Informe Técnico Final del Proyecto

Mecanismo de la regulación del cotransportador renal de NaCl por angiotensina II

Responsable Técnico: Dr. Gerardo Gamba Ayala

A continuación se destacan lo logros del proyecto de investigación titulado "Mecanismo de la regulación del cotransportador renal de NaCl por angiotensina II" con No. De Registro NMM-25.

Este proyecto se inició en el año de 2010 y fue financiado con un donativo de la Fundación Wellcome Trust No. 091415. Por tanto, en los agradecimientos de los artículos producidos este es el número que identifica el apoyo otorgado a este proyecto.

El proyecto se extendió por un periodo de tiempo mayor al esperado porque hubo con adquirir equipo y montar técnicas que no teníamos en el laboratorio, como el caso de la medición de presión arterial por telemetría.

I. Artículos Publicados.

Con este proyecto se produjeron seis artículos en revistas de alta circulación internacional. Cuatro de ellos reportaron resultados originales y dos de ellos fueron de revisión para difundir los resultados de este y otros proyectos a una mayor audiencia.

Artículo 1.
Castañeda-Bueno M, Cervantes-Pérez LG, Vázquez N, Uribe N, Kantsaria S, Morla L, Bobadilla NA, Doucet A, Alessi DR & Gamba G. The activation of the renal Na-Cl cotransporter by angiotensin II is a WNK4 dependet process in the mouse. *Proc Natl Acad Sci USA*, 109:7929-7934, 2012. Factor de Impacto 9.7

Artículo 2
Melo Z, de los Heros P, Cruz-Rangel S, Vazquez N, Bobadilla NA, Alessi DR, Morales-Pasantes H, Mercado A & Gamba G. N-terminal serine dephosphorylation is required for full activation of KCC3 during cell swelling. *J Biol Chem*, 288:31468-31476, 2013. Factor de Impacto 4.6

Artículo 3
Castañeda-Bueno M, Cervantes-Perez L, Rojas-Vega L, Arroyo-Garza I, Vázquez N, Moreno E, & Gamba G. Modulation of NCC activity by low and high K^+ intake: insight into

the signaling pathways involved. *Am J Physiol Renal Physiol*, 306:F1507-F1519, 2014. Factor de Impacto 3.6

Artículo 4
Cervantes- Pérez LG, Castañeda-Bueno M, Jimenez V, Vázquez N, Rojas-Vega L, Alessi D, Bobadilla NA & Gamba G. Disruption of the with no lysine kinase-STE20-proline alanine-rich kinase pathway reduces the hypertension induced by angiotensin II. *Journal of Hypertension*, 18:361-367, 2018 Factor de Impacto 4.6

Artículo 5
Arroyo JP, Kahle KT & Gamba G. The SLC12 family of electroneutral cation-coupled chloride cotransporters. *Molec Asp Med*, 34:288-298, 2013. Factor de Impacto 10.8

Artículo 6
Gamba G. Regulation of the renal NaCl cotransporter by phosphorylation and ubiquitylation. *Am J Physiol Renal Physiol*, 303:F1573-F1583, 2012. Factor de Impacto 3.6

Artículo 7
Castañeda-Bueno M & Gamba G. Mechanisms of NCC modulation by angiotensin II. *Curr Op Nephrol Hyperten*, 21:516-522, 2012. Factor de impacto 3.2

II. Formación de recursos humanos.

Gracias en buena parte a este este proyecto se formaron tres alumnos. Dos de Doctorado y uno de posdoctorado.

María Castañeda Bueno obtuvo el Doctorado en Ciencias Bioquímicas de la Facultad de Química de la UNAM con Mención Honorífica en 2013. Los artículos 1 y 3, en donde ella es primer autor, fueron parte de su tesis doctoral y en ambos se menciona en agradecimientos la beca de conacyt que hizo posible los estudios de la Dra. Castañeda.

Zesergio Melo obtuvo el Doctorado en Ciencias Bioquímicas de la Facultad de Química de la UNAM con Mención Honorífica en 2014. El artículo 2 es parte de su tesis doctoral y en se mención en agradecimientos la beca de conacyt que hizo posible los estudios de la Dr. Melo.

Luz Graciela Cervantes Pérez fue estudiante de pos-doctorado y es la primera autora del artículo No. 4. El posdoctorado de la Dra. Cervantes fue cubierto por el proyecto de Wellcome Trust (por dos años) y por una beca de DAGAPA de la UNAM, por un tercer año, como consta en los agradecimientos del artículo NO. 4

III. Presentación en congresos

Los trabajos arriba mencionados fueron presentados en los congresos anuales de la Sociedad Americana de Nefrología o de la Sociedad Americana de Fisiología por los primeros autores. Los mismos resultados fueron también presentados en los congresos anuales del Instituto Mexicano de Investigaciones Nefrológicas.

IV. Resultados obtenidos

Demostramos que la angiotensina II estimula al transportador de NaCl en el riñón y que para hacer esto es necesaria la presencia de las cinasas WNK4 y SPAK (artículo 1). Este trabajo ha sido importante y aunque fue publicado en 2012 tiene ya a la fecha 147 citas.

Demostramos que al no existir la cinasa SPAK en un modelo knockin, el efecto hipertensor de la angiotensina II se reduce a la mitad, lo que implica a la vía WNK4-SPAK-NCC en la producción de la hipertensión (artículo 4).

Demostramos que para la completa activación del cotransportador de K-Cl KCC3 se requiere defosforilar un sitio extra, no antes considerado. Trabajos previos de otros autores mostraron que para activar a KCC3 se requiere defosforilar las treoninas 991 y 1048. En este trabajo mostramos que para la activación total se requiere también defosforilar a la serina 96 (artículo 3). Tiene a la fecha 15 citas.

Demostramos que la actividad del NCC se regula por la concentración extracelular de potasio y que la vía WNK4-SPAK está implicada en este mecanismo (artículo 4). Tiene 42 citas a la fecha.

De los artículos de difusión, el número de citas recibidas a la fecha son 51 al número 5, 42 al número 6 y 29 al número 7.

Se anexan las publicaciones producidas.

Atentamente,

Dr. Gerardo Gamba
Director de Investigación
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Review

The SLC12 family of electroneutral cation-coupled chloride cotransporters ☆

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ABSTRACT

The SLC12 family encodes electroneutral cation-coupled chloride cotransporters that are critical for several physiological processes including cell volume regulation, modulation of intraneuronal chloride concentration, transepithelial ion movement, and blood pressure regulation. Members of this family are the targets of the most commonly used diuretic drugs, have been shown to be the causative genes for inherited disease such as Gitelman, Bartter and Andermann syndromes, and potentially play a role in polygenic complex diseases like arterial hypertension, epilepsy, osteoporosis, and cancer.

Contents

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1. Introduction

The SLC12 family of solute cotransporters encodes electroneutral cation-coupled chloride cotransporters. This gene family is made up of nine members of which the functional characteristics of seven members are well known (Table 1). They are classified within the APC super family as the 2.A.30 Cation-Chloride-Cotransporter (CCC) Family within the Milton Saier transporter classification system. The SLC12/CCC family is divided in two functional branches. The first includes the Na^+-coupled chloride cotransporters, including the Na^+:K^+:$2Cl^-$ cotransporters 1 and 2 (NKCC1 and NKCC2) and the Na^+:Cl^- cotransporter (NCC), exhibiting about 50% identity amongst each other. The second branch is K^+-driven and encompasses four genes encoding the K^+:Cl^- cotransporters (KCC1 through KCC4), which exhibit about 70% identity. The functional characteristics of two remaining members of the SLC12 family, CCC9 and CIP, are not completely known. This review will focus on the recent advances within the SLC12 family, focusing on their regulation, as well as on their relation to health and disease. For an in-depth review of the extensive amount of information for this family, readers are referred to recent reviews on the subject (Ares et al., 2011; Blaesse et al., 2009; Delpire and Austin, 2010; Gamba, 2005, 2009; Hebert et al., 2004; Kahle et al., 2008; Pacheco-Alvarez and Gamba, 2011; Russell, 2000).

The SLC12A family members (Table 1) are membrane transporters with a proposed Kyte-Dolittle topology of a hydrophilic short amino-terminal and long carboxyl-terminal domain. The amino and carboxyl domains flank 12 putative transmembrane (TM) spanning segments, with an extracellular loop, containing N-linked glycosylation sites, located between TM segments 7 and 8 for NKCC1/2 and NCC and between segments 5 and 6 for KCCs (Fig. 1). The proposed model has been experimentally corroborated only for NKCC1 and glycosylation in the expected sites has been documented for NCC and NKCC2 (Gamba, 2005; Paredes et al., 2006). The lack of *N*-glycosylation of NKCC2 and NCC leads to decreased cell surface protein expression as well as an increased affinity for Cl^- ions in NKCC2 and for thiazides in NCC (Gamba, 2005; Paredes et al., 2006). A direct and/or indirect association has been found between the dysfunction of several SLC12A members and disease (Table 1).

2. The Na^+–(K^+)–Cl^- cotransporters: SLC12A1 to SLC12A3

2.1. *Slc12a1* – *NKCC2*

SLC12A1 encodes NKCC2 (also known as BSC1) which is the major salt transport pathway in the Thick Ascending Limb of Henle (TALH) and plays a key role in the maintenance of the countercurrent mechanism responsible for urinary concentration, blood pressure regulation, and divalent cation (Ca^{2+}, Mg^{2+}) reabsorption. Type I Bartter syndrome represents the clinical phenotype (hypotension, hypokalemia, hypercalciuria and metabolic alkalosis) due to inactivating mutations of the *SLC12A1* gene (Gamba, 2005) (Fig. 2). Additionally, rare mutations in one allele of *SLC12A1* in subjects of the Framingham cohort study (Ji et al., 2008) reduce the activity of NKCC2 (Acuna et al., 2011), which correlates with a decreased risk of hypertension and protects against cardiovascular events, highlighting the important role of NKCC2 in the maintenance of blood pressure and salt-water balance. NKCC2 is also important in the treatment of cardiovascular disorders. It is specifically inhibited by loop diuretics (bumetanide and furosemide), which are the most potent diuretics clinically available today and are extensively used for the treatment of edematous states that are associated with congestive heart failure among other diseases.

Expression of NKCC2 is limited to the kidney and more particularly to the TALH (Fig. 2).This cotransporter has 1095 amino acids and shares the predicted membrane topology of the sodium coupled SLC12 family members. Multiple isoforms, including variants A, B, and F, are generated by three exclusive cassettes in exon that change half of TM2 and part of the interconnecting segment between TM2 and TM3 The isoforms are expressed along the TALH with varying ion affinity (B > A > F). NKCC2 variant B is particularly expressed in the cortical cells and the macula densa and thus plays a key role in regulating tubuloglomerular feedback (Carota et al., 2010). Additionally, in mouse, a carboxyl terminal truncated isoform that displays bumetanide sensitive Na^+:Cl^- transport is expressed in the medullary TALH, thereby explaining the switch between K^+-dependent and K^+-independent bumetanide-sensitive NaCl transport in the mouse TALH. This shorter isoform also exerts a dominant negative effect on Na^+:K^+:$2Cl^-$ transport (Gamba, 2005).

A particular 77 amino acid segment of the carboxyl terminal sequence in NKCC2 (amino acid 708–884) that is absent in NKCC1 is responsible for directing NKCC2 to the apical membrane in polarized MDCK cells (Carmosino et al., 2008). A dileucine motif in the carboxyl terminal domain that targets NKCC1 to the basolateral membrane (Nezu et al., 2009) is not present in NKCC2. Additionally, an LLV motif in the carboxyl terminal domain of NKCC2 was identified as an important endoplasmic reticulum (ER) retaining signal that plays a role in mediating transport from the ER to the cell surface (Zaarour et al., 2009). Using the NKCC2 carboxyl terminal domain as bait, the same group identified the protein SCMP2 which reduces NKCC2 expression through recycling endosomes and interfering with its exocytotic trafficking (Zaarour et al., 2011). Of interest, a Type I Bartter causing mutation lies close to the LLV motif (Adachi et al., 2007) additionally inferring that the glycosylation of NKCC2 is associated with lower expression at the cell surface (Paredes et al., 2006).

Although the carboxyl terminal domain of NKCC2 has been associated with maturation, glycosylation, and protein interactions, to date, all the regulatory phosphorylation sites have been mapped to the aminoterminal domain (Ponce-Coria et al., 2008; Richardson et al., 2011) (for an excellent review on NKCC2 regulation see (Ares et al., 2011)) (Table 1). Indeed, NKCC2 activity and aminoterminus phosphorylation of human NKCC2 at positions T100 and T105 (homologous to rat NKCC2

Table 1
SLC12 Family of Cl- coupled electroneutral co-transporters (KO – knockout mouse model, TALH – thick asceding loop of henle, DCT – distal convoaluted tubule, ACCPN – peripheral neuropathy with variable agenesis of the corpus callosum, HTN – hypertension). For detailed information about the SLC gene tables, please visit: http://www.bioparadigms.org.

Gene name	Protein name	Human gene locus	Ion co-transport coupling	Tissue expression	Functionally important phosphorylation sites	Direct disease association	KO phenotype	Potential role in complex disease	Sequence Accession ID
SLC12A1	**NKCC2**	15q21.1	$1Na^+:1K^+:2Cl^-$	Kidney specific	T100, T105, S130	Type I Bartter syndrome	Severe hypotension, hypokalemia, hypercalciuria, metabolic alkalosis	HTN	NM_000338
SLC12A2	**NKCC1**	5q23.3	$1Na^+:1K^+:2Cl^-$	Ubiquitous	T184,T189,S202	–	Multiple phenotypes: sensorineural deafness, alterations in endolymph secretion, reduced saliva production, sensory perception abnormalities and infertility; low blood pressure not consistent	Epilepsy	NM_001046
SLC12A3	**NCC**	16q13	$1Na^+:1Cl^-$	Kidney and bone	T53,T58,S71	Gitelman syndrome	Hypotension, hypocalciuria, hypomagnesemia, hypokalemia (with low K^+ diet)	HTN, osteoporosis	NM_000339
SLC12A4	**KCC1**	16q22	$1 K^+:1Cl^-$	Ubiquitous	–	–	No phenotype	Sickle cell anemia?	NM_005072
SLC12A5	**KCC2**	20q13	$1 K^+:1Cl^-$	Neurons	–	–	Complete – death due to absent respiratory drive. incomplete KO (5% of function remains). Status epilepticus, death ± 15 days after birth.	Epilepsy	NM_020708
SLC12A6	**KCC3**	15q14	$1 K^+:1Cl^-$	Extensive	T991, T1048	ACCPN (Andermann syndrome)	Deafness, hypertension and Tumor biology	Renal tubular acidosis, epilepsy, sickel cell anemia	NM_133647 NM_005135
SLC12A7	**KCC4**	5p15	$1 K^+:1Cl^-$	Extensive; limited in brain	–	–	Sensorineural deafness and renal tubular acidosis	Renal tubular acidosis, epilepsy, sickel cell anemia	NM_006598
SLC12A8	**CCC9**	3q21	Unknown	Extensive	–	–	–	Psoriasis	AF319951 AF389851
SLC12A9	**CIP**	7q22	Polyamines?	Extensive	–	–	–	–	NM_020246

T96,T101) are induced by hypertoxicity, WNK3, growth hormone, and hypotonic low-chloride stress (Ares et al., 2011) which are known to reduce intracellular chloride concentration (Lytle and McManus, 2002) (Fig. 2). It had been proposed that activity of NKCC2 in oocytes was the end result of a balance between WNK3, SPAK, and $[Cl^-]_i$, thus suggesting that the WNK3-SPAK system could play an important role in sensing changes in $[Cl^-]_i$ there by modulating co-transport activity. In this regard, it was recently shown that the Tamm-Horsfall protein renders NKCC2 more sensitive to activation secondary to changes in $[Cl^-]_i$ (Mutig et al., 2011).

Membrane trafficking of NKCC2 has been studied by Ortiz's group (Ares et al., 2011) (Fig. 1) who showed that Vesicle Associated Membrane Proteins (VAMP 2 and 3) when blocked by botulinum toxin can inhibit the cAMP mediated NKCC2 membrane trafficking. The effect of sorting proteins (SORLA/SORL1) was recently studied (Reiche et al., 2010). It was

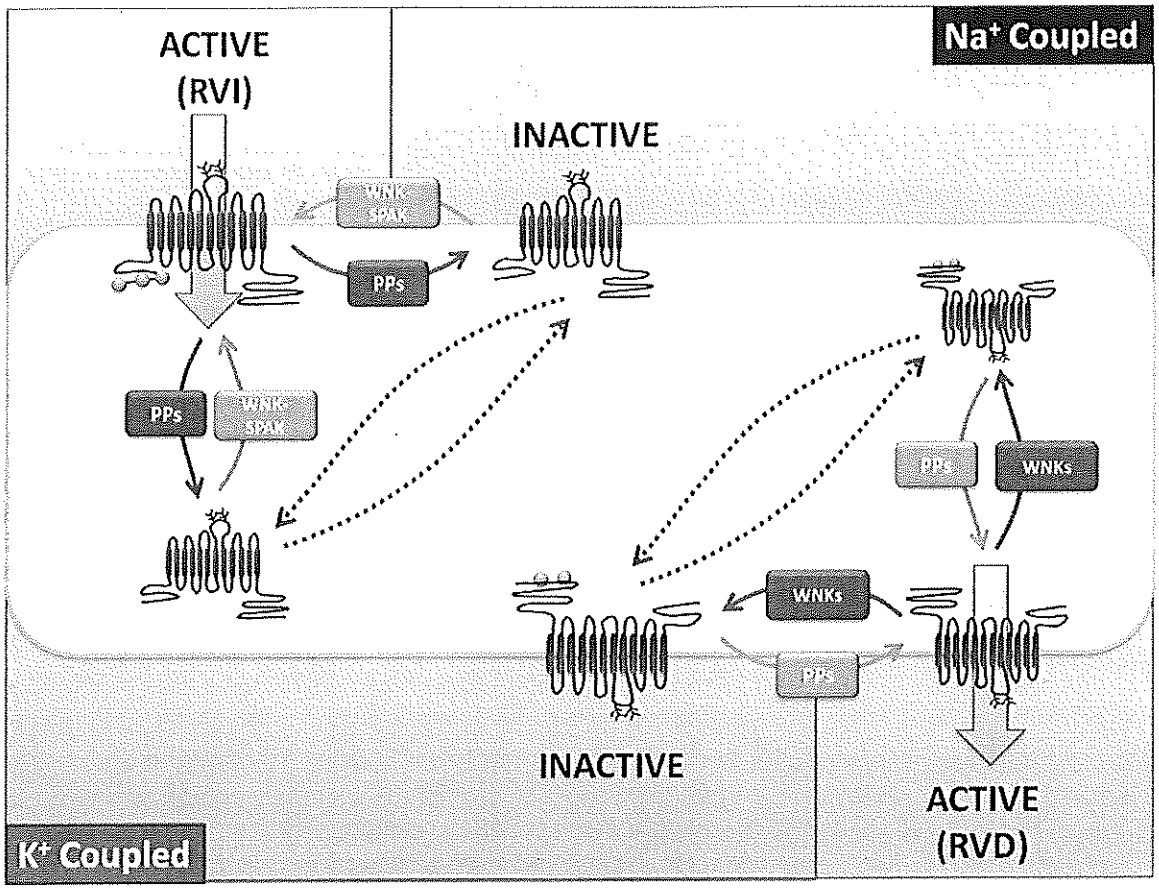

Fig. 1. Membrane expression and regulation of the SLC12A family members differ between Na^+ coupled and K^+ coupled cotransporters. WNK3 is an activator of the Na^+ coupled members, but an inhibitor of the K^+ coupled members. Protein phosphatases (PPs) function inversely by activating KCC's, but inhibiting Na^+ coupled transporters. WNK4 can exert biphasic effects on NCC, but behaves as an inhibitor of KCCs. The question of whether phosphorylation occurs at or before the membrane is still unresolved. However, the final effect of activation of the Na^+ coupled co-transporters favors Regulatory Volume Increase (RVI) in the presence of extracellular hypertonicity (particularly via NKCC1), while the activation of the K^+ coupled cotransporters favors the Regulatory Volume Decrease (RVD), like during hypotonicity. See text for additional details on specific modulation of activation and inhibition of transport activity.

observed that SORLA interacts with STE20 kinases (SPAK). Lack of the SORLA receptor results in a miss-sorting of SPAK, and inactivation of NKCC2. Thus, the SORLA-SPAK pathway plays a role in activation/deactivation of NKCC2 trafficking.

2.2. *Slc12a2 – NKCC1*

SLC12A2, positioned on human chromosome 5, encodes the ubiquitously-expressed, bumetanide-sensitive NKCC1 (BSC2) (Gamba, 2005). One alternatively-spliced isoform of *SLC12A2* exists, lacking exon 21, which encodes a carboxyl-terminal domain required for the differential sorting of NKCC1 in polarized epithelial cells. NKCC1 is expressed on the basolateral membrane of numerous polarized secretary epithelia where it is important for loading Cl^- into cells (Fig. 1 and 3). Driven by electrochemical gradients, Cl^- is then secreted across the apical membrane via Cl^- channels like CFTR, with water and solute following suit (Russell, 2000).

The diverse phenotypes of *SLC12A2* knockout mice illustrate the importance of this transporter in numerous physiological processes (Delpire and Mount, 2002). *SLC12A2* KO mice exhibit a number of alterations including deafness due to a combination of a sensorineuronal defects and disrupted epithelial secretion in the labyrinth, cecum bleeding and blockade of the colon due to impaired intestinal secretion, salivation impairment due to parotid gland dysfunction, infertility due to a deficiency in spermatocyte production, and low blood pressure due to vascular and renal effects (Orlov et al., 2010).

