylation of NKCC1 [53], NKCC2 [54], and NCC [55] is associated with activation of the cotransporters by either cell shrinkage or intracellular chloride depletion. In contrast, phosphorylation of KCCs is associated with inhibition of the cotransporters [56]. In this regard, several conserved threonine/serine residues in the amino terminus of NKCC1/2 and NCC have been shown to be phosphorylated in response to activating stimuli [54, 57-61]. Similarly, phosphorylation of at least two threonine residues in the carboxyl terminal domain (T991 and T1048)

Fig. 2. Effects of wild type and catalytically inactive WNK3 on the activity of electroneutral cotransporters. The activity of all cotransporters was assessed in *Xenopus laevis* oocytes injected with the appropriate cRNA for each cotransporter alone or together with wild type WNK3 or catalytically inactive WNK3-DA, as stated, by measuring $^{86}\text{Rb}^+$ uptake, except for NCC, which was assessed by $^{22}\text{Na}^+$. The function of the cotransporters was determined in the absence or presence of specific inhibitors (thiazide for NCC or bumetanide for NKCC1 or NKCC2) or extracellular chloride (for KCCs), and the activity of each cotransporter was obtained by subtracting the uptakes observed in the presence of the inhibitor or in the absence of chloride. Uptake experiments were performed in isotonic, hypertonic or hypotonic conditions as stated. The figure was reconstructed from data in references [66], [67], and [69].

in KCC3 have been shown to be associated with inactivation of the cotransporters [62]. In contrast, dephosphorylation of NKCC1/2 or NCC results in inactivation, whereas dephosphorylation of KCCs is associated with activation. Consistent with this, protein phosphatase inhibitors such as calyculin A, okadaic acid, or cyclosporine prevent activation of KCCs [63] or inhibition of NKCC1/2 [64]. Consistent with these data, it has been suggested that activation/deactivation of the Na^+ -coupled and K^+ -coupled branches of the SLC12 family could be regulated by a kinase/phosphatase pair that is sensitive to cell volume and/or $[\text{Cl}^-]_i$ [51, 57]. That is, an intracellular soluble kinase and phosphatase pair could be activated and inhibited, respectively, by a decrease in $[\text{Cl}^-]_i$ or cell shrinkage, resulting in phosphorylation of SLC12 family members, activating NKCCs and inhibiting KCCs. In contrast, this kinase/

phosphatase pair could be inhibited and activated, respectively, by an increasing $[\text{Cl}^-]_i$ and by cell swelling, leading to dephosphorylation of all SLC12 family members in the cell and promoting activation of KCCs and inhibition of NKCCs.

WNK3 effects on the SLC12 family members

The effect of WNK3 on SLC12 cotransporters has been studied using the *Xenopus laevis* oocyte heterologous expression system [65]. Microinjections of cRNA from any of the cotransporters in the absence or presence of wild type or catalytically inactive forms of human WNK3 cRNA revealed interesting effects of this kinase on the cotransporters. WNK3 is expressed all along the nephron, from the proximal tubule to the

collecting duct. It is coexpressed with NKCC2 in the thick ascending limb of the loop of Henle and with NCC in the distal convoluted tubule [66]. Therefore, interaction between these proteins could occur *in vivo*. Catalytically inactive WNK3 was obtained by substituting aspartic acid 294 for alanine (D294A). WNK3 is also expressed in many epithelial and non-epithelial cells, as well as in many regions of the central nervous system where it is found in GABAergic neurons [67]. As shown in Figure 2A, coexpression of NCC, NKCC2 or NKCC1 with wild type WNK3 resulted in considerable activation of the cotransporters, even when the uptake experiment is performed in hypotonic conditions in which these proteins, particularly NKCC1, are normally inhibited [67]. It was observed that activation of NCC was associated with an increased amount of the protein on the cell surface, suggesting that WNK3 modulates trafficking of the cotransporter to the plasma membrane. As for NKCC1 and NKCC2, it was demonstrated that WNK3-induced activation of these cotransporters was associated with increased phosphorylation of T96 and T101 on NKCC2 [66] or the corresponding T212 and T217 for NKCC1 [67]. Similar experiments were performed using the catalytically inactive WNK3 cRNA (WNK3-DA) in order to determine whether the catalytic activity of WNK3 is required for the activation of NKCC2 or NCC. Surprisingly, as shown in Figure 2B, the activation of these cotransporters was not only prevented by eliminating the catalytic activity. Instead, WNK3-DA acted as a strong inhibitor of the three cotransporters, even when the uptake experiments were performed under hypertonic conditions, in which NKCC1/2 are maximally activated. The opposite effects of WNK3-DA were also observed for the surface expression and phosphorylation of the cotransporters. It was observed that coexpression of NCC with WNK3-DA reduced the amount of NCC protein on the cell surface [12, 14]. Furthermore, for NKCC2 or NKCC1, WNK3-DA induced dephosphorylation of the amino terminal threonines.