NKCC1 is also important for the function of the central and peripheral nervous systems. In micro vascular endothelial cells of the blood–brain–barrier (BBB) and in the choroid plexus, NKCC1 is localized to the apical (luminal) membrane (Blaesse et al., 2009). Here, the activity of NKCC1 helps maintain the milieu of the brain's extracellular spaces and CSF, respectively, thereby protecting glial and neuronal cell volume against changes of extracellular osmolality and intracellular solute. This

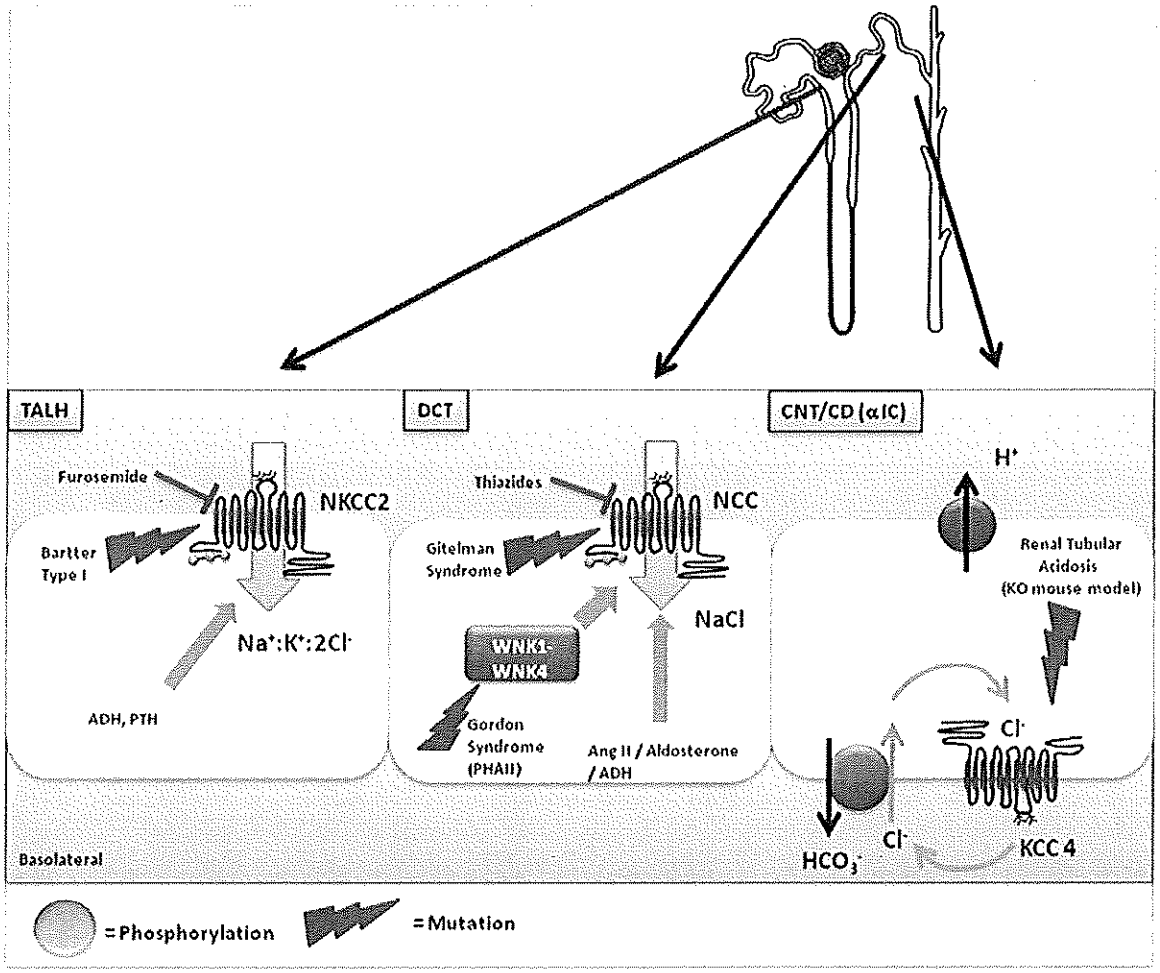

Fig. 2. NKCC2 is expressed in the Thick Ascending Limb of Henle (TALH), inhibited by loop diuretics (furosemide), and mutations result in Bartter type I syndrome. It is activated by antidiuretic hormone (AVP/ADH) and PTH. NCC is expressed in the Distal Convoluted Tubule (DCT) and inhibited by thiazide type diuretics. Mutations in NCC result in Gitelman syndrome while mutations in WNK1 or WNK4 result in hyperactivation of NCC (Gordon Syndrome, PHAII). It is activated by Angiotensin II, Aldosterone and AVP/ADH among other hormones. KCC4 is expressed in α intercalated cells of the collecting duct (CD). Elimination of KCC4 in mice results in an alteration of Cl^- recirculation which causes Renal Tubular Acidosis (RTA).

suggests that bumetanide, a more specific inhibitor of NKCC1 than furosemide, could be of greater utility in the treatment of idiopathic intracranial hypertension (Kahle et al., 2011).

Expression of NKCC1 in neurons is developmentally regulated, with high expression in immature neurons and lower expression in mature neurons (Blaesse et al., 2009; Kahle et al., 2008) (Fig. 3). The depolarizing action of GABA is likely to result from an efflux of chloride through $GABA_A$-gated anion channels, the driving force for Cl^- efflux being generated and maintained in part by high expression of NKCC1, as well as low expression of KCC2 (see below). Consistent with its importance in maintaining high Cl^- in immature neurons, a role for NKCC1 in neonatal seizures in both mice and humans has been proposed in which GABA is pathologically depolarizing (Kahle and Staley, 2008). Moreover, certain adult seizure syndromes, like mesial temporal sclerosis, appear to show a recapitulation of the "immature" neuronal phenotype with high NKCC1 expression thereby rendering GABA activity depolarizing and even excitatory.

In the adult peripheral nervous system, NKCC1 is heavily expressed in primary sensory neurons and root ganglia (DRG). Here, the presence of NKCC1 also makes GABA a depolarizing neurotransmitter that is important for presynaptic inhibition, a mechanism in the regulation of pain sensation. Indeed, the nocioceptive threshold of NKCC1 null mice in the hot plate test is increased, as well as the touching evoked pain test (allodynia) (Blaesse et al., 2009; Kahle et al., 2008).

Regulation of NKCC1 is predominately achieved via serine/threonine phosphorylation. Cell shrinkage and low intracellular chloride activates NKCC1, while the opposite is inhibitory (Fig. 3) (Lytle and McManus, 2002). Activation of NKCC1 is associated with phosphorylation of the three threonines located in the amino terminal domain (Table 1) that are the target of SPAK (Table 1) (Figs. 1 and 3) (Delpire and Austin, 2010). SPAK in turn lies downstream of other pathways, including the WNK serine–threonine kinases (with no lysine kinases) (Kahle et al., 2010; Vitari et al., 2005). Inactivation of NKCC1 is

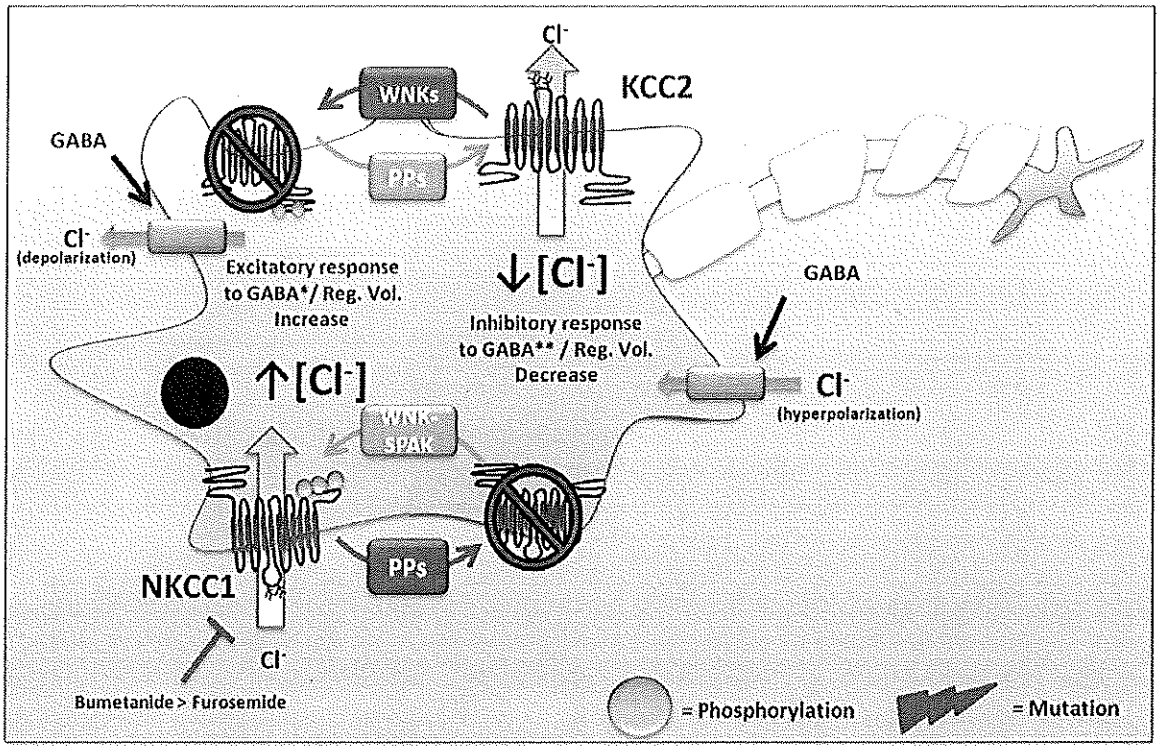

Fig. 3. Balance between the function of NKCC1 and KCC2 in neurons will dictate the $[Cl^-]_i$. If NKCC1 and KCC2 are phosphorylated, for instance, by WNK-SPAK, NKCC1 will promote influx of Cl^- into the neuron and KCC2 will remain inactive, preventing Cl^- efflux. This will increase $[Cl^-]_i$ generating an excitatory response to GABA. Conversely if phosphatases (PPs) become active, NKCC1 will be inhibited and KCC2 will activate, thus reducing $[Cl^-]_i$, mediating the inhibitory response to GABA. Similarly, increased expression of NKCC1 over KCC2 during embryonic development favors GABA mediated depolarization*, while increased expression of KCC2 over NKCC1after birthfavors GABA mediated hyperpolarization**.

associated with dephosphorylation of the amino terminal threonines by protein phosphatase 1 (Gagnon and Delpire, 2010) (Fig. 1).

2.3. *Slc12a3 – NCC*

The major salt transport pathway in the distal convoluted tubule is the thiazide-sensitive Na^+:Cl^- Cotransporter, NCC (also known as TSC) encoded by the *SLC12A3* gene located in human chromosome 16 (Fig. 2). This gene has recently gained attention due the understanding of its increasingly important role in blood pressure regulation. The possibility of studying the activity of NCC using an *in vivo* model was advanced by the demonstration that activation of NCC is associated with phosphorylation of key threonine and serines at the amino terminal domain, similar to NKCC1 and NKCC2 (Table 1) (Pacheco-Alvarez et al., 2006; Richardson et al., 2008). In addition to the kidney, NCC is expressed in bone cells with a possible role in the mineralization process (Gamba, 2009).

In the kidney, expression of NCC is limited to the Distal Convoluted Tubule (DCT) (Gamba, 2009). NCC loss-of-function mutations are the cause of Gitelman Syndrome, which presents with hypotension, hypocalciuria, hypokalemia, and metabolic alkalosis. The phenotypical mirror image of Gitelman Syndrome is Pseudohypoaldosteronim Type II (PHAII), also known as Familial Hyperkalemic Hypertension (FHHt) (Fig. 2). These patients present with hypertension, hyperkalemia, hypercalciuria, and metabolic acidosis which are completely corrected with low dose thiazide treatment, suggesting a hyperactive NCC could be responsible. Mutations in the With No Lysine kinases WNK1 and WNK4, that presumably increase NCC activity, were identified as the cause of the PHAII phenotype (Wilson et al., 2001) (Fig. 2).

Extensive research with the WNKs and STE20 serine/threonine kinases points to a complex picture behind the regulation of salt transport pathways among other effects. Further analysis of the pathophysiology of PHAII led to the realization that intronic mutations in the *WNK1*gene altered the balance between the NCC-activating long WNK1 isoform (L-WNK1) and the NCC-inhibiting kidney-specific variant (KS-WNK1), thus leading to increased NCC activity (Fig. 2). This model, however, awaits definite confirmation by *in vivo* models (Hadchouel et al., 2010; Huang and Kuo, 2007). With WNK4 the situation is different. Several groups have shown that wild-type WNK4 inhibits NCC, while the PHAII harboring mutant WNK4 loses this inhibitory capacity (Gamba, 2009). Activation of NCC by mutant WNK4 has been observed in genetically altered mice strains (Lalioti et al., 2006; Yang et al., 2007b). *WNK4* mutations however cannot be classified as simple loss-of-function

mutations because of the effects PHAII-WNK4 exerts on other renal transport proteins (Ring et al., 2007) and because Lalioti et al. (2006) demonstrated that the presence of mutant WNK4 produces the PHAII phenotype, even in the presence of two normal alleles of *WNK4*. WNK4 harboring PHAII mutations seem to result in NCC, ENaC, and paracellin activation, along with ROMK inhibition, resembling the functional state of the nephron during volume depletion which favors salt reabsorption and minimizes K^+ excretion (Gamba, 2009)(Fig. 2). Since activation of the renin angiotensin system is the hallmark of volume depletion it was suggested that mutations in WNK4 could be mimicking the physiological actions of Angiotensin II (Ang II) (San Cristobal et al., 2009). In *Xenopus laevis* oocytes, AngII was able to activate NCC only in the presence of wild type WNK4 in a SPAK dependent mechanism. Supporting this possibility, the activation of NCC by low salt diet or AngII infusion is not longer present in the WNK4 total knockout mice (Castañeda-Bueno et al., 2012; Na et al. (2012) recently reported that the kinase activity of WNK4, towards SPAK and NCC, is modulated by calcium concentration. Additionally, Sandberg et al. demonstrated that AngII modulates membrane trafficking of NCC in rat DCT (Sandberg et al., 2007; Van der Lubbe et al. (2011) showed that in the absence of aldosterone (adrenalectomized rats) AngII infusion increases phosphorylation of NCC. Thus, AngII and aldosterone can exert independent effects on NCC (Fig. 2). Supporting the role of SPAK in the process of AngII-WNK4 regulation of NCC, the generation of an inactive SPAK Knock-In mouse model showed decreased blood pressure levels and NCC phosphorylation vs. control animals (Rafiqi et al., 2010). Alternative proposals to explain the effects of wild type and mutant WNK4 on NCC have been advanced. Ellison's group (Yang et al., 2007a) made observations suggesting that interactions between WNK3 and WNK4 could be behind the mechanism of PHAII, while Uchida's group (Ohta et al., 2009) proposed that WNK4 is a direct activator of NCC and that this effect is increased by mutations.

The mechanism by which aldosterone increases NCC expression without affecting *SLC12A3* transcription rate has been elucidated, at least in part, by Arroyo et al. (2011) who demonstrated that NCC protein levels and activity are modulated by the SGK1-Nedd4-2 pathway in a similar way to that of ENaC. NCC activity is increased by intracellular chloride depletion (Pacheco-Alvarez et al., 2006). WNK3 is also a powerful activator of NCC (Gamba, 2009) in a SPAK dependent manner (Pacheco-Alvarez et al., 2012). It has been suggested that WNK3-SPAK could serve as the intracellular chloride sensitive kinases (Pacheco-Alvarez and Gamba, 2011). Another physiological condition that has been described which increases NCC phosphorylation and thus is a positive regulator of the cotransporter is vasopressin hormone (Mutig et al., 2010; Pedersen et al., 2010). Additionally, recent studies have shown that protein phosphatases also play a role in regulating NCC activity (Glover et al., 2010) (Figs. 1 and 2).

3. The K^+:Cl^- Cotransporters: SLC12A4 to SLC12A7

KCCs are involved in a myriad of functions including cell volume regulation, transepithelial salt transport, as well as central and peripheral nervous activity (Hebert et al., 2004). KCC proteins are approximately 1100 amino acids in size (with variations between family members) and the glycosylation-dependent molecular weights oscillate between 130 and 180 kDa (Gamba, 2005). Unlike the Na^+ coupled members (NKCC1, NKCC2 and NCC) it is generally accepted that activation of KCC mediated transport requires dephosphorylation rather than phosphorylation (Fig. 1). Although KCC1, KCC3, and to a lesser extent KCC4 are expressed in the brain, their expression is not limited to the nervous system, unlike KCC2 which is neuronal-specific (Table 1). KCCs have been associated with neurological disease, as well as with sickle cell anemia and cancer growth and invasiveness.

3.1. *Slc12a4 – KCC1*

SLC12A4 is located in human chromosome 16 and encodes KCC1 which was the first member of the K^+:Cl^- cotransporters to be identified. KCC1 expression is ubiquitous and is generally considered to be involved in volume regulation. KCC1 expression in the erythrocytes is particularly important as it is suggested to regulate cell volume and maturation of early erythroid precursor cells (Adragna and Lauf, 2007). The *SLC12A4*$^{-/-}$ mouse model (Rust et al., 2007) did not display a distinct phenotype, as it was associated with up regulation of KCC3 in erythrocytes which was presumably able to fully compensate for the lack of KCC1. The generation of a *SLC23A4*$^{-/-}$, *SLC12A6*(KCC3)$^{-/-}$, and SAD$^{-/-}$ mouse model (a transgenic sickling human hemoglobin S variant) by the same group, showing no K^+:Cl^- co-transport activity, revealed increased mean corpuscular volume, osmotic resistance, and a decrease in mean corpuscular hemoglobin concentration (Rust et al., 2007), demonstrating the role of KCC1 and KCC3 in regulation erythrocytes volume.

3.2. *Slc12a5 – KCC2*

SLC12A5, located on human chromosome 20, encodes the neuronal-specific KCC2 (Gamba, 2005). There are two alternatively spliced isoforms, KCC2a and KCC2b, varying in 40 residues in the amino terminal domain (Uvarov et al., 2007). KCC2 expression is high in pyramidal neurons of the hippocampus, cerebellum, brain stem, and retina (Blaesse et al., 2009). The highly restricted expression profile of KCC2 is likely due to negative transcriptional regulation in all other cells, since the *SLC12A5* gene in both human and mouse possesses a neuronal-restrictive silencing element positioned 3′ of KCC2's exon 1. KCC2 expression can be detected in embryonic life at E12.5 in the ventral horns of the developing spinal cord, and increases in the early days of postnatal life in other neurons along in the central nervous system (CNS) in a caudo-cranial

direction. The high expression of KCC2 in CNS neurons is maintained throughout adult life in both brain and spinal cord neurons. KCC2 does not appear to be expressed in the peripheral nervous system (PNS), including the DRG.

Among KCCs, KCC2 is unique in that it is active in isotonic conditions (Gamba, 2005) due to a region of the carboxyl terminal domain (Mercado et al., 2006), consistent with its role as a Cl^- extrusion pathway in neurons that maintains Cl^- potential below equilibrium (Blaesse et al., 2009; Kahle et al., 2008). KCC2 also exhibits the highest affinity for K^+ and Cl^- relative to other KCCs. Thus, if extracellular K^+ is increased during neuronal activity to values as high as 10–12 mM, a range wherein KCC2 is highly active, the driving force for net K^+-Cl^- cotransport will switch from efflux to influx (Fig. 3).

By determining the level of intracellular Cl^- in neurons, KCC2 dictates the strength and polarity of neuronal signaling by glycine and GABA during development and maturation. In embryonic and early postnatal life, NKCC1 activity is robust in hippocampal neurons, which display minimal activity of KCC2, resulting in a positive ECl relative to Vm, such that GABA receptor activation mediates an outwardly-directed Cl^- current prompting neuronal depolarization (Fig. 3). A negative shift in the GABA reversal potential (EGABA) is paralleled by a robust increase in KCC2 expression near the end of the second postnatal week in rat cortical neurons (Blaesse et al., 2009; Kahle et al., 2008). In the human cortex, KCC2 expression begins to increase at 40 weeks after conception. This expression profile of KCC2 has major implications for GABA signaling. siRNA reduction of KCC2 expression in pyramidal cells from rat hippocampus markedly shifts the reversal potential of GABA response, and complete knockout of *Slc12a5* results in early neonatal death secondary to apneic respiratory failure (Delpire and Mount, 2002). An *Slc12a5* hypomorphic mouse with 95% knockout lived for a maximum of 17 days. These mice experience frequent, massive seizures triggered by slight stimuli (Delpire and Mount, 2002). *Slc12a5* heterozygote animals exhibit no overt phenotype, but possess a lower threshold for pharmacologically-induced seizures. Reduced KCC2 expression in mouse models of intractable epilepsy and human patients with temporal lobe epilepsy is additional evidence for its role in regulation of neuronal excitability.

As with other KCCs, KCC2 has been shown to be inhibited by the WNK kinase pathway in oocytes, and a catalytically inactive form of WNK3 is a potent activator of KCC2, likely through inhibition of PP1 (Pacheco-Alvarez and Gamba, 2011). KCC2 is also modulated by WNK4 (Gagnon et al., 2006). Thus, WNKs are potential potent modulators of intraneuronal chloride concentration because of their reciprocal phosphoregulation of NKCC1 and KCC2 (Kahle et al., 2008; Pacheco-Alvarez and Gamba, 2011).

3.3. *Slc12a6* – *KCC3*

SLC12A6, is located in human chromosome 15, and gives rise two variants of KCC3 differing in the amino terminal domain due to the existence of two mutually exclusive cassette exons 1,KCC3a and KCC3b. KCC3a is expressed in brain, muscle, lung, erythrocytes and heart among other tissues and KCC3b is predominantly expressed in the kidney. Additionally, a truncated isoform of KCC3a has been described in rat neurons (Le Rouzic et al., 2006).

Inactivating mutations of *SLC12A6* are the cause of a rare and complex neurological disease known as peripheral neuropathy associated with agenesis of the corpus callosum (ACCPN or Anderman syndrome). *SLC12A6* mutations truncating part of the carboxyl terminal domain apparently impair the transit of the cotransporter to the plasma membrane (Salin-Cantegrel et al., 2011). The *Slc12a6* knockout model recapitulates the phenotypic characteristics of the syndrome, including peripheral and central nervous system neurodegeneration as well as a decreased seizure threshold (Delpire and Mount, 2002). Additionally, the *Slc12a6* null mouse model exhibits arterial hypertension, apparently secondary to increased sympathetic tone, and deafness via a degenerative process (Rust et al., 2006).

KCC3 has been shown to be regulated by two members of the WNK kinase family, WNK3 (Pacheco-Alvarez and Gamba, 2011) and WNK4 (Garzon-Muvdi et al., 2007), in a kinase dependent manner. Co-expression of KCCs with WNK3 or WNK4 result in prevention of the hypotonically induced activation of the cotransporters. Furthermore, there are two carboxyl terminal threonine residues (T-991 and T-1048) that are key for regulating KCC3 (Rinehart et al., 2009). When these threonines are phosphorylated, KCC3 remains inhibited and when exposed to hypotonicity, phosphorylation of these threonines is reduced, with the concomitant activation of the cotransporter. Supporting this conclusion, substitution of these threonines for alanine residues turns KCC3 to active in isotonic conditions. Interestingly, it was also shown that phosphorylated KCC3 is located at the plasma membrane, suggesting that exposure to hypotonicity results in dephosphorylation/activation of the KCC3 that is already present in the plasma membrane, rather than trafficking cotransporters from internal sources. Supporting this conclusion, dephosphorylation/activation of KCC4 also seems to occur in the cotransporter that is already in the plasma membrane (Bergeron et al., 2009).

Additional roles for KCC3 and particularly KCC3b involve the regulation of renal function and blood pressure. In this regard, a lack of KCC3 leads to decreased reabsorption in the proximal tubule pointing to KCC3 as a regulator of renal reabsorption mechanisms. Additionally, there is evidence that KCC3 is important in the maintenance of blood pressure as $SLC12A6^{-/-}$ animals present with neurogenic hypertension (Rust et al., 2006) and alterations in the NO pathway, along with reduced vasorelaxation that has been associated with mutations in *SLC12A6* (Adragna and Lauf, 2007).

An additional role for KCC3 has been identified. Over-expression of KCC3 is associated with tumor cell invasion and proliferation (Chen et al., 2010). It was first demonstrated that KCC3 regulates cell cycle progression. The observation that KCC3 is the most abundant isoform present in primary cervical carcinoma and correlates with tumor size, strongly suggests that expression of KCC3 is extremely important in cell growth regulation and thus plays a potential important role in cancer

growth and invasion. Furthermore, there is evidence that KCC3 plays a key role in the epithelial-mesenchymal transition required for malignant transformation through the down regulation of the E-cadherin/β-catenin complex.

3.4. *Slc12a7 – KCC4*

SLC12A7, located in human chromosome 5, encodes for KCC4 that is expressed in the kidney, heart, lung and liver. In the kidney expression is localized to the basolateral membrane of the proximal tubule and the α-intercalated cells of the collecting duct (Gamba, 2005). The similarity between KCC2 and KCC4 led to the identification of 4 conserved cysteine residues in the large-extracellular loop located between TM5 and TM6. Elimination of these residues completely abolished KCC2 activity, but did not affect KCC4 function (Hartmann et al., 2010) demonstrating that even highly conserved amino acids can have different functions among KCC family members. Another study explored functional differences to analyze structure–function relationships in KCCs. KCC2 is partially active in isotonic conditions in which KCC4 is completely inactive. Using a chimeric and point mutagenesis strategy, a stretch of 15 residues in the carboxyl terminal domain responsible for these differences was revealed (Mercado et al., 2006).

Recent studies report KCC3 and KCC4 expression in gastric parietal cells. KCC3 is co-expressed with sodium pump and thus is present in the basolateral membrane, while KCC4 is co-expressed with H^+/K^+ ATPase and is thus present in the apical membrane of gastric parietal cells. These results suggest that coordinated activation of KCCs is involved in modulating acid secretion in the stomach (Fujii et al., 2011). Supporting this hypothesis, it is known that KCC activity is modulated by acid base status. Mutations of both the H^+-ATPase (Karet et al., 1999) and KCC4 exhibited renal tubular acidosis (Boettger et al., 2002) pointing to KCC4 as an important regulator of $[Cl^-]_i$ recycling in α-intercalated cells and general acid base balance (Fig. 2), as well as deafness due to degeneration of the outer hair cells of the cochlea.

A role for KCC4 in tumor biology has also recently begun to be elucidated. KCC4 is associated with metastasis as cancer cell invasion increases with up-regulation of this cotransporter (Chen et al., 2010), purportedly through the regulation of the IGF-1 and EGF. By altering KCC4 trafficking and membrane expression, the IGF1 and EGF dependent cancer cell invasiveness was decreased. The identification of KCC3 and KCC4 as key players in tumor growth and invasiveness has put them at the forefront of possible pharmacological targets that could aid in the treatment of cancer.

4. The orphan members: SLC12A8 and SLC12A9

The *SLC12A8* gene is located in human chromosome 3 and encodes CCC9, a protein of 714 amino acid residues with a unique predicted membrane topology with a central core of eleven membrane spanning domains that may end in a glycosylated extracellular loop. *SLC12A8* has been identified as a psoriasis susceptibility gene by two independent groups (Hewett et al., 2002; Huffmeier et al., 2005). The function of this protein has not been clearly defined, but one report shows that a splicing variant of CCC9 translocates polyamines and aminoacids across the plasma membrane of HEK-293 cells, suggesting that could be a polyamine transporter (Daigle et al., 2009).

SLC12A9, located in human chromosome 7, encodes a 918 residue cotransporter originally defined as cotransporter interacting protein (CIP) for its ability to specifically interact with, and modulate the activity of, NKCC1 (Gamba, 2005). *SLC12A9* encodes a 914 amino acid protein with predicted 12 TM domains, two *N*-glycosilation sites between TM5 and TM6, and a carboxyl terminus PKC phosphorylation site. It topological similarity and the 25% identity with other members of the family suggest that its likely that CIP transport substrates that has not been identified.

5. Concluding comments

Members of the SLC12 family of solute cotransporters are involved in many different physiological processes, are the target for the most potent diuretics used today, and are the cause of inherited diseases in which blood pressure is affected. Future studies with the SLC12A family members could reveal important roles in further understanding the physiology and pathophysiological processes associated with brain electrical activity, blood pressure, and cell proliferation, making them interesting molecular targets for the development of new drugs aimed at correcting seizures, hypertension and cancer.

Conflict of interest

No conflicts of interest are declared.

Acknowledgments

We apologize to the authors of many studies on SLC12 cotransporters whose work was not directly discussed owing to the specific focus of this review. Due to limited references space, many original articles that already are discussed in the reviews we cite, were intentionally omitted to give space for more recent original work, not included in aforementioned reviews. Supported in part by the Wellcome Trust grant 091415, the Transatlantic Network on Hypertension funded by the

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Activation of the renal Na^+:Cl^- cotransporter by angiotensin II is a WNK4-dependent process

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Pseudohypoaldosteronism type II is a salt-sensitive form of hypertension with hyperkalemia in humans caused by mutations in the with-no-lysine kinase 4 (WNK4). Several studies have shown that WNK4 modulates the activity of the renal Na^+Cl^- cotransporter, NCC. Because the renal consequences of WNK4 carrying pseudoaldosteronism type II mutations resemble the response to intravascular volume depletion (promotion of salt reabsorption without K^+ secretion), a condition that is associated with high angiotensin II (AngII) levels, it has been proposed that AngII signaling might affect WNK4 modulation of the NCC. In *Xenopus laevis* oocytes, WNK4 is required for modulation of NCC activity by AngII. To demonstrate that WNK4 is required in the AngII-mediated regulation of NCC in vivo, we used a total WNK4-knockout mouse strain ($WNK4^{-/-}$). WNK4 mRNA and protein expression were absent in $WNK4^{-/-}$ mice, which exhibited a mild Gitelman-like syndrome, with normal blood pressure, increased plasma renin activity, and reduced NCC expression and phosphorylation at T-58. Immunohistochemistry revealed normal morphology of the distal convoluted tubule with reduced NCC expression. Low-salt diet or infusion of AngII for 4 d induced phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and of NCC at S-383 and T-58, respectively, in $WNK4^{+/+}$ but not $WNK4^{-/-}$ mice. Thus, the absence of WNK4 in vivo precludes NCC and SPAK phosphorylation promoted by a low-salt diet or AngII infusion, suggesting that AngII action on the NCC occurs via a WNK4-SPAK–dependent signaling pathway. Additionally, stimulation of aldosterone secretion by AngII, but not by a high-K^+ diet, was impaired in $WNK4^{-/-}$ mice.

distal tubule | diuretics | thiazide | renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system (RAAS) is a key modulator of blood pressure. The blockade of this system has proven to be effective in the treatment of arterial hypertension. Recent studies suggest that hypertension induced by angiotensin II (AngII) results primarily from the renal effects of this hormone. An elegant set of renal transplant experiments between wild-type and AngII type 1 (AT1) receptor-knockout mice has revealed that arterial hypertension induced by AngII requires the presence of AT1 receptors in the kidney but not in any other tissue (1) and that hypertension mostly is the consequence of an increase in renal salt reabsorption (2). To date, all monogenic diseases featuring altered blood pressure in which the causative gene has been uncovered have been found to be caused by mutations in genes encoding proteins that are either the effectors or the modulators of salt-reabsorption pathways (3). The study of one of such disease has revealed interesting molecular mechanisms for the regulation of distal nephron salt reabsorption.

Pseudohypoaldosteronism type II (PHAII), also known as "familial hyperkalemic hypertension" or "Gordon's syndrome," is an autosomal dominant disease featuring arterial hypertension and hyperkalemia with metabolic acidosis and hypercalciuria (4). PHAII is the mirror image of Gitelman's syndrome, an autosomal recessive disease exhibiting arterial hypotension, hypokalemic metabolic alkalosis, and hypocalciuria, which is caused by inactivating mutations of the renal thiazide-sensitive Na^+Cl^- cotransporter, NCC (5). One type of PHAII is caused by missense mutations in the with-no-lysine kinase 4 (WNK4) (6). Numerous studies in *Xenopus laevis* oocytes, Cos-7 cells, HEK-293 cells, and BAC transgenic mice have shown that WNK4 is a negative modulator of NCC activity that becomes an activator when its primary structure is changed by the PHAII- type mutations (for extensive review, see ref. 7). It has been shown that WNK4 interacts with and activates SPAK/OSR1 (8). The active WNK4–SPAK complex thus is able to phosphorylate and activate NCC, at least in part, by increasing its traffic to the plasma membrane (9–11). The ability of WNK4 to phosphorylate SPAK/OSR1, and thus, NCC could be a subject to modulation: An inactive state would result in the highjacking of nonphosphorylated NCCs, whereas an active state would result in phosphorylation and activation of NCC. This active state could be what PHAII mutations in WNK4 mimic (12). The observation that NCC activity is associated with its phosphorylation of N-terminal threonines 53 and 58 and serine 71 (13) opened the possibility to assess NCC "activity" in vivo indirectly using specific phospho antibodies (14).

In addition to increasing the activity of the NCC, the PHAII-mutant WNK4 increases activity of the apical epithelial sodium channel (ENaC) (15) and distal paracellular chloride transport (because of its action on claudins) (16, 17) while strongly inhibiting the renal outer medullary potassium channel (ROMK) (18). The combination of these effects induces salt reabsorption and prevents K^+ secretion, mimicking what occurs in the distal nephron during a low-salt diet or hypovolemia, conditions characterized by the activation of the RAAS. We thus proposed that PHAII-type mutations confer a gain of function to WNK4 that mimics the effect produced by AngII upon the WNK4–SPAK–NCC pathway (19). This hypothesis was supported by observations made in *Xenopus laevis* oocytes and murine distal convoluted tubule (mpkDCT) cells in which AngII induces a WNK4-SPAK–dependent increase in NCC phosphorylation and activity that can be prevented with the specific AT1 receptor blocker, losartan (19). The positive effect of AngII on NCC activity was suggested previously by Sandberg et al. (20), who demonstrated that AngII increases NCC trafficking to the apical plasma membrane in rat distal convoluted tubule (DCT) cells. Using adrenalectomized rats, Van der Lubbe et al. (21) reported that AngII increases NCC T53 and T58 phosphorylation, implying that AngII is able to activate the NCC by an aldosterone-independent mechanism. The goal of the present study was to

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use an in vivo model to determine whether WNK4 is required for the AngII-induced activation of SPAK and NCC.

Results

WNK4$^{-/-}$ Knockout Mice Exhibit a Mild Gitelman-Like Syndrome. From crosses of heterozygous WNK4$^{+/-}$ mice, wild-type, heterozygous, and homozygous mice were born at the expected Mendelian frequencies and showed normal growth and development (Table S1). WNK4$^{+/+}$ and WNK4$^{-/-}$ mice were identified by PCR assays on DNA from tail biopsies (Fig. S1*B*). The absence of WNK4 expression in the WNK4$^{-/-}$ mice was confirmed by real-time PCR and Western blot analysis (Fig. S1 *C* and *D*).

On a normal diet, WNK4$^{-/-}$ mice displayed mild hypokalemia, hypochloremia, metabolic alkalosis, and hypomagnesemia compared with wild-type littermates (Table 1). WNK4$^{-/-}$ mice showed greater urinary Na^+, K^+, and Cl^- excretion than wild-type mice. No difference was observed in urinary Ca^{2+} excretion. WNK4$^{-/-}$ mice exhibited a higher urinary volume, but no difference in body weight was observed. Thus, WNK4$^{-/-}$ mice did not appear to be dehydrated (Table 1). The ability to concentrate urine was similar in wild-type and WNK4$^{-/-}$ mice, as suggested by a dehydration test (Fig. S2). Hence, polyuria was probably the consequence of an increased thirst drive in the WNK4$^{-/-}$ mice, perhaps because of hypokalemia (22).

Similar systolic blood pressure was observed in the wild-type and WNK4$^{-/-}$ groups (117.2 ± 2.6 vs. 116.8 ± 3.2 mmHg; P = NS) (Fig. 1*A*); however, higher levels of plasma renin activity (PRA) were observed in the WNK4$^{-/-}$ mice (24.7 ± 1.8 in ng·mL^{-1}·h^{-1} WNK4$^{-/-}$ mice vs. 15.7 ± 1.3 ng·mL^{-1}·h^{-1} in WNK4$^{+/+}$; $P < 0.05$) (Fig. 1*B*). Interestingly, aldosterone levels in the two groups were similar despite elevated PRA (Fig. 1*C*). Thus, the phenotype of WNK4$^{-/-}$ mice is reminiscent of a mild Gitelman syndrome without arterial hypotension and hypocalciuria.

NCC Expression, Phosphorylation, and Activity Are Reduced in WNK4$^{-/-}$ Mice. Under basal conditions, the expression levels of NKCC2, Nedd4-2, OSR1, and SPAK were similar for WNK4$^{+/+}$ and WNK4$^{-/-}$ mice. Additionally, levels of SPAK phosphorylation at the T loop and S motif of the kinase (T-243 and S-383, respectively) were similar in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice (Fig. 2*A*). In contrast, NCC expression and phosphorylation at T-58 were markedly lower in WNK4$^{-/-}$ mice (~10% of wild-type levels for NCC and practically undetectable for phosphorylated NCC). Phosphorylation of T-58 has been shown to be critical for cotransporter activity (13, 14). NCC expression also was lower at the mRNA level (44% of wild-type expression level) (Fig. 2*B*). Immunohistochemistry of kidney sections confirmed the lower levels of phosphorylated NCC. No differences in number, apical surface, or cell size were detected in NCC-positive tubules (Fig. 2*C* and Table S2).

Decreased NCC activity was confirmed by showing that the natriuretic effect of hydrochlorothiazide was almost abolished in WNK4$^{-/-}$ mice (Fig. 2*D*). Na^+ excretion in the WNK4$^{+/+}$ mice was decreased between 6–24 h after thiazide injection; this decrease is explained as the response elicited after a period of loss (0–6 h) to restore the animal's Na^+ store. As expected, this decrease was not observed in the WNK$^{-/-}$ mice. Interestingly, the inhibition of NCC was compensated, at least in part, by activation of ENaC in downstream nephron segments, as demonstrated in vivo by the increased natriuretic effect of amiloride in WNK4$^{-/-}$ mice (Fig. 2*E*) and in vitro by the increased rate of amiloride-sensitive sodium reabsorption measured in microperfused kidney collecting ducts (CCDs) (Fig. 2*F*). In addition, the increased rate of K^+ excretion (Table 1) was consistent with activation of ENaC.

Table 1. Plasma and urine electrolytes of WNK4$^{+/+}$ and WNK4$^{-/-}$ mice on a normal diet

Electrolyte	WNK4$^{+/+}$	WNK4$^{-/-}$
Plasma		
Na^+ (mM)	152.61 ± 0.53 (15)	153.19 ± 1.06 (19)
K^+ (mM)	4.00 ± 0.08 (15)	3.46 ± 0.1 (19)***
Cl^- (mM)	118.24 ± 0.52 (15)	113.92 ± 1.28 (19)*
CO_2 (mM)	13.93 ± 0.65 (15)	16.02 ± 0.58 (19)*
Ca^{2+} (mg/dL)	8.43 ± 0.09 (15)	8.38 ± 0.15 (19)
Mg^{2+} (mg/dL)	2.31 ± 0.07 (15)	1.97 ± 0.07 (19)**
Creatinine (mg/dL)	0.15 ± 0.02 (15)	0.17 ± 0.02 (19)
Urine		
Na^+ (mmol/mmol Cr)	40.88 ± 1.78 (9)	49.07 ± 2.3 (7)*
K^+ (mmol/mmol Cr)	70.44 ± 5.87 (9)	109.27 ± 5.03 (7)**
Cl^- (mmol/mmol Cr)	58.82 ± 4.12 (9)	81.42 ± 3.36 (7)**
Ca^{2+} (mg/mg Cr)	0.11 ± 0.01 (9)	0.11 ± 0.014 (7)
Mg^{2+} (mg/mg Cr)	1.24 ± 0.09 (9)	1.66 ± 0.21 (7)
Creatinine (mg/dL)	37.7 ± 6.26 (9)	18.64 ± 2.25 (7)*
Urinary volume (mL)	0.84 ± 0.11 (6)	1.98 ± 0.10 (10)***
Creatinine clearance (mL/min)	0.25 ± 0.07 (8)	0.18 ± 0.06 (6)
Weight (g)	25.94 ± 2.57 (19)	25.32 ± 0.61 (13)
Food intake (g)	3.07 ± 0.15 (9)	3.32 ± 0.034 (7)
Water intake (mL)	3.83 ± 0.64 (6)	5.25 ± 0.43 (10)

Values are presented as the mean ± SE. The number of animals per group (n) is given in parentheses. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

Response of WNK4$^{-/-}$ Mice to Low-Salt Diet. During a low-salt diet, urinary Na^+ excretion was reduced similarly in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice (Fig. S3*A*). No difference in Ca^{2+} excretion was observed at any time point during the experiment (Fig. S3*B* but increased Cl^- and K^+ excretion were observed at several time points (Fig. S3 *C* and *D*). No differences were observed in systolic blood pressure. At days 0 and 4, blood pressure in WNK4$^{+/+}$ mice was 104 ± 5 and 106 ± 11 mmHg, respectively and in WNK4$^{-/-}$ mice was 112 ± 2 and 108 ± 2 mmHg, respectively (P = NS). Thus, the ability to reach Na^+ balance was not affected in the WNK4$^{-/-}$ mice, suggesting that the increased activity of ENaC in the connecting tubule and/or collecting duct (Fig. 2) probably is sufficient to allow the WNK4$^{-/-}$ mice to reach balance.

As previously reported (23), the low-salt diet promoted an increase in NCC expression and phosphorylation in the WNK4$^{+/+}$ mice (Fig. 3*A*). Total levels of SPAK remained similar, but SPAK phosphorylation increased with the low-salt diet in the WNK4$^{+/+}$ mice. In contrast, increased expression and/or phosphorylation of the NCC or SPAK were not observed in WNK4$^{-/-}$ mice exposed to the low-salt diet. The absence of an increase in NCC phosphorylation in WNK4$^{-/-}$ mice was not due to the complete absence of NCC protein, as demonstrated in Figs. 2*C* and 3*B*.