Interestingly, although WNK3 catalytic activity is required for its effects on the SLC12 cotransporters, one study showed that the carboxyl terminal domain of WNK3 by itself, which does not contain a kinase domain, can also induce activation of NCC [68]. The authors suggested that one potential mechanism could be that the fragment of WNK3 activates NCC by interacting with and inhibiting WNK4. It is not known, however, if such a WNK3 fragment is present in the distal convoluted tubule, where NCC and WNK4 are expressed. Another

study investigating the difference between WNK3 and WNK4 activity on SLC12 cotransporters developed a chimeric strategy to determine which WNK domains are responsible for their effects [31]. In *Xenopus oocytes*, WNK4 inhibits NCC as well as KCCs. In contrast, WNK3 activates NCC and inhibits KCCs, suggesting that the different effects of WNK3 and WNK4 upon NCC depend on differences in their residue sequences. The chimeric approach involved swapping the amino and carboxyl terminal domains of WNK3 and WNK4, and the resultant chimeras were tested to determine their effects on NCC and KCCs. The results showed that NCC-activating or NCC-inhibitory properties of WNK3 or WNK4, respectively, are located within the amino terminal domain [31] because a chimera with the amino terminal domain of WNK3 inserted into the kinase and carboxyl terminal domain of WNK4 increased NCC activity, whereas the construct with the amino terminal domain of WNK4 inserted into the rest of WNK3 inhibited the cotransporter.

KCCs are also modulated by WNK3, but in a completely opposite direction than the effects of WNK3 on NKCC1/2 or NCC [67, 69]. Co-expression of any of the KCCs with wild type WNK3 resulted in complete inhibition of the cotransporters' activity when uptake experiments were performed in hypotonic medium in which KCCs are normally activated (Fig. 2C). The activities of KCC1 through KCC4 in hypotonic environments were reduced by more than 90% when coexpressed with wild type WNK3. As was observed for the Na⁺-coupled cotransporters, the effect of the catalytically inactive WNK3-DA was completely opposite to that of its wild type form. As shown in Figure 2D, KCCs are totally inhibited in isotonic conditions. The only exception is KCC2, which exhibits minimal but significant activity. When oocytes are exposed to hypotonicity, KCCs are activated, as shown in Figure 2C. Interestingly, the presence of WNK3-DA was enough for KCCs to behave as if the cells were exposed to a hypotonic medium (Fig. 2D). Thus, in modulating the activity of the SLC12 family members, WNK3/WNK3-DA bypasses the tonicity requirements for activation/deactivation of the cotransporters.

There are four potential WNK3 variants due to the combination of two alternative splicing mechanisms. The presence of two mutually exclusive exons, 18a or 18b, combined with the presence or absence of exon 22, lead to these splice variants. Exon 18b is exclusively present in transcripts from the CNS and introduces 47 residues not present in 18a variant. Exon 18a is present

in most of the studied tissues. One study suggests that variants 18a and 18b have opposite effects on NCC: activation by 18a and inhibition by 18b. This effect is similar to wild type WNK3 vs. catalytically inactive WNK3-DA [70]. These results led to the attractive hypothesis that different isoforms of WNK3 modulate activity of the cotransporters by differential splicing mechanisms. However, other study in which the four potential variants of WNK3 (18a or 18b, with or without exon 22) were analyzed with respect to their effects on all SLC12 cotransporters using *Xenopus laevis* oocytes as the heterologous expression system reported no differences among isoforms in effects on each of the SLC12 family members [71].