NCC Response to AngII Infusion Is Impaired in WNK4$^{-/-}$ Mice. Mice were infused with AngII at a nonpressor dose (400 μg·kg^{-1}·d^{-1}) (24) for 4 d through miniosmotic pumps. As expected, no changes were observed in blood pressure in either WNK4$^{+/+}$ or WNK4$^{-/-}$ mice. Correct hormone infusion was confirmed by measuring AngII levels in the urine. As observed in previous reports (21), AngII induced an increase in NCC (T-58) and SPAK (S-383) phosphorylation in the WNK4$^{+/+}$ mice (379 ± 94% and 217 ± 13%, respectively; $P < 0.05$ relative to vehicle-infused mice) (Fig. 4*A*). Total NCC and SPAK protein increased slightly, but the difference did not reach significance (Fig. 4*A*). The effect of AngII infusion on NCC phosphorylation was observed even in the context of mineralocorticoid receptor blockade achieved through treatment with spironolactone (Fig. 4*B*). This result is consistent with previous observations (21) suggesting that AngII promotes NCC phosphorylation in an aldosterone-independent manner. Interestingly, no such increases in NCC or SPAK phosphorylation were observed in WNK4$^{-/-}$ mice (Fig. 4*A*). Notably, although SPAK expression was normal in the WNK4$^{-/-}$ mice, AngII did not induce its phosphorylation. In agreement with these data, AngII infusion decreased urinary salt excretion at day 1 in WNK4$^{+/+}$ mice but not in WNK4$^{-/-}$ mice (Fig. 4*C*). The difference in the response to AngII between

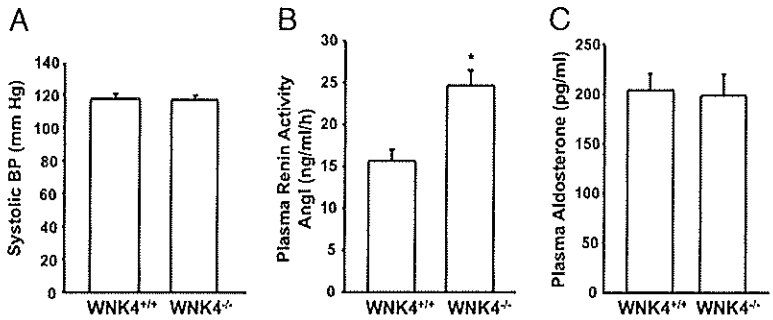

Fig. 1. Normal systolic blood pressure (BP) (*A*) with increased PRA (*B*) and normal aldosterone (*C*) in WNK4$^{-/-}$ mice. Values are presented as the mean ± SE; *P < 0.001 vs. wild type.

WNK4$^{+/+}$ and WNK4$^{-/-}$ mice was not caused by altered AT1 expression in the WNK4$^{-/-}$ mice kidney (Fig. 4*D*). Intriguingly, AngII infusion did not promote the expected increase in plasma aldosterone concentration in the WNK4$^{-/-}$ mice, which was indeed observed in the WNK4$^{+/+}$ mice (Fig. 4*E*).

Response of WNK4$^{-/-}$ Mice to Changes in Potassium Intake. Mice were fed with normal (1.2%), low- (0%), or high- (5%) K^+ diets for a period of 4 d. At the end of this period, plasma K^+ levels remained within physiological limits in WNK4$^{+/+}$ mice on low- and high-K^+ diets. In contrast, plasma K^+ levels were lower in WNK4$^{-/-}$ mice kept on a normal diet (3.26 ± 0.17 mM vs. 3.99 ± 0.12 mM in wild-type mice). Hypokalemia was aggravated further in WNK4$^{-/-}$ mice subjected to a low-K^+ diet (2.03 ± 0.11 mM vs. 3.44 ± 0.2 mM in wild-type mice). On a high-K^+ diet, the difference between genotypes in plasma K^+ no longer was observed (Fig. 5*A*). In contrast to observations with AngII, the high-K^+ diet increased the plasma aldosterone concentration in both WNK4$^{+/+}$ (866 ± 135 pg/mL vs. 232 ± 33 pg/mL on a normal diet) and WNK4$^{-/-}$ mice (1,467 ± 343 pg/mL vs. 234 ± 30 pg/mL on a normal diet) (Fig. 5*B*).

Discussion

In this study, we characterized a WNK4-total knockout mouse strain to define the role of this kinase in renal ion handling. Surprisingly, the total absence of WNK4 resulted in an incomplete form of Gitelman's disease with mild hypokalemia, metabolic alkalosis, hypochloremia, and hypomagnesemia but without hypocalciuria or arterial hypotension. Biochemical analysis revealed a significant decrease in the expression, phosphorylation, and activity of NCC without changes in NKCC2, Nedd4-2, SPAK, or OSR1 expression. Sodium balance was maintained, at least in part, through increased activity of ENaC in the distal nephron.

Surprisingly, different phenotypes similar to Gitelman result from two opposite WNK4 models: the complete absence (this study) or an overexpression of WNK4 (11). Absence of WNK4 results in hypokalemia, metabolic alkalosis, hypochloremia, and hypomagnesemia without changes in blood pressure or urinary Ca^{2+} excretion (Table 1). In contrast, overexpression of WNK4 leads to hypotension with hypocalciuria without changes in the other electrolytes (11). Intriguingly, NCC is reduced in both models. The difference could be caused by the consequences of the absence versus overexpression of WNK4 in other renal transport pathways. In the absence of WNK4, ENaC is activated

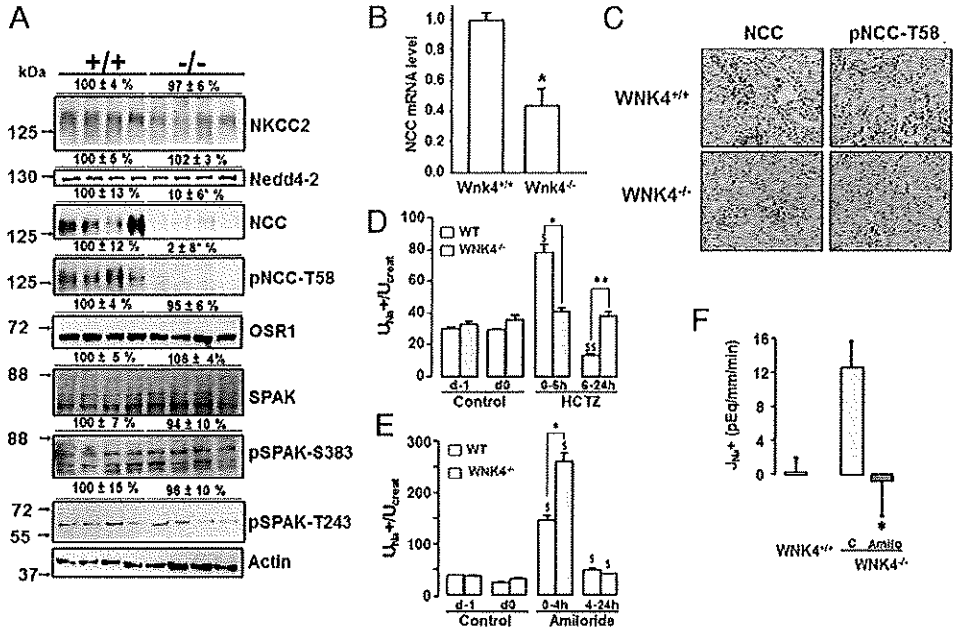

Fig. 2. Effects of WNK4 absence on distal nephron Na^+ transport pathways. (*A*) Western blot analysis of kidney protein samples of WNK4$^{+/+}$ and WNK4$^{-/-}$ mice on a normal diet. Representative blots are shown for each protein analyzed. The numbers displayed are the results of the densitometric analysis of at least two blots per assay, expressed as a percentage ± SE of wild type (100%). *P < 0.0001 vs. wild type. (*B*) NCC expression at the mRNA level measured through real-time PCR. RNA samples extracted from WNK$^{+/+}$ (n = 12) or WNK4$^{-/-}$ (n = 10) kidneys were used. Values shown are the relative abundance of NCC mRNA to 18S mRNA. (*C*) Immunohistochemical analysis of NCC and phosphorylated NCC (pNCC). Kidney slices from WNK4$^{+/+}$ and WNK4$^{-/-}$ mice were treated simultaneously with the same antibody and detection solutions. (Magnification: 400×.) (*D* and *E*) Effect of (*D*) hydrochlorothiazide (HCTZ) and (*E*) amiloride on Na^+ excretion. Urinary excretion of Na^+ before (days −1 and 0) and after a single administration at time 0 of HCTZ (50 mg/kg body weight, i.p.) (*D*) or amiloride (5 mg/kg body weight, i.p.) (*E*) to WNK4$^{+/+}$ mice (open bars) and WNK4$^{-/-}$ mice (gray bars). n = 6 mice per group except in the WNK4$^{+/+}$ amiloride group (n = 9), *P < 0.05 and **P < 0.005 vs. WNK4$^{+/+}$; $^{\$}P$ < 0.005 and $^{\$\$}P$ < 0.001 vs. control. (*F*) CCDs from WNK4$^{-/-}$ mice display amiloride-sensitive Na^+ reabsorption. Sodium flux (JNa^+) in microperfused CCDs from WNK$^{+/+}$ mice (n = 6) and WNK4$^{-/-}$ mice (n = 4) treated or not treated with 10 μM amiloride (amilo). *P < 0.05 vs. CCDs not treated with amiloride. All data are presented as the mean ± SE.

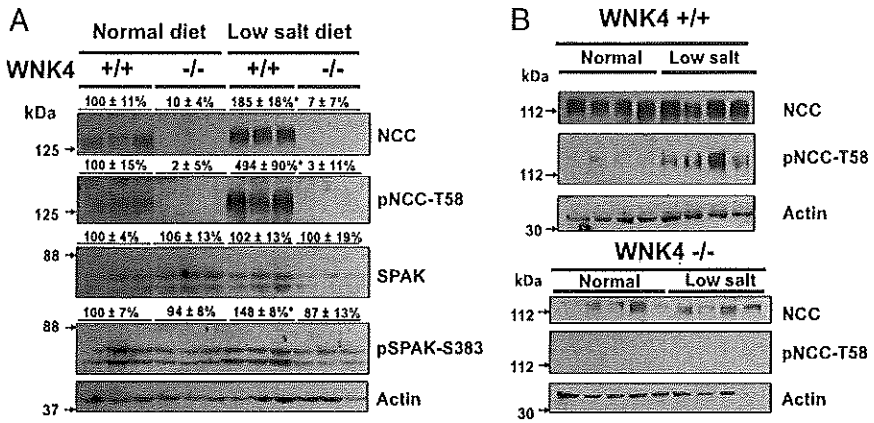

Fig. 3. NCC expression and phosphorylation are not stimulated in WNK4$^{-/-}$ mice on a low-salt diet. (*A*) Representative Western blots of kidney proteins from WNK4$^{+/+}$ and WNK4$^{-/-}$ mice on a normal or low-salt diet (4 d). The results from the densitometric analysis in which at least three blots were included per assay and at least six samples from different mice were analyzed per group are shown above each blot as the mean ± SE. $P < 0.0005$ vs. normal diet. Two bands are seen using the SPAK antibodies. These bands were reported previously in kidney (36) and represent isoforms of SPAK (34). Thus, both bands were used for the densitometric analysis. (*B*) WNK4$^{+/+}$ and WNK4$^{-/-}$ mice were studied separately to allow longer exposure times during the film scan for the WNK4$^{-/-}$ blots so that NCC expression could be detected. No increase with a low-salt diet was observed. NCC phosphorylation (at T58) could not be detected even on a low-salt diet.

(Fig. 2 *E* and *F*), and, presumably, ROMK is activated also [WNK4 is an inhibitor of this channel (18)], further contributing to the K^+ wasting already expected from the decreased NCC activity. Increased ENaC activity probably is sufficient to compensate for NCC deficiency in terms of Na^+ reabsorption; thus, these animals are normotensive. In contrast, WNK4 overexpression will exert some degree of inhibition not only on NCC but also upon ENaC and ROMK (15, 18). Thus, K^+ (and H^+) wasting in response to NCC loss probably is counteracted by a decreased activity of ENaC/ROMK, preventing important urinary K^+ losses. However, decreased Na^+ reabsorption by NCC cannot be compensated by ENaC, leading to hypotension. Regarding hypocalciuria, WNK4 has been shown to increase TRPV5 activity (25). Thus, an overexpression of WNK4 would increase Ca^{2+} reabsorption in the DCT, leading to hypocalciuria.

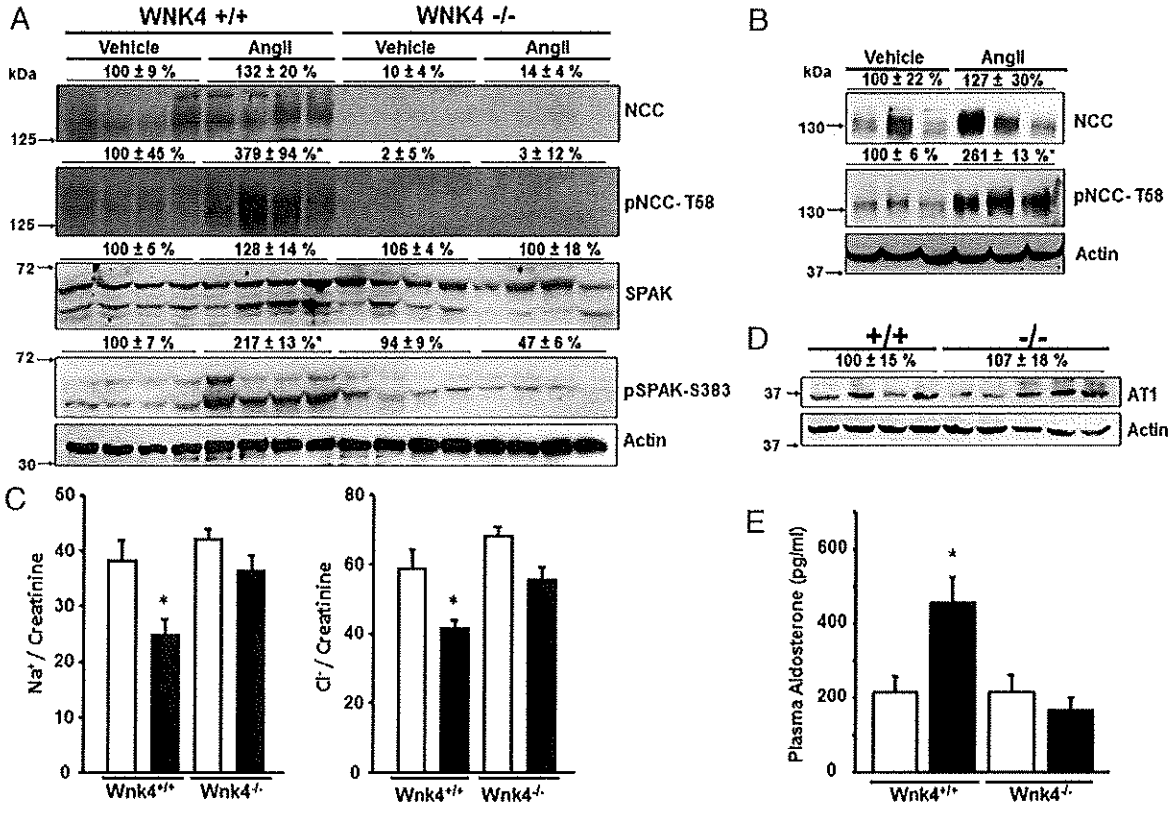

Fig. 4. Response to AngII infusion is altered in WNK4$^{-/-}$ mice. (*A*) Representative Western blots of kidney proteins from WNK4$^{+/+}$ and WNK4$^{-/-}$ mice infused with vehicle or AngII (400 $\mu g \cdot kg^{-1} \cdot d^{-1}$). The results from densitometric analysis are expressed as percentages of wild-type levels above each blot (100%). At least two blots were included per assay, and at least six samples from different mice were analyzed per group. *$P < 0.05$ vs. vehicle. (*B*) NCC expression and phosphorylation (T58) in wild-type mice treated with spironolactone and infused with vehicle or AngII. For densitometric data shown above the blot, two blots were used for a total of six samples from per group. *$P < 0.05$ vs. vehicle. (*C*) Urinary Na^+ and Cl^- excretion of WNK4$^{+/+}$ ($n = 6$) and WNK4$^{-/-}$ ($n = 6$) mice at day 1 of infusion with vehicle (open bars) or AngII (black bars). Values are presented as the mean ± SE; *$P < 0.05$ vs. vehicle. (*D*) Kidney AT1 expression in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice. (*E*) Plasma aldosterone concentration (mean ± SE) of WNK4$^{+/+}$ and WNK4$^{-/-}$ mice infused with vehicle (open bars) or AngII (black bars). *$P < 0.05$ vs. vehicle. Measurements were performed in duplicate. Samples from six different animals per group were studied.

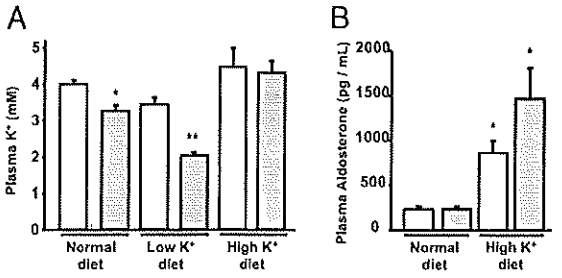

Fig. 5. Response of WNK4$^{-/-}$ mice to changes in dietary K^+ content. (*A*) Plasma K^+ concentration in WNK4$^{+/+}$ (open bars) and WNK4$^{-/-}$ (gray bars) mice kept on a normal, low-, or high-K^+ diet. Samples from nine different animals were studied per group, except for the WNK4$^{+/+}$ normal-diet group (n = 8) and WNK4$^{-/-}$ low-K^+ group (n = 10). Values are presented as the mean ± SE; $^*P < 0.005$ and $^{**}P < 0.00005$ vs. WNK4$^{+/+}$. (*B*) Plasma aldosterone concentration of WNK4$^{+/+}$ (open bars) and WNK4$^{-/-}$ (gray bars) mice subjected to a normal or high-K^+ diet. From left to right, n = 7; n = 10; n = 8; n = 9; $^*P < 0.005$ vs. normal diet.

The effect of WNK4 harboring PHAII-type mutations on the distal nephron ion transport systems (NCC, ENaC, ROMK, Claudins) resembles what occurs during a low-salt diet or hypovolemia (26) in which the RAAS is activated. Thus, we proposed in a previous study that AngII could be a hormonal signal involved in switching WNK4 to the functional state promoting NCC-ENaC activation, with increased ROMK inhibition stimulating volume retention without K^+ wasting. This hypothesis was supported by observations in *Xenopus* oocytes in which WNK4 was required for AngII to increase the activity of NCC in a SPAK-dependent fashion and in mpkDCT cells in which AngII induced SPAK and NCC phosphorylation (19). Additionally, AngII increased the surface expression and phosphorylation of NCC by an aldosterone-independent mechanism in the distal tubule of rats (20, 21, 27). Supporting this proposal, we observed that renal sodium excretion and blood pressure were normal in WNK4$^{-/-}$ mice, but the activity of AngII was increased, constituting the expected physiological response when a component of a negative feedback system is lost. Additionally, our data confirmed that absence of WNK4 precluded the increase in NCC and SPAK phosphorylation induced by a low-salt diet or AngII infusion, suggesting that a WNK4–SPAK complex is part of the pathway through which AngII induces activation of NCC in the DCT.

These results may be controversial because of the low expression of NCC, even though we showed that NCC expression is not lost completely (Fig. 3*C*). Nevertheless, observations on SPAK help us resolve this issue. Under basal conditions, SPAK expression and phosphorylation in the kidney were similar in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice. However, a low-salt diet or AngII infusion increased SPAK phosphorylation in the WNK4$^{+/+}$ mice but not in the WNK4$^{-/-}$ mice (Figs. 3 and 4), strongly supporting the idea that WNK4 is implicated in SPAK and therefore NCC activation through AngII. The conservation of basal SPAK phosphorylation in the WNK4$^{-/-}$ mice suggests that kinases other than WNK4 mediate this phosphorylation. SPAK phosphorylation, however, cannot be traduced into NCC activation. Thus, a WNK4–SPAK–NCC complex apparently is required for SPAK to phosphorylate NCC.

As discussed above, PHAII mutations in WNK4 may be mimicking the NCC-activating state of WNK4 that is induced by AngII. In this scenario, WNK4 harboring PHAII mutations behaves as if AngII were constitutively acting upon the WNK4–SPAK–NCC pathway, activating NCC in the DCT and inhibiting ROMK in the collecting duct (28), resulting in hypertension and hyperkalemia. If so, the NCC-inhibiting and NCC-activating forms of the WNK4–SPAK–NCC complex could coexist under physiological conditions, with the ratio of the two depending on the activity of the RAAS. A recent study supports this possibility by showing that the NCC phosphorylation by the WNK4–SPAK/OSR1 complex is modulated by calcium concentration in the wild-type WNK4 but not in the PHAII-mutant WNK4 (12). An increase in calcium concentration shifts the kinase activity of WNK4 toward SPAK/OSR1–NCC into high gear. Accordingly, at low calcium, WNK4 may have an inhibitory effect on NCC, because the complex is less active, high-jacking nonphosphorylated NCCs, whereas at higher calcium levels NCC becomes active as the ability of the complex to phosphorylate SPAK/OSR1 and NCC increases. The PHAII mutations in the acidic motif of WNK4 disrupt the Ca^{2+}-sensing mechanism, probably locking the kinase at the state induced by elevated calcium.

The observation that both the absence (in this study) and the overexpression of WNK4 (11) lead to decreased mRNA levels of NCC is intriguing and reveals that modulation of the *SLC12A3* gene transcription rate/mRNA stability is another poorly studied pathway for WNK4 regulation of NCC activity. A recent work shows that variations in salt diet in rats induce opposite effects in NCC and WNK4 mRNA levels (29). Interestingly, contradictory observations also exist in adult mice in which WNK4 expression is changed by pharmacologic interventions. Norepinephrine induces salt-sensitive hypertension with increased NCC expression and phosphorylation associated with WNK4 down-regulation (30). In contrast, tacrolimus induces salt-sensitive hypertension with increased NCC phosphorylation but is associated with WNK4 up-regulation (31). These results imply that in wild-type mice similar effects on NCC can also result from opposite changes in WNK4. These two models are expected to differ in RAAS activity because norepinephrine is known to stimulate renin secretion directly (32). Additionally, WNK4 may have other pathways for NCC and ion transport system regulation in the kidney. For instance, it is known that WNK4 inhibits the transient receptor potential canonical 3 channel, modulating the vascular tone, which in turn could modulate pressure natriuresis mechanisms (33).

In this study, we observed in the basal state that, although PRA was higher in WNK4$^{-/-}$ mice, aldosterone levels were similar in WNK4$^{+/+}$ and WNK4$^{-/-}$ mice, suggesting a deficient response of the adrenal glands to AngII. This notion was supported by the observation that AngII infusion resulted in more than a twofold increase in plasma aldosterone in WNK4$^{+/+}$ mice but did not induce an increase in aldosterone in the WNK4$^{-/-}$ mice. However, a high-K^+ diet increased aldosterone secretion in both genotypes. These observations suggest that WNK4 may be implicated in the intracellular pathway through which AngII regulates aldosterone secretion. In this regard, SPAK-knockout mice also display decreased aldosterone secretion in the context of increased AngII levels, suggesting that SPAK is implicated in aldosterone secretion (34). Further investigation is required to clarify this issue.

In conclusion, the present study shows that total absence of WNK4 in mice is associated with an impaired ability of low-salt diet or AngII to promote phosphorylation of SPAK and NCC, suggesting that in the DCT the AngII-positive effect on the NCC is a WNK4-SPAK–dependent process.

Methods

Animal Studies. All experiments involving animals were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (35) and were approved by the Animal Care and Use Committee at our institutions. For every study performed, only male mice aged 12–16 wk were used.

Initial Characterization (Basal State). Mice were housed in metabolic cages with free access to food and water and were given a powder diet containing 0.49% NaCl (0.2% Na^+) for a period of 12 d during which three 24-h urine collections were performed. The first two collections were intended for adaptation to the cages. The results reported correspond to samples from the third collection. At the end of the experiment, mice were killed, and blood and kidneys were collected.

Salt-Balance Studies. Teklad custom normal diet (containing 0.49% NaCl) and NaCl-deficient diet (TD.96208 and TD.90228) were used. During a 4-d period, mice were given a powder diet (0.49% NaCl) and placed in metabolic cages daily for 2- to 3-h periods to adapt to the cages and diet. Then a 12-h urine

collection was performed while mice were on a normal diet. Mice then were switched to a NaCl-deficient diet, and four 12-h collections were performed over the next 4 d. The first collection began immediately after the diet was changed. All collections were used for urine analysis. Tail-cuff blood pressure measurements were done every day. At the end of the experiment, mice were killed, and blood and kidneys were collected.

Low- and High-Potassium Diets. Control (1.2% K^+), low- (0% K^+), and high- (5% K^+) potassium diets were obtained from TestDiet. Diets were prepared by modification of an AIN-93M semipurified diet. Tribasic potassium citrate was added to the 0% K^+ diet to make the 1.2% K^+ and 5% K^+ diets. After the 4-d period of adapting to the 1.2% K^+ powder diet, the diet was changed to 0% and 5% K^+ for some animals and kept at 1.2% K^+ for others. Four days later, mice were killed for urine and blood collection.

Response to Diuretics. Mice were acclimatized to individual metabolic cages (Techniplast) for at least 4 d before the study. Mice received a single dose of hydrochlorothiazide (50 mg/kg body weight, i.p.) or amiloride (5 mg/kg body weight, i.p.) after the first day. Then urine was collected for several periods (6- and 18-h urine collections for hydrochlorothiazide and 4- and 20-h urine collections for amiloride), and Na^+ and creatinine concentrations were determined.

Blood Pressure Measurement. Systolic blood pressure was measured when mice were awake using the noninvasive volume-pressure recording CODA system (Kent Scientific). For each mouse, sessions of 10 acclimation cycles and 20 measurement cycles were performed daily for a period of 5 d. Before this 5-d period, three measurements were performed over consecutive days for the purpose of adaptation. The blood pressure reported corresponds to the average of all the accepted measurement cycles according to software parameters.

AngII Infusion. Micro-osmotic pumps (Model 1007; Alzet, DURECT) were implanted s.c. to infuse AngII at a rate of 280 $ng \cdot kg^{-1} \cdot min^{-1}$ (400 $\mu g \cdot kg^{-1} \cdot d^{-1}$), a dose previously reported to lack pressor effects (24). The infusion lasted 4 d, during which two 24-h urine collections were performed, one starting the day after implantation and the second starting 24 h before the animal was killed. After the animals were killed, blood and kidneys were collected. Some animals were treated with spironolactone during the infusion period. Spironolactone was dissolved in olive oil and administered s.c. at 50 $mg \cdot kg^{-1} \cdot d^{-1}$.

Details on the generation of WNK4-knockout mice, immunoblot assays, immunohistochemistry, real-time PCR, in vitro microperfusion, and statistical analysis are provided in *SI Methods*.

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N-terminal Serine Dephosphorylation Is Required for KCC3 Cotransporter Full Activation by Cell Swelling*

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Background: KCC3 lacking the two known phosphorylation sites is still regulated by cell swelling and WNK3.
Results: Dephosphorylation of serine 96 is necessary for full activation of the cotransporter.
Conclusion: Serine 96 is a third phospho-site involved in KCC3 regulation.
Significance: The finding of new phosphorylation sites sheds light on an increasingly complex regulation of K^+:Cl^- cotransporters.

The K^+:Cl^- cotransporter (KCC) activity is modulated by phosphorylation/dephosphorylation processes. In isotonic conditions, KCCs are inactive and phosphorylated, whereas hypotonicity promotes their dephosphorylation and activation. Two phosphorylation sites (Thr-991 and Thr-1048) in KCC3 have been found to be critical for its regulation. However, here we show that the double mutant KCC3-T991A/T1048A could be further activated by hypotonicity, suggesting that additional phosphorylation site(s) are involved. We observed that *in vitro* activated STE20/SPS1-related proline/alanine-rich kinase (SPAK) complexed to its regulatory MO25 subunit phosphorylated KCC3 at Ser-96 and that in *Xenopus laevis* oocytes Ser-96 of human KCC3 is phosphorylated in isotonic conditions and becomes dephosphorylated during incubation in hypotonicity, leading to a dramatic increase in KCC3 function. Additionally, WNK3, which inhibits the activity of KCC3, promoted phosphorylation of Ser-96 as well as Thr-991 and Thr-1048. These observations were corroborated in HEK293 cells stably transfected with WNK3. Mutation of Ser-96 alone (KCC3-S96A) had no effect on the activity of the cotransporter when compared with wild type KCC3. However, when compared with the double mutant KCC3-T991A/T1048A, the triple mutant KCC3-S96A/T991A/T1048A activity in isotonic conditions was significantly higher, and it was not further increased by hypotonicity or inhibited by WNK3. We conclude that serine residue 96 of human KCC3 is a third site that has to be dephosphorylated for full activation of the cotransporter during hypotonicity.

The K^+:Cl^- cotransporters (KCCs)[4] constitute a branch of the electroneutral cation-coupled chloride cotransporter family SLC12 composed of four members: KCC1 to KCC4. Following the driving force imposed by the Na^+:K^+:ATPase, the KCCs translocate ions from the inside to the outside of the cell participating in fundamental physiological processes such as regulatory volume decrease, trans-epithelial ion transport, and reduction of intracellular chloride concentration. Erythroid function and differentiation, cancer cell growth and invasiveness, arterial blood pressure regulation, and neuronal excitability are some of the important roles of these membrane proteins (1–3). Inactivating mutations of the *Slc12A6* gene encoding KCC3 are the cause of a complex neurological disease known as agenesis of the corpus callosum with peripheral neuropathy, also referred as Andermann syndrome (Online Mendelian Inheritance in Man (OMIM) 218000) (4–6). Moreover, in addition to reproducing this disease, the KCC3 knock-out mouse model also develops arterial hypertension (7).

The other branch of the SLC12 family is composed of the Na^+-coupled chloride cotransporters, generally called N(K)CCs, that following the driving force imposed by the Na^+:K^+:ATPase translocate ions from the outside to the inside of the cell, thus having similar important roles as the KCCs in many physiological aspects, but in the opposite direction (1). As expected, NKCC and KCC activity is reciprocally regulated. Phosphorylation promoted by cell shrinkage, intracellular chloride depletion, or protein phosphatase inhibitors increases NKCCs and reduces KCC activity, whereas, dephosphorylation

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[4] The abbreviations used are: KCC, K^+:Cl^- cotransporter; N(K)CC, Na^+-coupled chloride cotransporter; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; WNK, with no lysine kinase; SPAK, STE20/SPS1-related proline/alanine-rich kinase.

associated with cell swelling, intracellular chloride accumulation, or protein phosphatases decreases NKCC function and triggers KCCs (8).

For NKCCs, a cluster of 3–5 highly conserved threonine/serine residues, localized in the N-terminal domain, has been identified as a key regulator of the cotransporter activity (9, 10). Phosphorylation of these sites by kinases SPAK and oxidative stress-responsive kinase 1 (OSR1) in response to osmotic stress increases their activity. In contrast, for the KCC branch, two threonine residues at the C-terminal domain have been shown to be critical for KCC3 activity regulation (11). Phosphorylation of these sites renders the cotransporter inactive, and it becomes active after dephosphorylation. Here we show, however, that the N-terminal domain serine 96 of human KCC3a fulfills the characteristics for a third phosphorylation site involved in regulation of this cotransporter.

EXPERIMENTAL PROCEDURES

Mutagenesis and Constructs—Mutant constructs were prepared with the QuikChange mutagenesis system (Stratagene) and custom-made primers (Sigma). All mutations were confirmed by sequencing and subcloned back into the appropriate expression constructs.

Functional Expression of KCCs—We assessed the activity of wild type or mutant KCC3 or KCC4 using the heterologous expression system of *Xenopus laevis* oocytes as described previously (12–15). In brief, mature oocytes were injected with wild type or mutant KCC3 or KCC4 cRNA at 10 ng/oocyte, and 3 days later, the activity of the cotransporter was determined by assessing the Cl^--dependent, $^{86}Rb^+$ uptake in isotonic or hypotonic conditions. cRNA for injection was transcribed *in vitro* from KCCs cDNA linearized at the 3′ using the T7 RNA polymerase mMESSAGE kit (Ambion). All experimental data are based on a minimum of three different experiments. Our institutional committee on animal research approved the use of *X. laevis* frogs.

Expression and Purification of GST-tagged SPAK DA, KCC3a(1–175), and NKCC2(1–174) in Escherichia coli—All pGEX-6P-1 constructs were transformed into BL21 *E. coli* cells, and 1-liter cultures were grown at 37 °C in LB medium (100 μg/ml ampicillin) until the absorbance at 600 nm was 0.8. Isopropyl β-D-thiogalactopyranoside (30 μM) was then added, and the cultures were grown for further 16 h at 26 °C. Cells were isolated by centrifugation, resuspended in 40 ml of ice-cold lysis buffer, and sonicated (Branson Digital Sonifier; ten 15-s pulses with a setting of 45% amplitude) to fragment DNA. Lysates were centrifuged at 4 °C for 15 min at 26,000 × *g*. The GST fusion proteins were affinity-purified on 0.5 ml of glutathione-Sepharose and eluted in buffer A containing 0.27 M sucrose and 20 mM glutathione.

KCC3a Phosphorylation Site Mapping by SPAK—GST-KCC3a(1–175) and GST-NKCC2(1–174) (10 μg) purified from *E. coli* were incubated with active SPAK (Carna Biosciences STLK3 (STK39), product number: 07-130) (1 μg) or kinase-inactive SPAK GST-SPAK(D212A) (1 μg) also purified from *E. coli* at 30 °C for 60 min in buffer A containing 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (~15,000 cpm/pmol) in a total reaction volume of 25 μl. The reaction was terminated by the addition of LDS sample buffer. Dithiothreitol (DTT) was added to a final concentration of 10 mM, and the samples were heated at 95 °C for 4 min and cooled for 20 min at room temperature. Iodoacetamide was then added to a final concentration of 50 mM, and the samples were left in the dark for 30 min at room temperature to alkylate cysteine residues. The samples were subjected to electrophoresis on a Bis-Tris 10% polyacrylamide gel, which was stained with colloidal blue and then autoradiographed. The phosphorylated GST-KCC3a(1–175) and GST-NKCC2(1–174) bands were excised, cut into smaller pieces, and washed sequentially for 10 min on a vibrating platform with 1 ml of the following: water; 1:1 (v/v) mixture of water and acetonitrile; 0.1 M ammonium bicarbonate; 1:1 mixture of 0.1 M ammonium bicarbonate and acetonitrile; and finally acetonitrile. The gel pieces were dried and incubated for 16 h at 30 °C in 25 mM triethylammonium bicarbonate containing 5 μg/ml trypsin as described previously (10, 16). Following tryptic digestion, >95% of the ^{32}P radioactivity incorporated in the gel bands was recovered, and the samples were chromatographed on a Vydac 218TP5215 C_{18} column (Separations Group, Hesperia, CA) equilibrated in 0.1% trifluoroacetic acid in water. The column was developed with a linear acetonitrile gradient (see Fig. 2*B*, *diagonal line*) at a flow rate of 0.2 ml/min, and fractions of 0.1 ml were collected.

Phospho-peptide Sequence Analysis—Isolated phospho-peptides were analyzed by LC-MSMS on a Thermo Scientific Orbitrap Classic. Raw files were processed using the Raw2msm application, and the resultant data were searched against an in-house database using Mascot search engine software. The site of phosphorylation of all the ^{32}P-labeled peptides was determined by solid-phase Edman degradation on an Applied Biosystems 494C sequencer of the peptide coupled to Sequelon-AA membrane (Applied Biosystems) as described previously (17).

Antibodies—The following antibodies were raised in sheep and affinity-purified on the appropriate antigen: KCC3 total (residues 1–175 of human KCC3a), KCC3 phospho-Ser-96 (residues 80–94 or 89–103 of human KCC3a), KCC3 phospho-Thr-982 (residues 975–989 or 984–998 of human KCC3a), and KCC3 phospho-Thr-1039 (residues 1032–1046 or 1041–1055 of human KCC3a). Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Pierce. Specificity of phospho-antibodies was assessed using protein extracts from HEK293 transfected with KCC3 cDNA.

Stable and Transient Cell Lines and Cell Culture—A stably transfected HEK293 cell line developed for a previous study (19) that had pCDNA3.1 expression vector with the open reading frame of full-length human with no lysine kinase 3 (WNK3) was used to transiently transfect full-length human KCC3a. To generate the transient cell lines, pre-cultures of HEK293-WNK3 underwent passages every 2–3 days to keep the cells in their exponential growth phase. 2 h before transfection, the cell suspension was centrifuged (300 × *g* for 5 min), resuspended in fresh medium at 5 × 10^6 cell/ml, and plated on 60-mm diameter dishes (60% confluence). Then, cells were transfected at 37 °C with the plasmid DNA or empty vector using the calcium phosphate method. Briefly, 5 μg of the full-length KCC3a was suspended in $CaCl_2$ solution at a final concentration of 0.3 M

and mixed with 150 μl of 2× HEPES-buffered saline (in mM: 50 HEPES, 280 NaCl, and 1.5 Na_2HPO_4, pH 7.05) dropwise while mixing gently. The mixture was left at room temperature for 30 min and then incubated with the cells for 6 h in 37 °C. Cells were then washed two times with 1× phosphate-buffered saline (PBS) and placed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1 mg/ml G418 in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. 48 h after transfection, cells were exposed to isotonic (in mM: 160 NaCl, 5 KCl, 1.17 $MgSO_4$, 1 $CaCl_2$, 5 glucose, and 10 HEPES, pH 7.4) or hypotonic 30% solutions (obtained by reduction of NaCl concentration) for 30 min. After treatment, the cells were harvested in ice-cold lysis buffer (in mM: 20 Tris/HCl, pH 7.4, 1 EDTA, 50 NaCl, 1 EGTA, 0.5 Na_3VO_4, 1 2β-glycerophosphate, and 1% Triton X-100), incubated for 10 min and then scraped. Cells homogenates were sonicated and centrifuged (11,000 × *g* for 20 min), and finally the supernatants were collected.

Immunoblotting—Groups of 10–20 oocytes were injected with cRNA from various constructs, transferred to Eppendorf tubes, and homogenized at 4 °C in lysis buffer (in mM 10 Tris-HCl, 150 NaCl, 1 EDTA, 1% Triton (10 μl/oocyte)) supplemented with protease and phosphatase inhibitor mixture (Roche Diagnostics). After clearing the lysate by centrifugation at 10,000 × *g* at 4 °C for 30 min, total protein was collected from the supernatant. Western blotting was carried out using previously characterized anti-KCC3 rabbit polyclonal antibodies (14) and the specific phospho-antibodies mentioned above. Total protein equivalent to one oocyte was separated in 7.5% polyacrylamide gels, transferred onto membranes (PVDF; Amersham Biosciences), blocked, and incubated overnight with the specific antibody at 3 μg/ml in TBST, 5% milk at 4 °C. After several washes with TBST (Tris-buffered saline/Tween 20, in mM: 100 Tris/HCl, 150 NaCl, and 0.1% Tween 20, pH 7.5), membranes were incubated with horseradish peroxidase-conjugated secondary antibody in blocking solution (1:7000, GE Healthcare Bioscience) and visualized by chemiluminescence (Amersham Biosciences ECL-Plus, GE Healthcare).

Cells cultured on 60-mm dishes were washed and then scraped into lysis buffer. Cell homogenates were sonicated and clarified by centrifugation (11,000 × *g* for 5 min), and protein concentration was determined by the Bradford method. Then, 25 μg of protein was separated by SDS-PAGE (7.5% acrylamide gel) and transferred onto PVDF membranes (Bio-Rad). Membranes were blocked with TBST containing 5% (w/v) nonfat dried milk and incubated overnight at 4 °C, with rabbit primary antibodies anti-KCC3 (1:1000) (14), anti-KCC3 phospho-Ser-96, anti-KCC3 phospho-Thr-991 (3 μg/ml plus 2 μl/ml non-phospho-peptide), or anti-β-actin (Santa Cruz Biotechnology). After further washing, blots were incubated with HRP-conjugated goat anti-rabbit IgG antibody (1:5000; Zymed Laboratories Inc.), except for KCC3 phospho-Ser-96 and phospho-Thr-991 that were incubated with an anti-sheep secondary antibody for 1 h at room temperature. Chemiluminescent reaction was assayed using ECL®-Plus Western blot detection reagents (GE Healthcare) according to the manufacturer's recommendations, and bands were visualized with exposure to Kodak BioMax light films (Sigma).

Statistical Analysis—Statistical significance is defined as two-tailed $p < 0.05$, and the results are presented as mean ± S.E. The significance of the differences between two groups was tested by Student's *t* test, and the significance of the differences for three or more groups was tested by one-way analysis of variance with multiple comparisons using Bonferroni's correction.

RESULTS

Evidence for Additional Phosphorylation Sites for KCC3 Regulation—The functional properties of wild type KCC3 (Fig. 1, *A* and *B*) were compared with those of the double mutant KCC3-T991A/T1048A (Fig. 1, *A* and *C*), in both isotonic and hypotonic conditions. As shown previously (14), wild type KCC3 was inactive in isotonic conditions (0.26 ± 0.053 nmol/oocyte/h) and incubation in hypotonicity dramatically increased its activity (7.83 ± 0.94 nmol/oocyte/h; $p > 0.001$). As reported by Rinehart *et al.* (11), we observed that $^{86}Rb^+$ uptake in oocytes injected with the mutant KCC3-T991A/T1048A cRNA revealed a significant activity in isotonic conditions (2.0 ± 0.13 nmol/oocyte/h; $p < 0.01$ *versus* wild type in isotonicity). However, when oocytes were incubated in hypotonicity, a further increase in activity was observed (3.45 ± 0.40 nmol/oocyte/h; $p < 0.0001$ *versus* uptake in isotonicity). Additionally, as depicted in Fig. 1, *B* and *C*, and reported previously for wild type KCC3 (15, 18, 19), the activity of both wild type KCC3 and KCC3-T991A/T1048A in hypotonic conditions was significantly reduced by co-expression with the WNK3 (2.98 ± 0.37 and 2.87 ± 0.046 nmol/oocyte/h, respectively, $p < 0.05$ *versus* the absence of WNK3). These observations suggested that there might be an additional phosphorylation site(s) that has to be dephosphorylated to achieve full activation of the cotransporter. This proposal differs from that of Rinehart *et al.* (11) where dephosphorylation of threonines 991 and 1048 was suggested to be sufficient for full activation of the cotransporter. In their study, however, wild type KCC3 was incubated in hypotonic conditions for a total of 8 min, whereas it has been reported that KCCs require longer incubation times to reach full activation (20–22). Thus, it is possible that the activity of the double mutant KCC3-T991A/T1048A in isotonicity was higher than wild type KCC3 in hypotonicity because this latter was not yet fully active at the time of the uptake assay. To assess this possibility, we measured the activity of wild type KCC3 and the double mutant KCC3-T991A/T1048A after 5 or 30 min of pre-uptake incubation followed by 15–60 min of uptake (Fig. 1*D*). Thus, total time of exposition to hypotonicity was 20 min in the shorter groups and 90 min in the longer groups. As shown in Fig. 1*D*, when using a shorter period of hypotonic incubation, the Cl^--dependent $^{86}Rb^+$ uptake was similar between wild type and double mutant KCC3-T991A/T1048A injected oocytes (0.45 ± 0.25 *versus* 0.54 ± 0.35 nmol/oocyte/h; p = not significant), whereas when using a longer period of exposition to hypotonic medium, the Cl^--dependent, $^{86}Rb^+$ uptake induced by wild type was significantly higher than the double mutant.