Modulation of the electroneutral cation chloride cotransporters by WNK3 has many implications in several cells and tissues. At the cellular level, for instance, the NKCC1/KCC1 pair is known to play an important role in cell volume regulation. In contrast, the NKCC1/KCC2 pair is critical in defining the $[Cl^-]_i$ in neurons (for reviews see [72-75]). This is important because the effect of neurotransmitters acting on chloride channels associated with membrane receptors in postsynaptic membranes, such as gamma amino butyric acid, GABA, depends on the electrical potential of chloride. If $[Cl^-]_i$ is above potential, GABA produces a depolarizing, excitatory effect by extruding chloride from the neuron. In contrast, if $[Cl^-]_i$ is below potential, the effect of GABA will be inhibitory by hyperpolarizing the cell. WNK3 modulates cotransporters that are expressed in many epithelial cells, including NKCC2 and NCC in the nephron. In this location, activities of these proteins play a role in blood pressure regulation. Furthermore, NKCC1 is present in many secretory epithelia, and KCCs are present in both absorbing and secretory epithelia. Thus, WNK3 is a potential modulator of all these physiological processes. A recent study shows that in glioma cells, WNK3 co-localizes with NKCC1 upon changes in cell volume, and knocking down WNK3 reduces the activity of NKCC1, with the consequent reduction in migration and invasion of contiguous tissues, suggesting that WNK3 is a potential target for reducing the capacity of these types of tumors to invade other regions of the central nervous system [76].

Similar effects of WNK3 towards SLC12 cotransporters have recently been reported to occur with WNK2 [29]. That is, WNK2 also activates NKCC1 and inhibits KCCs. However, some important differences between WNK2 and WNK3 were observed. First, WNK2-DA does not induce effects that are opposite to

Fig. 3. Hypothesis for WNK3 modulation of electroneutral cotransporter activity. In the presence of cell swelling or high intracellular chloride, WNK3 is inactive, and as a consequence, the cotransporters becomes dephosphorylated, which increases or decreases the activity of KCCs and NKCCs, respectively. In contrast, during cell shrinkage or low intracellular chloride, WNK3 becomes autophosphorylated and promotes phosphorylation of all cotransporters, inhibiting KCCs, but activating NKCCs.

those of WNK2, as WNK3-DA does relative to WNK3. Second, WNK2 expression is enriched in the CNS, and is present by embryonic day 10.5, whereas WNK3 expression begins after birth.

In addition to SLC12 cotransporters, it has been shown that WNK3 modulates the activity of other ion transport proteins, such as ROMK [77], the transient potential regulators TRPV5 and TRPV6 [78], and the bicarbonate cotransporters [79].

WNK3 is the putative cell volume/chloride sensitive kinase

The activity of NKCC1/2 and NCC is clearly modulated by $[Cl^-]_i$. It has been demonstrated that manipulations that induce intracellular chloride depletion activates NKCC1 [53], NKCC2 [58], and NCC [55], with concomitant phosphorylation of the conserved threonine/serines in the amino terminal domain. Further study of this phenomenon in NKCC2 strongly suggested that modulation of NKCC2 by $[Cl^-]_i$ occurs via WNK3 and SPAK/OSR1 [58]. In this study involving *Xenopus laevis* oocytes, the authors observed that activation of NKCC2 by intracellular chloride depletion is disrupted by either overexpressing WNK3-DA (working as a dominant negative), by eliminating the three threonines that become phosphorylated in NKCC2 (T96, T101, T111) or by reducing the interaction between WNK3 and SPAK/

Fig. 4. Molecular model to propose a mechanism for the dual effects of WNK3. Wild type WNK3 (A) and the catalytically inactive WNK3-DA (B) are able to form protein complexes with SPAK/OSR1, protein phosphatases (PPs), and the cotransporters. This complex allows WNK3 to activate SPAK and inhibit the PPs, resulting in phosphorylation of the cotransporters. However, when WNK3 is not active, the complex is still formed, but cotransporters are no longer phosphorylated due to inactivity of SPAK/OSR1, together with the activity of PPs. Questions marks denote interactions that have not yet been demonstrated to occur.

OSR1 by removing one SPAK binding site on WNK3 that appears to be required for appropriate interaction between WNK3 and SPAK (WNK3-F1337A). Thus, is possible that intracellular chloride concentration is transduced by means of activation/inactivation of the WNK3-SPAK pathway. In this regard, it has been clearly shown that SPAK is a chloride-sensitive kinase [57]. This could be due to a direct effect of $[Cl^-]_i$ in SPAK, or in WNK3, or other upstream kinase that in turn phosphorylates SPAK.