SPAK Kinase Phosphorylates KCC3 at Ser-96—The evidence shows that phosphorylation and activation of the NKCCs in the N-terminal domain by SPAK and OSR1 mediate WNK kinase signaling (23–26). Therefore we decided to analyze the effect of

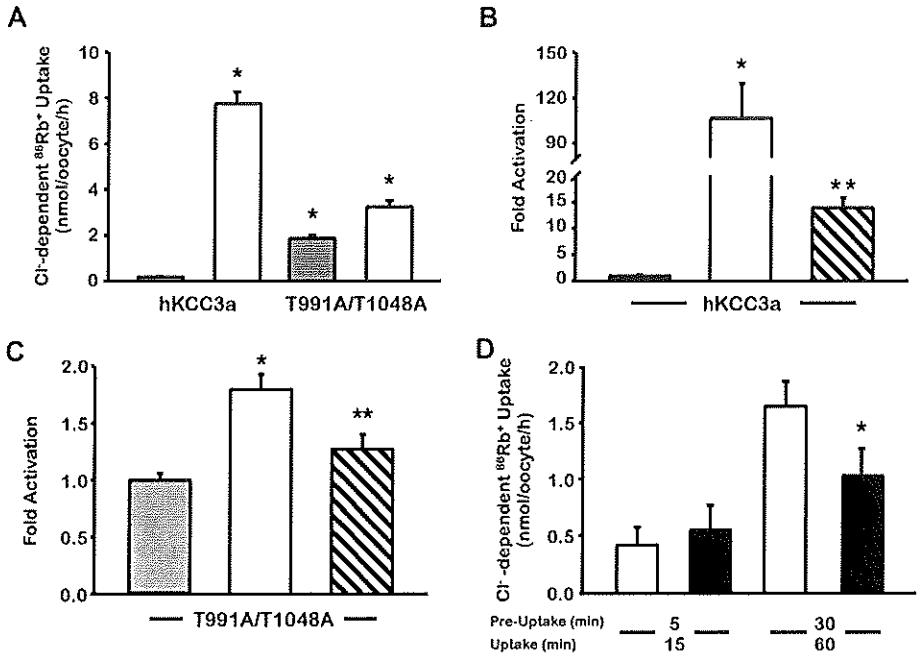


FIGURE 1. **Evidence for additional phosphorylation sites in KCC3.** *A*, functional expression assay shows the activity of wild type (*WT*) KCC3 and KCC3-T991A/T1048A under isotonic (*gray bars*) and hypotonic (*open bars*) conditions. *, $p < 0.01$ *versus* human KCC3a (*hKCC3a*) in isotonic condition. *B* and *C*, -fold activity of WT KCC3a (*B*) or KCC3a-T991A/T1048A (*C*) taking isotonic conditions as 100% (*gray bars*) and normalizing accordingly the effect of hypotonic conditions alone (*open bars*) or hypotonic conditions plus co-injection with WNK3 cRNA (*hatched bars*). *, $p < 0.01$ *versus* isotonic control. **, $p < 0.05$ *versus* hypotonicity without WNK3. *D*, time dependence of WT KCC3a activity (*open bars*) when compared with KCC3a-T991A/T1048A (*black bars*) in hypotonic conditions was measured after 5 or 30 min of pre-uptake incubation. *, $p < 0.01$ *versus* wild type.

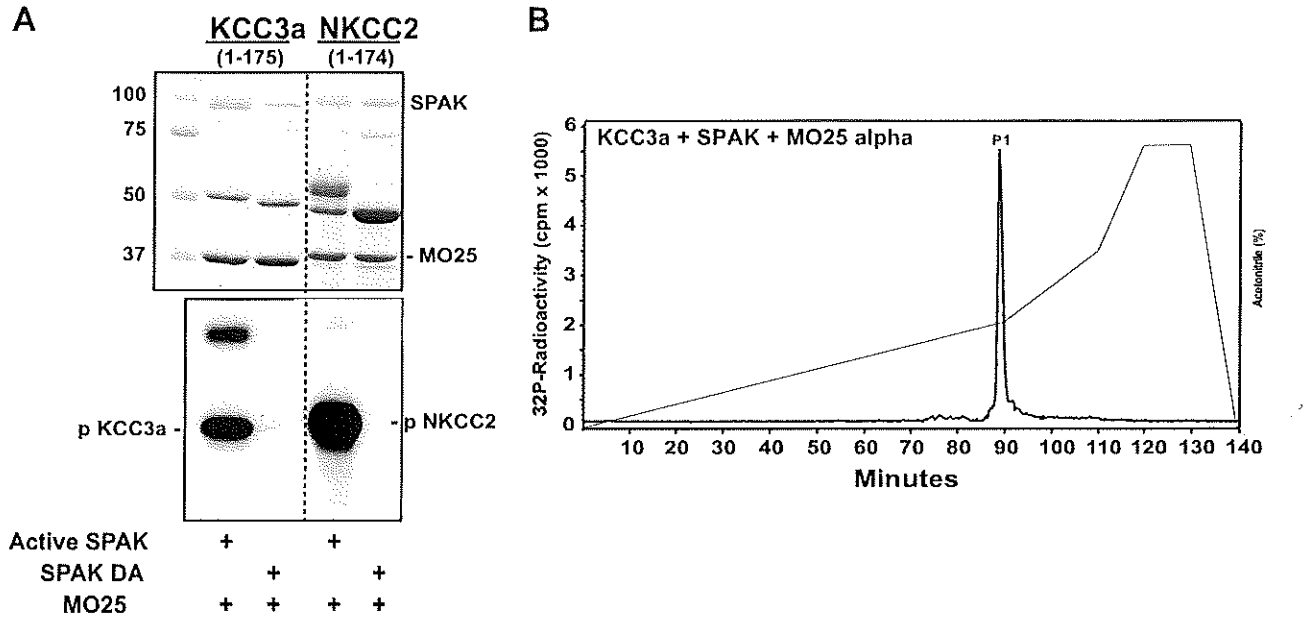
SPAK on the phosphorylation of KCC3. To test this, a fragment of the cotransporter encompassing the N-terminal cytoplasmic domain, equivalent to the region that SPAK phosphorylates on NCC, NKCC1, and NKCC2 (10, 16), was phosphorylated in the presence of catalytically active recombinant SPAK complexed to its regulatory MO25 subunit (27). Strikingly, this revealed that active wild type but not kinase-inactive SPAK directly phosphorylated the N-terminal fragment of KCC3 encompassing residues 1–175 to a similar extent as the phosphorylation of the N-terminal domain of NKCC2 (Fig. 2*A*).

SPAK phosphorylated KCC3(1–175) in a time-dependent manner, showing an ~18-fold increase in phosphorylation when compared with phosphorylation by active SPAK in the absence of MO25 (data not shown). ^{32}P-labeled KCC3(1–175) was digested with trypsin and analyzed by chromatography on a C_{18} column for phospho-site mapping. One major ^{32}P-labeled phospho-peptide was observed (Fig. 2*B*). A combination of solid-phase Edman sequencing and mass spectrometry (Fig. 2*C*) revealed that this encompassed a peptide phosphorylated at Ser-96. Mutating Ser-96 to Ala prevented phosphorylation of KCC3 by SPAK, thereby confirming that this residue represents the major site of phosphorylation (data not shown).

The sequence alignment of the four K^+:Cl^- cotransporters shows that the potential phosphorylation site Ser-96 is unique to KCC3 because it is only present in KCC3a and KCC3b isoforms (Fig. 3*A*), but not in the other KCCs. The double mutant KCC4-T926A/T980A, which is homologous to KCC3-T991A/T1048A, but lacks the Ser-96 residue, is also active in isotonic conditions, but cannot be further increased in hypotonicity (Fig. 3*B*). Thus, in contrast to KCC3, in the KCC4 isoform that lacks the Ser-96 site, elimination of the two known sites at the C-terminal domain results in full activation of the cotransporter in isotonicity, suggesting that the presence of Ser-96 in KCC3 is responsible for the different functional behavior between KCC3-T991A/T1048A and KCC4-T926A/T980A toward hypotonicity.

KCC3 Serine 96 Becomes Dephosphorylated during Cotransporter Activation by Hypotonicity—We next substituted the serine 96 on wild type KCC3 to produce the KCC3-S96A mutant. As shown in Fig. 4*A*, similar to wild type KCC3, the KCC3-S96A was inactive in isotonic conditions and was dramatically stimulated by incubation in hypotonicity. The activation was fully prevented by co-expression with WNK3 (Fig. 4*B*). Thus, mutation of Ser-96 by itself has no effect on the behavior of KCC3 toward isotonic or hypotonic conditions, as well as to the known inhibitory action of WNK3 on the cotransporter (15, 18, 19).

We next generated phospho-specific antibodies that efficiently recognize KCC3 phosphorylated at Ser-96, Thr-991, or Thr-1048. Mutation of the phosphorylated residue to a non-phosphorylatable Ala residue prevented recognition, confirming the specificity of the phospho-specific antibodies (Fig. 5*A*). As shown in Fig. 5*B*, we were able to demonstrate that wild type KCC3 overexpressed in *X. laevis* oocytes is indeed phosphorylated at these three residues in isotonic conditions, when the cotransporter is inactive, and is considerably dephosphorylated following incubation in hypotonic conditions, when KCC3 becomes active. We also observed the expected effect of WNK3 kinase co expression; in the presence of WNK3, dephosphory-



C

Sample ID	Phosphopeptide	m/z (observed)	Cycle of ^{32}P release	Phosphorylation site
P1	TSHPQDVIEDLSQN(p)SITGEHSQLLDDGHKK	1094.16	15	Ser96

FIGURE 2. ***In vitro* identification of KCC3 phosphorylation sites by SPAK.** *A*, GST-KCC3(1–175) and GST-NKCC2(1–174) were expressed in *E. coli* and phosphorylated with the active and kinase inactive (*DA*) forms of SPAK in the presence of 10 μM MO25α. *Dotted lines* between autoradiographs and gels indicate that these were undertaken on separate gels. *p*, phosphorylated. *B*, phosphorylated GST-KCC3(1–175) was digested with trypsin and chromatographed on a C18 column. The peak fraction containing the major ^{32}P-labeled peptides is labeled *P1*. *C*, summary of the mass spectrometry and solid-phase Edman sequencing data obtained after phospho-peptide analysis.

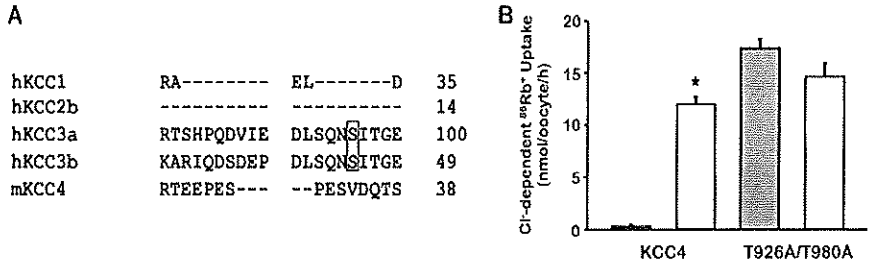


FIGURE 3. **Serine 96 is unique to KCC3.** *A*, KCCs sequence alignment of the N-terminal domain fragment revealed that Ser-96 of KCC3a (Ser-45 in KCC3b as shown the *gray shaded box*) is not present in other KCCs and thus is unique to KCC3. *h*, human; *m*, mouse. *B*, KCC4 Rb^+ uptake of WT KCC4 and KCC4-T926A/T980A under isotonic (*gray bars*) and hypotonic (*open bars*) conditions. $n = 8–9$ experiments, *, $p < 0.0001$ *versus* KCC4 in isotonicity.

lation of the three sites was partially precluded. This correlates with the uncompleted activation of KCC3 in hypotonic conditions that occurs in the presence of this kinase (15, 18, 19). Similarly, in the double mutant KCC3-T991A/T1048A, Ser-96 also appeared phosphorylated in isotonic conditions and dephosphorylated after hypotonicity, and this was partially prevented by WNK3. These observations were corroborated using HEK293 cells transiently transfected with KCC3 cDNA and HEK293 cells stably transfected with WNK3 cDNA that were, in addition transiently transfected with KCC3 cDNA (19). As shown in Fig. 5*C*, HEK293 cells expressed KCC3 protein after transfection. In isotonic conditions, both Ser-96 and Thr-991 were phosphorylated, and the signal for each phospho-antibody disappeared when cells where incubated in hypotonicity, in which KCC activity is known to be increased (19). In addition, in HEK293 cells stably transfected with WNK3, Ser-96 and Thr-991 were phosphorylated in both isotonic and hypotonic conditions, consistent with the known inhibition that WNK3 exerts on KCC3 activity (15, 18, 19). All these observations together suggested that although Ser-96 is not a unique or a master site for KCC3 regulation, it behaves as phospho-sites Thr-991 and Thr-1048 and it seems to be a third site playing a role in full activation/inhibition of the cotransporter.

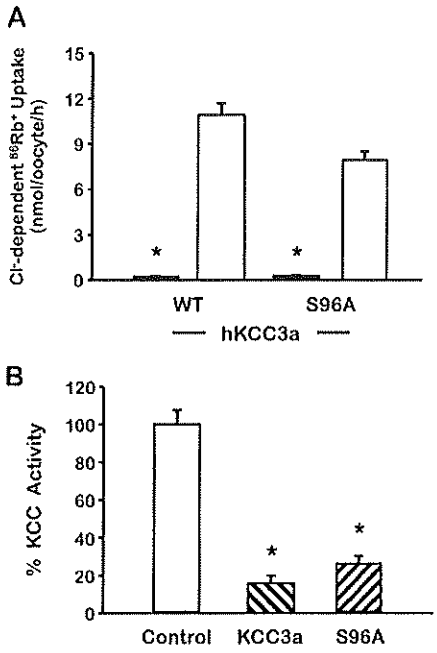


FIGURE 4. **Elimination of serine 96 by itself had no effect on the KCC3 behavior.** *A*, functional expression assay of WT KCC3a and mutant KCC3a-S96A shows no difference in hypotonic activation (*open bars*), with no basal expression under isotonic conditions (*gray bars*). $n = 4$, $p < 0.0001$ *versus* hypotonicity. *B*, similarly to WT KCC3a, the activity of mutant KCC3a S96A in hypotonic conditions is inhibited by WNK3 (*hatched bars*). $n = 4$, *, $p < 0.0001$ *versus* control.

Serine 96 Is a Third Site Involved in Regulation of KCC3 Activity—Because Ser-96 residue in the double mutant KCC3-T991A/T1048A was phosphorylated and dephosphorylated similarly as in the wild type KCC3a, but elimination of this site alone had no effect on the cotransporter behavior (Fig. 4), to find out the role of serine 96 on the regulation of KCC3, we substituted the Ser-96 for alanine in the double mutant KCC3-T991A/T1048A to create the triple mutant KCC3-S96A/T991A/T1048A. In parallel experiments, functional expression of the double and triple mutant revealed that both were active in isotonic conditions (Fig. 6*A*). However, the level of activity was significantly higher in the triple mutant than in the double mutant (4.97 ± 0.57 *versus* 1.86 ± 0.15 nmol/oocyte/h; $p <$ 0.0001), suggesting that the absence of phosphorylation in serine 96 contributed to higher activity of the triple mutant KCC3 in isotonic medium. This is supported by the findings observed during incubation of oocytes in hypotonic conditions. Further activation of the double mutant was noted (Figs. 1, *A* and *C*, and 6*B*), whereas no additional effect of hypotonicity was detected in the triple mutant (Fig. 6*B*). The Cl^--dependent, $^{86}Rb^+$ uptake of the triple mutant in isotonicity and hypotonicity was 4.97 ± 0.57 and 5.23 ± 0.57 nmol/oocyte/h, respectively (p = not significant). In addition, co-injection of oocytes with WNK3 cRNA was able to significantly reduce the activity of wild type and double mutant KCC3 in hypotonic conditions, but no inhibitory effect was observed upon the triple mutant (Fig. 6*C*).

DISCUSSION

In the present study, we provide functional and biochemical evidences suggesting that full activation by cell swelling of the

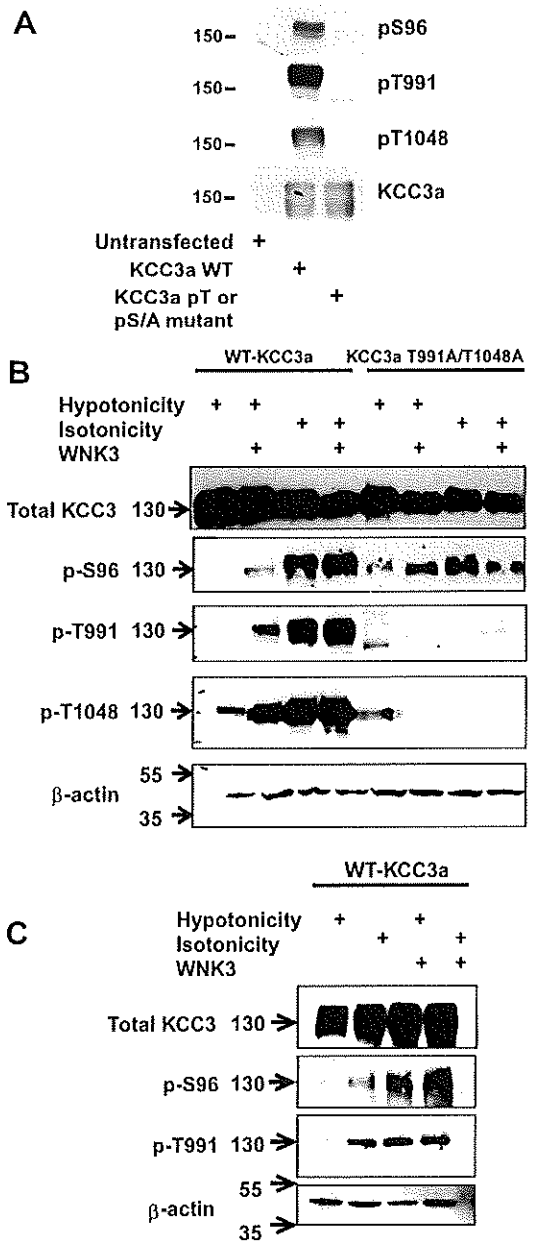


FIGURE 5. **Serine 96 becomes phosphorylated under isotonic conditions and in co-expression with WNK3.** *A*, characterization of KCC3a phosphorylation sites using phospho-specific antibodies. HEK 293 cells were transfected with WT human KCC3a or the indicated mutant forms (*pT* or *pS*). At 36 h after transfection, cells were lysed, and total cell extracts were immunoblotted with KCC3a total and phospho-specific antibodies. Similar results were obtained in 2 separate experiments. *B*, representative immunoblotting of total protein extracted from oocytes injected with WT KCC3a or KCC3a-T991A/T1048A cRNA with or without WNK3 cRNA and under hypotonicity or isotonicity maneuvers, as stated. *C*, representative immunoblotting of total proteins extracted from HEK293 cells transfected as stated. Blots were performed using specific antibody against total KCC3 and phospho-antibodies directed to Ser-96, Thr-991, and/or Thr-1048 of KCC3a and β-actin as loading control.

K^+:Cl^- cotransporter isoform KCC3 is only achieved after dephosphorylation of three distinct residues, two of which are located at the C-terminal domain (threonine residues 991 and 1048) and were reported previously by Rinehart *et al.* (11). The third residue, proposed in this study, is the serine 96 located at the N-terminal domain.