The proposed mechanisms for WNK3 functional properties are shown in Figures 3 and 4. It has been demonstrated that WNKs possess an autoinhibitory domain that can prevent autophosphorylation of WNKs, thereby preventing their activation [32]. In the absence of autophosphorylation, WNKs have no catalytic activity and are thus expected to perform similarly to catalytically inactive WNK3-DA, as shown above. In other words, it is possible that the effects observed using the mutant WNK3-DA could actually occur *in*

vivo when WNK3 has no catalytic activity due to autoinhibition (Fig. 3). Cell volume and $[Cl^-]_i$ could be activators/inhibitors of WNK3 autophosphorylation, either alone, making WNK3 a cell volume and/or chloride-sensitive kinase, or by interaction with other unknown kinases upstream of WNK3. Therefore, when cell volume or $[Cl^-]_i$ is increased, WNK3 autophosphorylation is lost, inactivating WNK3, and the cotransporters will no longer be phosphorylated, reducing the activity of NKCCs but increasing that of KCCs. In contrast, when cell volume or $[Cl^-]_i$ is reduced, WNK3 autophosphorylation is activated, promoting phosphorylation of the cotransporters, with the expected effects on their activity (Fig. 3).

The effects of WNKs on target proteins do not depend only on kinase catalytic activity. Instead, the ability of WNKs to interact with other proteins is critical. For instance, inhibition of the potassium channel ROMK by WNK4 does not require the catalytic activity of the kinase but is dependent on the interaction between WNK4 and ROMK [20]. WNKs interact with each other

and with many of their target proteins, such as NCC [12], ROMK [20], ENaC [19], and claudins [22]. SPAK and OSR1 are proteins that are known to interact with and lie downstream of WNKs [36-40]. WNK3 forms a protein complex with SPAK that is reduced by eliminating one of the WNK3 SPAK binding sites [58]. As shown above, catalytically inactive WNK3-DA exhibits opposite effects towards SLC12 cotransporters as those obtained with wild type WNK3. It is possible that these effects can be explained by the ability of the kinase to form the appropriate protein complex but lacking catalytic activity.

As shown in Figure 4, we propose a working model to explain both the wild type and the WNK3-DA effects. We suggest that WNK3 forms a protein complex with SPAK, the SLC12 cotransporter and potentially, a protein phosphatase, which according to the effects of calyculin, cyclosporine, and fostreicin could be PP1, PP2B, and/or PP4, respectively [69, 80]. When the protein complex is formed with active WNK3, the kinase is able to phosphorylate SPAK and probably also the protein phosphatase, promoting simultaneous phosphorylation of the cotransporter and inhibition of the phosphatase. The consequence would be to increase phosphorylation of the cotransporter due to the combination of the SPAK activity and phosphatase inactivation. If the cotransporter is NKCC1, NKCC2 or NCC, the consequence would be activation, whereas for any of the KCCs, the result would be inhibition. Elimination of the catalytic activity of WNK3, which could occur physiologically by preventing autophosphorylation or experimentally by introducing the D294A substitution, does not affect the ability of WNK3 to form

protein complexes. Thus, the WNK3, SPAK, cotransporter and protein phosphatase complex would be formed, but WNK3 could not phosphorylate SPAK and protein phosphatases, resulting in inhibition of SPAK but allowing the phosphatase to be active. In this circumstance, phosphorylation of the cotransporter would decrease for two reasons: first, because the cotransporter is "hijacked" in a protein complex that cannot phosphorylate, it and second, because in this complex, there is an active phosphatase that eliminates phosphate groups from serine/threonines residues nearby, resulting in the dephosphorylation of the cotransporter. As shown in Figure 4B, NKCC1, NKCC2, or NCC would be inhibited, whereas KCCs will be activated.

In summary, WNK3 and the catalytically inactive form WNK3-DA modulates the activity of SLC12 cotransporters and in doing so, bypasses the changes of $[Cl^-]_i$ or of tonicity that are normally required for modulation of the activity of these membrane proteins. These observations suggest that WNK3 is an important kinase for the regulation of cell volume and/or intracellular chloride concentration.

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