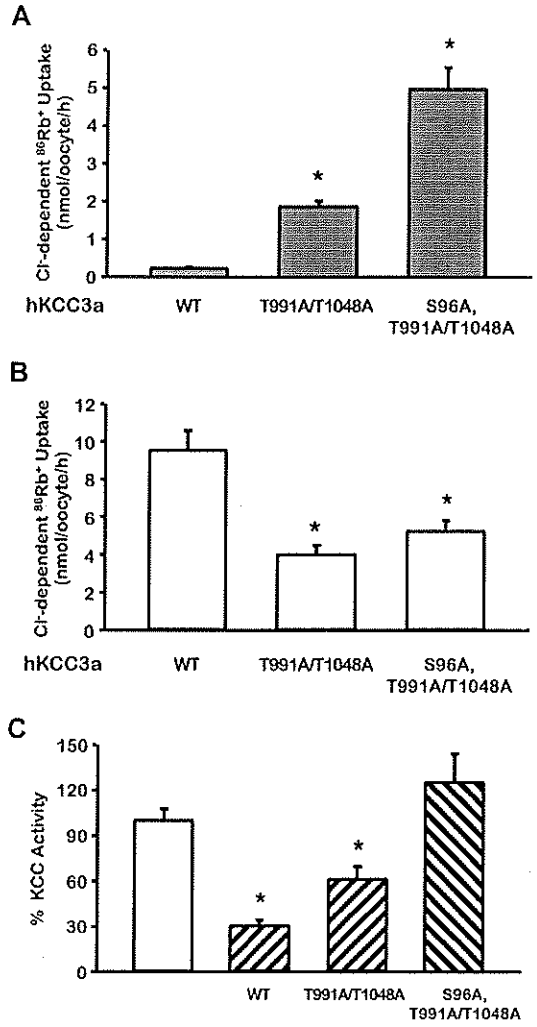


FIGURE 6. **Serine 96 is a third site involved in regulation of KCC3a activity.** *A* and *B*, functional expression assay was assessed in isotonic (*A*) or hypotonic (*B*) conditions in oocytes injected with cRNA for WT KCC3, KCC3 double mutant (T991A/T1048A), or KCC3 triple mutant (S96A/T991A/T1048A) as stated. *, $p < 0.001$ *versus* WT. *C*, the triple mutant KCC3a S96A/T991A/T1048A is no longer sensitive to WNK3 (*hatched bars*) inhibition. The *open bar* shows the control uptake for each clone taken as 100%. The *hatched bars* show the effect of WNK3 upon wild type or mutants KCC3, as stated. $n = 3$, *, $p < 0.001$ *versus white bar.*

Our proposal for the requirement of a third site dephosphorylation to achieve complete activation of KCC3 differs from conclusions presented by Rinehart *et al.* (11). They observed, on the one hand, that double mutant KCC3-T991A/T1048A exhibited similar activity in both isotonicity and hypotonicity, and on the other hand, that activity of the double mutant in isotonicity was higher than that shown for wild type KCC3 when cells were incubated in hypotonic conditions. Thus, they suggested that Thr-991 and Thr-1048 were the only two residues required for cell swelling to induce KCC3 activation. However, although we observed significant activity of the double mutant KCC3-T991A/T1048A in isotonic conditions, we also documented that incubation of oocytes in hypotonicity further increased the activity of the double mutant KCC3-T991A/T1048A (Fig. 1*A*). The observed increase was sensitive to WNK3 (Fig. 1*C*). Because WNK3 cannot phosphorylate neither Thr-991 nor Thr-1048 in the double mutant KCC3-T991A/T1048A, this observation suggested that it was probably reducing the cotransporter activity by phosphorylating Ser-96. In addition, we showed that wild type KCC3 activity in hypotonic medium was significantly higher than that of the double mutant KCC3-T991A/T1048A in the same conditions (Fig. 1*A*). The reason for the discrepancy appears to be the time of exposure to the hypotonic medium. Rinehart *et al.* (11) used a total of 8 min, whereas we used longer exposure times. In this regard, it has been suggested that time of exposure to hypotonicity is associated with activation of the cotransporter (20–22). This is supported by our observations in Fig. 1*D* in which no difference between wild type and double mutant KCC3-T991A/T1048A was observed at a short period of incubation, whereas a significant difference was observed with longer exposure to hypotonic medium. Because the double mutant KCC3-T991A/T1048A is already active in isotonicity, then at a short period of incubation in hypotonicity, the activation of wild type KCC3 is not yet enough to reveal a difference. Thus, our functional observations suggested that at least a third phosphorylation site was involved in the regulation of KCC3 by extracellular osmolarity.

The potential phosphorylation site Ser-96 in KCC3 was detected by mass spectrometry. Because in the Na^+ coupled-chloride cotransporters of the SLC12 family NCC, NKCC1, and NKCC2 regulatory phosphorylation sites have been detected at the N-terminal domain (28–31), and it has been demonstrated that SPAK is the kinase responsible for the phosphorylation of these sites (10, 16), we analyzed the effect of SPAK, complexed with its regulatory subunit MO25 (27), on the phosphorylation of the N-terminal domain of KCC3. NKCC2 N-terminal domain was used as positive control. Following this strategy, phosphorylation of Ser-96 in the KCC3a N-terminal domain was detected. Rinehart *et al.* (11) did not observe this site, probably due to methodological differences. On the one hand, what they did was to compare KCC3 phosphorylation status in isotonicity *versus* hypotonicity, whereas we directly applied SPAK/MO25 into the *in vitro* reaction. On the other hand, they analyzed KCC3 extracted from cells after 5 min of exposure to hypotonic conditions, which, as was discussed above, could not be enough time to allow a complete dephosphorylation of the cotransporter to detect a difference in this site.

Specific phospho-antibodies against Ser-96, Thr-991, and Thr-1048 were raised and used to demonstrate that indeed, in isotonic conditions in which KCC3 is inactive, all three sites were phosphorylated. Incubation of oocytes in hypotonicity resulted in decreased phosphorylation of the three sites, suggesting that dephosphorylation of these sites is coupled with activation of the cotransporter. Supporting this conclusion, when oocytes were co-injected with WNK3 cRNA, a kinase that is known to inhibit the KCCs (15), the activity and dephosphorylation of KCC3 in the three sites were partially precluded. Similar observations were obtained for Ser-96 and Thr-991 sites in HEK293 cells. Finally, the behavior of mutant S96A alone and in the context of the triple mutant S96A/T991A/T1048A also supports that Ser-96 is a third site involved in the modulation of the cotransporter activity. The absence of Ser-96 alone had no effect on KCC3 activity in isotonic conditions or its behavior

toward activation by hypotonicity and inhibition by WNK3, suggesting that the C-terminal domain sites Thr-991 and Thr-1048 are hierarchically higher. However, elimination of Ser-96 in the absence of the other two sites resulted in a KCC3 that is more active than the double mutant in isotonic conditions and cannot be neither further activated by hypotonicity nor inhibited by WNK3, indicating that Ser-96 participates in the regulation of KCC3 activity.

The physiological relevance of a third site for regulation of the KCC3 cotransporter activity will require further investigation. This site is not present in other KCCs. Indeed, KCC4 in which both C-terminal domain threonine residues were eliminated (KCC4-T926A/T980A) resulted in full activation in isotonic conditions, suggesting that in this isoform no other sites are involved. Similarly, for NKCCs, between three and five different phosphorylation sites involved in their regulation have been located at the N-terminal domain, from which the master one is conserved (Thr-60 in human NCC), whereas some of the others are unique to one isoform (9, 24).

The presence of a third site suggests that KCC3 in some cells, tissues, or circumstances requires fine-tuning for its regulation. In this regard, it is worth noticing that we have shown before that an alternatively spliced isoform of KCC3a lacking exon 2 (KCC3a-x2M) is present in several human tissues (14). Exon 2 encodes for a 15-amino acid residue peptide in which Ser-96 is included. Thus, the major consequence of this splicing could be to produce KCC3 variants with and without Ser-96. Supporting the role of Ser-96 in the regulation of KCC3, we have reported previously that the speed of activation by hypotonicity of the KCC3a-x2M clone, lacking exon 2, is significantly faster than KCC3a containing exon 2 (14), suggesting that the absence of Ser-96 decreases the time required for full activation of the cotransporter because there is not a third site that has to be dephosphorylated. It will thus be interesting in future work to define which cells within a tissue are expressing variants with or without exon 2 or to raise knock-in mice of the different phosphorylation sites to explore the physiological consequence of the three different sites.

Phosphorylation/dephosphorylation processes modulate the activity of the SLC12 cotransporters. Several studies support that promoting phosphorylation by cell shrinkage, activating kinases, or inhibiting protein phosphatases increases the activity of the NKCCs and inhibits the KCCs, thus increasing intracellular chloride concentration, cell volume, and transcellular ion transport capacity. In contrast, promoting dephosphorylation by cell swelling, inhibiting kinases, or activating protein phosphatases reduces the NKCCs and activates the KCCs, thus decreasing intracellular chloride concentration, cell volume, and transcellular ion transport. Because the mode of operation of these secondary transporters is still far from being understood, it is not clear at the moment how the phosphorylation or dephosphorylation of one site can affect the transport activity of the protein, particularly because it is believed that this occurs with proteins that are already in the plasma membrane (9, 11, 32). Thus, affecting trafficking by the phospho-status of the protein does not seem to be the answer. One possibility could be that phosphorylating or not phosphorylating certain residues changes the structure of the protein, opening or closing pores or turning binding sites for ions more or less accessible, thus changing the transport capacity of the cotransporter. Another possibility is that phosphorylation on a given cotransporter could be modulating its ubiquitylation, thus indicating that phosphorylation/dephosphorylation processes also affect the amount of total cotransporter (33).

In summary, our data support that serine 96 in KCC3 is a third phosphorylation site implicated in the regulation of the cotransporter activity.

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Modulation of NCC activity by low and high K^+ intake: insights into the signaling pathways involved

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[1]*Molecular Physiology Unit, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán and Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Tlalpan, Mexico City, Mexico; and* [2]*Departamento de Farmacología, Instituto Nacional de Cardiología Ignacio Chávez, Tlalpan, Mexico City, Mexico*

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Castañeda-Bueno M, Cervantes-Perez LG, Rojas-Vega L, Arroyo-Garza I, Vázquez N, Moreno E, Gamba G. Modulation of NCC activity by low and high K^+ intake: insights into the signaling pathways involved. *Am J Physiol Renal Physiol* 306: F1507–F1519, 2014. First published April 23, 2014; doi:10.1152/ajprenal.00255.2013.— Modulation of Na^+-Cl^- cotransporter (NCC) activity is essential to adjust K^+ excretion in the face of changes in dietary K^+ intake. We used previously characterized genetic mouse models to assess the role of Ste20-related proline-alanine-rich kinase (SPAK) and with-no-lysine kinase (WNK)4 in the modulation of NCC by K^+ diets. SPAK knockin and WNK4 knockout mice were placed on normal-, low-, or high-K^+-citrate diets for 4 days. The low-K^+ diet decreased and high-K^- diet increased plasma aldosterone levels, but both diets were associated with increased phosphorylation of NCC (phospho-NCC, $Thr^{44}/Thr^{48}/Thr^{53}$) and phosphorylation of SPAK/oxidative stress responsive kinase 1 (phospho-SPAK/OSR1, Ser^{383}/Ser^{325}). The effect of the low-K^+ diet on SPAK phosphorylation persisted in WNK4 knockout and SPAK knockin mice, whereas the effects of ANG II on NCC and SPAK were lost in both mouse colonies. This suggests that for NCC activation by ANG II, integrity of the WNK4/SPAK pathway is required, whereas for the low-K^- diet, SPAK phosphorylation occurred despite the absence of WNK4, suggesting the involvement of another WNK (WNK1 or WNK3). Additionally, because NCC activation also occurred in SPAK knockin mice, it is possible that loss of SPAK was compensated by OSR1. The positive effect of the high-K^- diet was observed when the accompanying anion was citrate, whereas the high-KCl diet reduced NCC phosphorylation. However, the effect of the high-K^+-citrate diet was aldosterone dependent, and neither metabolic alkalosis induced by bicarbonate, nor citrate administration in the absence of K^+ increased NCC phosphorylation, suggesting that it was not due to citrate-induced metabolic alkalosis. Thus, the accompanying anion might modulate the NCC response to the high-K^+ diet.

with-no-lysine kinase 4; Ste20-related proline-alanine-rich kinase; aldosterone; distal convoluted tubule

RENAL K^+ EXCRETION is dependent on K^+ secretion in the distal nephron because the filtered K^+ is almost completely reabsorbed in the proximal convoluted tubule. In the distal nephron, the Na^+ reabsorption that occurs through the epithelial Na^+ channel generates a lumen negative potential that serves as the driving force for K^+ secretion through the renal outer medullary K^+ (ROMK) channel. In addition, the rate of luminal flow is associated with the activation of large-conductance Ca^{2+}-activated K^+ channels (13, 15). Thus, K^+ secretion in the distal nephron is a flow-dependent process that is coupled to Na^+ reabsorption.

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Salt reabsorption in the distal convoluted tubule (DCT) occurs through the thiazide-sensitive Na^+-Cl^- cotransporter (NCC), which plays a key role in modulating salt and fluid delivery to downstream portions of the nephron and, thus, in K^+ secretion. Two inherited diseases, which are the consequence of the elimination or activation of NCC, produce opposite effects on plasma K^+ concentration: Gitelman's disease features hypokalemia, which is due to inactivating mutations in the gene encoding NCC (*Slc12a3*), and familial hyperkalemic hypertension or pseudohypoaldosteronism type II, a disease that features hyperkalemia, which may be the consequence of NCC activation by altered activity of mutant kinases or ubiquitin ligases, because it can be abrogated by either thiazide-type diuretics (19, 40) or the elimination of NCC in genetically engineered mice (17). Thus, decreased or increased NCC activity promotes K^+ secretion or retention, respectively.

Therefore, it is not unexpected that the amount of ingested K^+ modulates NCC expression and/or activity. Vallon et al. (34) observed that mice fed a low-K^+ diet exhibited a significant increase in activating phosphorylation of NCC, an effect that would be expected to decrease Na^+ and fluid delivery to the distal nephron, negatively affecting K^+ secretion. In contrast, mice fed a high-K^+ diet exhibited no to moderate decreases in NCC phosphorylation compared with the levels of phosphorylation observed in mice fed a normal diet. In another study, Frindt and Palmer (10) observed a moderate decrease in NCC apical expression in rats maintained on a high-K^+ diet. In this regard, it is important to note that a high-K^+ diet is a strong stimulus for increasing the synthesis and secretion of the mineralocorticoid hormone aldosterone, which is known to promote increased NCC expression (16). The increase in NCC expression is due, at least in part, to increased activity of serum/glucocorticoid regulated kinase 1 (SGK1), which, in turn, inhibits the ubiquitylation of NCC by the E3 ubiquitin ligase Nedd4-2 (1, 28). In addition, independent of its effect on NCC expression, aldosterone promotes NCC phosphorylation (35, 36). Thus, the explanation for the decreased phosphorylation and surface expression of NCC observed in mice fed a high-K^+ diet in Vallon et al.'s and Frindt and Palmer's works remains elusive. However, Vallon et al. (34) observed that these effects were exacerbated in SGK1-deficient mice, possibly due to loss of the aldosterone-induced positive effect on NCC.

A low-salt diet increases and a high-salt diet decreases NCC activity (for reviews, see Refs. 5 and 11). We have shown that increased NCC and Ste20-related proline-alanine-rich kinase (SPAK) phosphorylation during maintenance on a low-salt diet

or administration of ANG II does not occur in with-no-lysine kinase 4 (WNK4) knockout ($WNK4^{-/-}$) mice, suggesting that the salt-modulated NCC regulatory pathway requires integrity of the WNK4-SPAK binomium (4, 29). Therefore, the present study was designed to analyze the roles of WNK4 and SPAK in K^+-induced changes in NCC expression and phosphorylation, taking advantage of recently developed total $WNK4^{-/-}$ (4) and SPAK knockin ($SPAK^{T243A/T243A}$) (25) mouse models. In our study, both low- and high-K^+ diets were associated with increased NCC phosphorylation. The observed effect of a high-K^+ diet was aldosterone dependent. The increase in NCC phosphorylation in mice fed with both diets was correlated with an increase in SPAK phosphorylation and occurred in $WNK4^{-/-}$ mice, suggesting that, in contrast to a low-salt diet, the response of NCC to changes in K^+ intake is a WNK4-independent process.

METHODS

Animal experiments. All experiments involving animals were approved by the Animal Care and Use Committee of our institution. For this work, we used two genetically altered mouse models that have been previously described: a $WNK4^{-/-}$ mouse strain (4) and a SPAK knockin mouse strain (25). For experiments conducted with each of these strains, the wild-type controls were littermates of the homozygous mice. Briefly, in $WNK4^{-/-}$ mice (4), WNK4 expression was knocked out by replacing exon 1 with a neomycin phosphotransferase cassette. In the SPAK knockin mouse strain (25), Thr^{243}, which is required for WNK-induced activation of SPAK, was replaced with alanine ($SPAK^{T243A/T243A}$). The genetic background of both strains was C57BL/6. Genotyping was performed as previously described (4, 25). Male mice between 12 and 16 wk old were used.

Low-K^+, control, and high-K^+ diets. Control (1.2% K^-), low-K^+ (0% K^+), and high-K^- (5% K^+) diets were obtained from TestDiet (St. Louis, MO) and were prepared by modifying the AIN-93M semipurified diet, as previously described (4). The 0% K^+ diet was used as the base, and tribasic K^+-citrate was added to generate the 1.2% K^+ and 5% K^+ diets. After a 2-day period of adapting to the 1.2% K^+ powder diet, during which time mice were also allowed to adapt to the metabolic cages, the diet was changed to 0% or 5% K^+ for some animals, whereas the control group continued to receive the 1.2% diet. On *days 1* and *4* after switching to these diets, mice were placed in metabolic cages for urine collection. At the end of *day 4*, mice were euthanized for urine and blood collection. The concentrations of urinary and plasma electrolytes were determined using a Synchron CX5 (Beckman Coulter, Miami, FL). The plasma aldosterone concentration was measured by ELISA (DRG), and plasma renin activity was measured by RIA (REN-CT2, RADIM). For only a particular experiment, K^+ was added to the diet as KCl, to obtain a high-K^+ diet (5%) with high Cl^- content instead of high citrate content.

Spironolactone treatment. To determine whether the findings for mice fed high-K^+ diets were due to a concomitant aldosterone increase, the mineralocorticoid receptor was blocked with spironolactone in mice placed in individual cages. Mice were given normal- or high-K^+ diets and simultaneously treated with spironolactone (Sigma) that had been dissolved in ethanol at 25 mg/ml and then added to the drinking water, as previously described (21). The calculated dose was 40 $mg \cdot kg^{-1} \cdot day^{-1}$. Water intake was monitored daily.

HCO_3^- loading. Protocol and doses were adapted from previous reports (22, 38). After mice underwent a period of adaptation to metabolic cages, 0.28 M $NaHCO_3$ + 1% sucrose was added to the drinking water of the alkalosis group, whereas 0.28 M NaCl + 1% sucrose was added to the drinking water of the control group to maintain Na^+ intake at similar levels. Mice were fed ad libitum with standard pelleted chow. Twenty-four-hour urine collections were done on the days before the start of treatment and on *day 7*. At the end of the second collection, mice were euthanized and kidneys were collected.

Citrate diet. Mice were maintained on normal chow and were given a drinking solution containing 100 mM citric acid and 0.5% sucrose for 4 days. NaOH was used to adjust the pH to 4. The amount of NaOH added was measured, and an equivalent amount of Na^+ was added as NaCl to the drinking water of the control group (also containing 0.5% sucrose). Mice were kept in metabolic cages to monitor water intake, which was similar between groups. According to the observed water intake, the citrate intake in this experimental group was ~70% of that observed in the high-K^+-citrate group. This corresponds to seven times the amount of citrate contained in the normal diet (in the high-K^+-citrate diet, the amount citrate content was 10 times higher).

ANG II infusion. The effect of ANG II infusion on NCC phosphorylation in $WNK4^{-/-}$ mice has been previously reported (4). In this study, we assessed the effect of ANG II in the SPAK knockin mouse strain using microosmotic pumps (model 1007, Alzet, DURECT) implanted subcutaneously to infuse ANG II at a rate of 280 $ng \cdot kg^{-1} \cdot min^{-1}$ (400 $\mu g \cdot kg^{-1} \cdot day^{-1}$) (4). It has been previously reported that this dose does not cause pressor effects (23). Two 24-h urine collections were performed on the first and last days of the 4-day infusion. After the last day of infusion, animals were euthanized and kidneys were collected.

Immunoblot assays. Kidney proteins and testes proteins were extracted with a lysis buffer containing 50 mM Tris·HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% (wt/vol) Nonidet P-40, 0.27 M sucrose, 0.1% (vol/vol) 2-mercaptoethanol, and protease inhibitors (Complete tablets, Roche). For the SDS-PAGE analysis, 50–80 μg of protein were loaded per lane. Proteins were transferred to polyvinylidene difluoride membranes, which were then blocked for 1.5 h in 10% (wt/vol) nonfat milk dissolved in Tris-buffered saline-Tween. Antibodies were diluted in Tris-buffered saline-Tween containing 5% (wt/vol) nonfat milk. Membranes were incubated with primary antibodies overnight at 4°C and with secondary antibodies at ambient temperature for 1.5 h. The immobilized antigens were detected by chemiluminescence using the Luminata Crescendo detection system from Millipore.

Phosphatase treatment of kidney protein samples. One-half of a mouse kidney was lysed following the usual protocol, whereas the second half was prepared with lysis buffer lacking phosphatase inhibitors. Protein was quantified, 40 μg of protein of each sample were transferred to new tubes (~2 μl of lysate), and samples were diluted ~10 times with 1× PMP NEB buffer and 1 mM $MnCl_2$. Four hundred units (1 μl) of lambda protein phosphatase (P0753, New England Biolabs) were added to the sample that was prepared without phosphatase inhibitors. Samples were incubated at 30°C for 30 min, and Western blot analysis was performed following the usual protocol.

Antibodies. Polyclonal antibodies against NCC, SPAK, oxidative stress response kinase 1 (OSR1), and phosphorylated (p)SPAK at Ser^{383} (S-motif) were raised in sheep. The concentrations used were 1–3 μg/ml. These antibodies were produced at the MRC phosphorylation unit in Dundee University, and their specificity has been previously demonstrated (25, 26, 30). A sheep antibody against pNCC at Thr^{44}, Thr^{48}, and Thr^{53} (in the mouse) was used. This antibody was also produced in the MRC phosphorylation unit of Dundee University and has been previously used by others (14). However, since the characterization of this antibody has not been published, we confirmed the specificity by performing Western blots with samples of $NCC^{+/+}$ and $NCC^{-/-}$ mice (31) (see RESULTS). In addition, for all phospho-blots, antibody incubation was done in the presence of nonphosphopeptide, following the recommendation of the manufacturer. This confirms that the antibody is specific for the phosphorylated epitope. The goat anti-actin polyclonal antibody conjugated to horseradish peroxidase and the donkey anti-sheep antibody were purchased from Santa Cruz Biotechnology.

Statistical analysis. Bands from different blots were scanned for densitometry. For the NCC blots, the nonspecific band observed slightly above the 130-kDa marker band was not included in the densitometric analysis. Statistical significance was defined as two-tailed $P < 0.05$, and results are presented as means ± SE. Differences between two groups were tested for significance using Student's *t*-test. Differences between three or more groups were tested for significance using one-way ANOVA with multiple comparisons using the Bonferroni correction.

RESULTS

NCC is activated by both low- and high-K^+ diets. Wild-type mice were fed diets with high or low K^+ content for a period of 4 days. To assess changes in the activity of NCC, K^+ was added to the diet as K^+-citrate instead of KCl to keep the Cl^- intake constant. The physiological parameters of wild-type mice fed low- or high-K^+ diets were modified as expected (Table 1). Variations in dietary K^+ content did not affect food intake, and, thus, the average weights of the low-, normal-, and high-K^+ groups were similar. In agreement with this finding, urinary Na^+ and Cl^- excretion was also similar. In mice fed a low-K^+ diet, urinary K^+ excretion was effectively reduced (0.02 ± 0.003 vs. 0.63 ± 0.09 mmol/24 h on the normal diet, $P < 0.00005$) and plasma aldosterone was decreased (90.5 ± 36.2 vs. 232.3 ± 88.4 pg/ml on the normal diet, $P < 0.001$). In contrast, urinary K^+ excretion increased with the high K^+ diet (2.57 ± 0.24 vs. 0.63 ± 0.09 mmol/24 h on the normal diet, $P < 0.05$) and the expected increase in plasma aldosterone was also observed (866.01 ± 383.49 vs. 232.3 ± 88.4 pg/ml on the normal diet, $P < 0.05$). However, plasma Na^+ and K^+ concentrations did not change significantly during the study. Notably, urinary volume was increased in the high-K^+ group compared with the normal-K^+ group (5.2 ± 1.2 vs. 2.1 ± 0.98 ml/24 h, $P < 0.00005$), but plasma renin activity was not affected. Finally, high-K^+ group developed metabolic alkalosis due to the high citrate intake, as revealed by the higher urinary pH values (9.07 ± 0.08 vs. 7.67 ± 0.3 on the normal diet, $P < 0.001$) and the higher plasma CO_2 concentration (22.15 ± 2.06 vs. 14.44 ± 2.48 on the normal diet, $P < 0.05$).

To compare NCC expression and phosphorylation levels of the three groups, we performed Western blot analysis of total kidney protein extracts with a previously described NCC-specific antibody and a phospho-antibody recognizing NCC phosphorylated on Thr^{44}, Thr^{48}, and Thr^{53}. The specificity of this antibody was confirmed by Western blot analysis of kidney protein samples from $NCC^{+/+}$ and $NCC^{-/-}$ mice. As shown in Fig. 1*A*, the signal was increased by the low-salt diet in wild-type mice and was not present in $NCC^{-/-}$ mice fed either a regular or low-salt diet. In addition, protein treatment with alkaline phosphatase almost entirely eliminated the detected signal, indicating the requirement of phosphorylation for antibody recognition (Fig. 1*B*). These are sites phosphorylated by SPAK/OSR1, which are important for NCC activation under various conditions (23, 26). As previously reported by others (34), NCC phosphorylation increased with the low-K^+ diet (Fig. 1, *C* and *D*). Despite the increase in total NCC, the pNCC-to-NCC ratio was higher in the low-K^+ group than in the normal-K^+ group, suggesting that the increase in phosphorylation levels was not only secondary to the increase in NCC expression.

Table 1. *Physiological parameters of $WNK4^{+/+}$ and $WNK4^{-/-}$ mice on NKD, LKD, or HKD*

	$WNK4^{+/+}$ Mice		$WNK4^{-/-}$ Mice	
	Means ± SE	*n*	Means ± SE	*n*
Food intake, g				
NKD	3.2 ± 0.5	8	3.0 ± 0.4	9
LKD	2.8 ± 1.1	9	2.9 ± 0.3	10
HKD	3.3 ± 0.9	8	3.5 ± 0.2	9
Water intake, ml				
NKD	7.3 ± 1.6	8	8.1 ± 3.3	7
LKD	8.9 ± 2.9	9	7.5 ± 1.9	9
HKD	14.0 ± 3.0†	7	16.1 ± 2.5†	9
Urinary volume, μl				
NKD	2.1 ± 1.0	8	2.7 ± 1.0	9
LKD	2.4 ± 1.0	9	2.4 ± 1.6	10
HKD	5.2 ± 1.2†	8	5.3 ± 0.5†	9
Weight, g				
NKD	26.8 ± 3.1	8	25.7 ± 2.2	9
LKD	25.5 ± 2.3	9	23.4 ± 1.9	10
HKD	22.6 ± 2.7	8	22.2 ± 1.5	9
Plasma aldosterone concentration, pg/ml				
NKD	232.33 ± 88.43	7	234.27 ± 96.38	10
LKD	90.48 ± 36.23†	9	89.09 ± 56.36†	10
HKD	866.01 ± 383.49†	8	1,181.89 ± 617.79†	8
Plasma renin activity, ng ANG I·ml^{-1}·h^{-1}				
NKD	13.64 ± 7.03	7	N.D.	
LKD	N.D.		N.D.	
HKD	13.93 ± 3.12	6	N.D.	
	Urine data			
Na^+, mmol/24 h				
NKD	0.20 ± 0.04	6	0.24 ± 0.02	6
LKD	0.19 ± 0.02	8	0.16 ± 0.02	6
HKD	0.17 ± 0.01	8	0.15 ± 0.01	6
K^+, mmol/24 h				
NKD	0.63 ± 0.09	6	0.70 ± 0.06	6
LKD	0.02 ± 0.003†	8	0.03 ± 0.004†	6
HKD	2.57 ± 0.24†	8	2.02 ± 0.08†	6
pH				
NKD	7.67 ± 0.3	8	N.D.	
LKD	N.D.		N.D.	
HKD	9.07 ± 0.08†	8	N.D.	
	Plasma data			
Na^+, mM				
NKD	152.85 ± 1.55	8	154.04 ± 6.12	9
LKD	152.28 ± 2.47	9	152.57 ± 2.85	10
HKD	155.14 ± 2.05	8	156.33 ± 2.45	9
K^+, mM				
NKD	3.99 ± 0.34	8	3.26 ± 0.46*	7
LKD	3.44 ± 0.60	9	2.03 ± 0.33*†	9
HKD	4.45 ± 1.42	7	4.31 ± 1.00†	9
CO_2, mM				
NKD	14.44 ± 2.48	8	16.18 ± 3.34*	9
LKD	13.48 ± 2.13	9	17.77 ± 2.67*	10
HKD	22.15 ± 2.06†	8	26.52 ± 4.41†	9

Values are presented as means ± SE; the number of animals per group (*n*) is also shown. WNK4, with-no-lysine kinase 4; NKD, normal K^+ diet; LKD, low-K^+ diet; HKD, high-K^+ diet. Urine collected on *day 4* of the treatment period was analyzed. *$P < 0.05$ vs. $WNK4^{+/+}$ mice on the same diet; †$P < 0.05$ vs. NKD (same genotype).

Surprisingly, the high-K^+ diet induced an increase in NCC phosphorylation (Fig. 1, *C* and *D*), in contrast to the mild decrease that has been previously reported in mice fed with a high-KCl diet (10, 34). In this study, we chose to administer

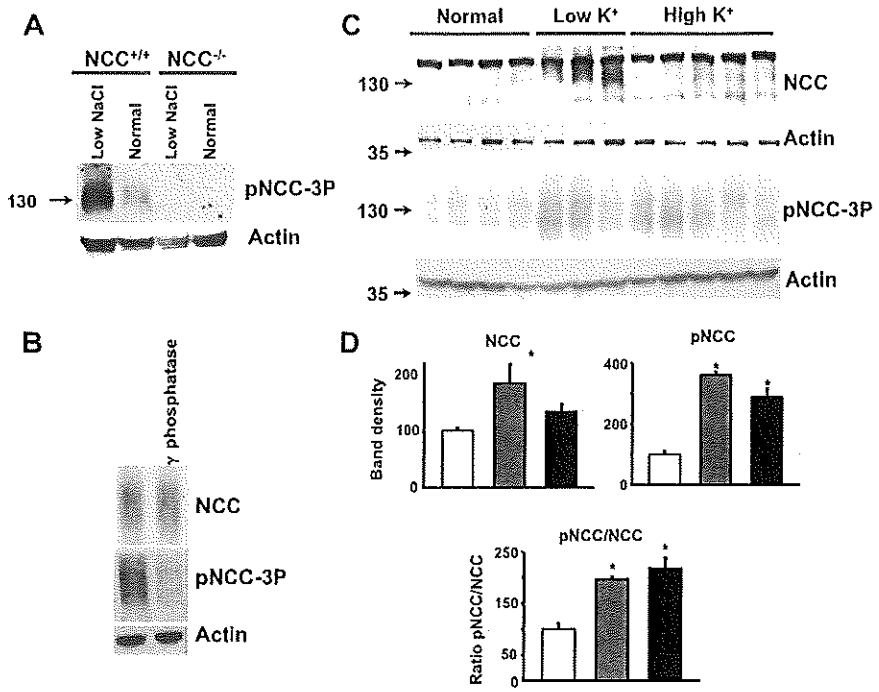

Fig. 1. Effects of varying dietary K^+ on Na^+-Cl^- cotransporter (NCC) expression and phosphorylation. *A*: specificity of the NCC phospho-antibody used in this work. The antibody recognizes three phosphorylated residues in NCC (Thr^{44}/Thr^{48}/Thr^{53}) [phosphorylated (p)NCC-3P]. Samples from wild-type ($NCC^{+/+}$) and NCC-deficient ($NCC^{-/-}$) mice kept on normal- or low-NaCl diets were analyzed by Western blot analysis. No signal was detected in lanes loaded with $NCC^{-/-}$ samples, and signal intensity was greatly reduced when samples were treated with λ-phosphatase (*B*). The low signal observed in the phosphatase-treated sample was due to incomplete dephosphorylation. Nonphosphorylated peptide was included in the antibody solution for all blots against phosphorylated epitopes. *C*: representative Western blot analysis of total kidney protein samples of wild-type mice maintained on diets with normal, low, or high K^+-citrate content. The solid band observed above the 130-kDa marker band in the NCC blots is a nonspecific band that was not included in the densitometric analyses. *D*: densitometric analyses were performed on at least two blots per assay, including samples of 6, 8, and 8 mice for the normal-K^+ (open bars), low-K^+ (shaded bars), and high-K^+ (solid bars) diet groups, respectively. The average value in the control group was fixed as 100%, and the effect of the diet was normalized accordingly. Results are expressed as mean percentages ± SE of the normal diet (100%). $^*P < 0.001$ vs. the normal diet.

K^+ as K^+-citrate to avoid possible confounding effects of high Cl^- intake. Indeed, when we fed mice with the high-KCl diet instead of high-K^+-citrate diet, we were able to reproduce the decrease in NCC expression and phosphorylation previously observed (Fig. 2). As shown in Table 1, with the high-K^+-citrate diet, mice developed a certain degree of metabolic alkalosis. It is unlikely, however, that the increased NCC phosphorylation was due to diet-induced metabolic alkalosis, because such an effect was not observed in wild-type mice in which metabolic alkalosis was induced through HCO_3^- loading (Fig. 3). In addition, it is unlikely that citrate by itself was responsible since high citrate intake, without K^+, did not stimulate an increase in NCC phosphorylation (Fig. 4). Thus, our data show that high K^+-citrate intake increased, whereas high KCl intake decreased, NCC phosphorylation, suggesting that the coadministered anions may also play a role in NCC regulation.

Low-K^+ diet promotes SPAK phosphorylation independently of WNK4. In an attempt to define the role of WNK4 in the modulation of NCC in the face of changes in dietary K^+ content, we studied the effects of low- and high-K^+ diets in $WNK4^{-/-}$ mice (4). The physiological parameters of $WNK4^{-/-}$ mice maintained on low- and high-K^+ diets were similar to those of wild-type mice, with the exception of plasma K^+ concentration (Table 1). As previously reported (4), $WNK4^{-/-}$ mice on a normal-K^+ diet displayed mild hypokalemia (3.26 ± 0.5 vs. 3.99 ± 0.3 mM in wild-type mice, $P < 0.01$), which was aggravated when mice were fed a low-K^+ diet (3.44 ± 0.6 vs. 2.03 ± 0.3 mM in wild-type mice, $P < 0.00005$). In contrast, the mild hypokalemia was corrected when mice were fed a high-K^+ diet (Table 1).

Western blot analysis of renal cortex protein extracts from $WNK4^{-/-}$ mice fed either a normal- or low-K^+ diet was performed in parallel to Western blot analysis of renal cortex protein extracts of $WNK4^{+/+}$ mice. In this set of blots, increases in NCC expression and phosphorylation in response to a low-K^+ diet were again observed in $WNK4^{+/+}$ mice (Fig. 5, *A* and *B*). Analysis of pNCC behavior was difficult in $WNK4^{-/-}$ mice because this colony exhibits low expression of the cotransporter and pNCC was undetectable in mice fed normal- and low-K^+ diets (Fig. 5, *A* and *C*). However, SPAK and OSR1 analysis was possible. Total SPAK and OSR1 expression was unchanged by diet in both $WNK4^{+/+}$ and $WNK4^{-/-}$ mice (Fig. 5). T-loop phosphorylation was not studied because it could not be detected by Western blot analysis, as previously reported (25). SPAK S-motif (Ser^{383}) phosphorylation levels increased in both $WNK4^{+/+}$ and $WNK4^{-/-}$ mice fed a low-K^+ diet. This site was originally identified as a target for WNK phosphorylation (37), and we have shown that the signal observed with this antibody

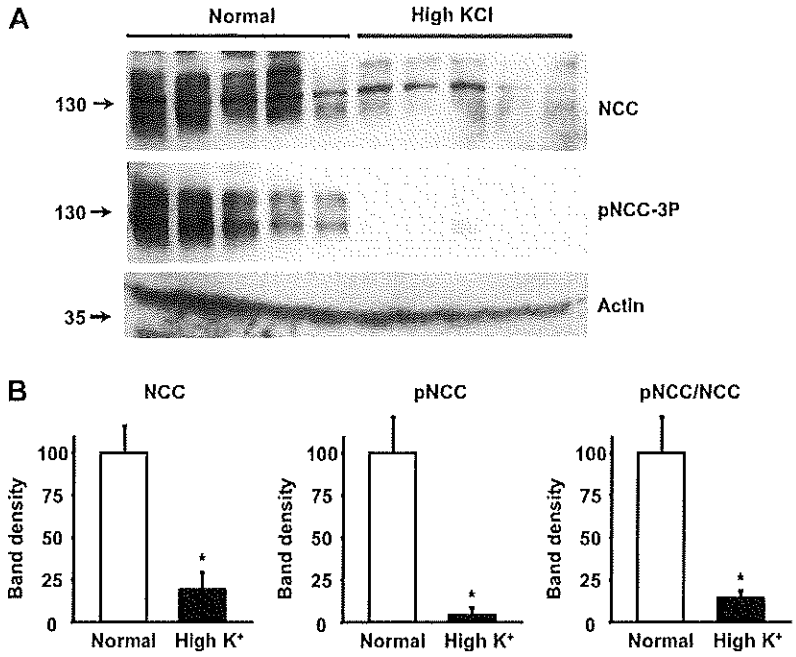

Fig. 2. Effect of the high-KCl diet on the expression and phosphorylation of NCC. *A*: representative Western blot analysis of total kidney protein samples of wild-type mice maintained on diets with normal or high KCl content. *B*: densitometric analysis of blots shown in *A*. Results are expressed as mean percentages ± SE of the normal diet (100%). *$P < 0.0001$ vs. the normal diet.

increases when SPAK is expected to be activated (4). Interestingly, this site is not only present in full-length SPAK but also in the shorter isoforms SPAK-2 and kidney-specific (KS-)SPAK (20).

Although the SPAK peptide used to generate this phospho-antibody is very similar to the corresponding OSR1 peptide (25), based on the following evidence we inferred that, in the kidney, this antibody mainly recognizes SPAK. Using the SPAK-specific antibody, we detected two bands in kidney samples, whereas in the testes, a tissue in which full-length SPAK expression is abundant (25), a single, larger band was observed (Fig. 6). Thus, the two bands observed in the kidney samples with total SPAK antibody very likely correspond to SPAK-2 and KS-SPAK (20). In contrast, with the OSR1-specific antibody, a single band of similar size was observed in both kidney and testes samples. The size of this band was clearly different from the size of the SPAK bands. With the pSPAK Ser^{383} phospho-antibody, two bands were observed in the testes, one presumably corresponding to full-length SPAK and the other one to OSR1, given their size and given that short SPAK isoforms are not expressed in the testes (20, 25). In the kidney samples, two bands were also detected with the pSPAK Ser^{383} phospho-antibody. Because these two bands were different in size from those detected in the testes but similar to those observed with total SPAK antibody, we inferred that they most likely correspond to SPAK-2 and KS-SPAK. In support of this conclusion, in a recent study, Saritas et al. (30) observed that the signal detected in the kidney with this pSPAK Ser^{382} antibody was not observed in a SPAK knockout mouse in immunofluorescence experiments, not even in the presence of vasopressin, which stimulates SPAK S-motif phosphorylation in wild-type mice. Together, the blots shown in Fig. 6 and the data presented by Saritas et al. (30) suggest that the bands observed with the pSPAK Ser^{382} phospho-antibody in the kidney samples mainly correspond to SPAK2 and KS-SPAK. In the testes samples, however, pOSR1 was indeed observed.

In our study, the intensity of the SPAK-2 and KS-SPAK bands observed with this phospho-antibody was increased with the low-K^+ diet in both $WNK4^{+/+}$ and $WNK4^{-/-}$ mice (Fig. 5). Thus, we concluded that phosphorylation of SPAK-2 and KS-SPAK increases in mice fed a low-K^+ diet and that this effect is independent of the presence of WNK4. Consistent with previous reports (20, 25), we did not observe full-length SPAK in our blots of kidney samples. However, we believe that phosphorylation of this isoform may also be increased, but nevertheless remains undetectable by this technique due to the low levels of expression.

NCC phosphorylation induced by a low-K^+ diet, but not ANG II infusion, is preserved in $SPAK^{T243A/T243A}$ mice. $SPAK^{+/+}$ and $SPAK^{T243A/T243A}$ mice fed either a normal- or low-K^+ diet had similar food intake, urinary Na^+ excretion, and weight by the end of the study period (Table 2). Mice maintained on the low-K^+ diet showed the expected reduction in urinary K^+ excretion. As previously reported, no difference was observed in plasma K^+ concentration between $SPAK^{+/+}$ and $SPAK^{T243A/T243A}$ mice on the normal diet, and both exhibited a decrease with the low-K^+ diet. However, the difference between the values observed in the normal- versus low-K^+ diets only reached significance in $SPAK^{T243A/T243A}$ mice. Immunoblot analysis of total kidney proteins showed that total NCC expression and NCC NH_2-terminal phosphorylation increased with the low-K^+ diet in both $SPAK^{+/+}$ and $SPAK^{T243A/T243A}$ mice (Fig. 7). Thus, the response of NCC expression and phosphorylation to the low-K^+ diet was present in mice expressing a catalytically inactive version of SPAK.

Given that the low-K^+ diet-induced phosphorylation of NCC is preserved in $SPAK^{T243A/T243A}$ mice, whereas the NCC phosphorylation induced by ANG II infusion is lost in

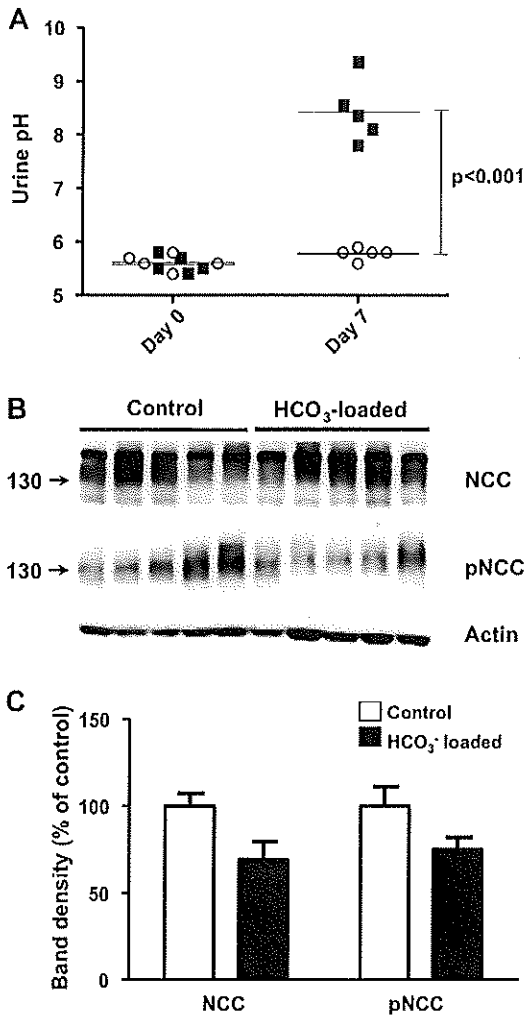

Fig. 3. Effect of HCO_3^- loading in expression and phosphorylation levels of NCC. *A*: urine pH of control mice (○) and HCO_3^--loaded mice (□) on the previous day to the beginning of treatment (*day 0*) and on *day 7* of high HCO_3^- intake. The mean urine pH for each group is indicated by the horizontal line. *B*: Western blot analysis of kidney samples from control and HCO_3^--loaded mice. *C*: densitometric analysis of the blot shown in *B*. No difference in NCC expression or phosphorylation was observed between groups.

$WNK4^{-/-}$ mice (4), we decided to analyze the effect of ANG II infusion on NCC phosphorylation in the $SPAK^{T243A/T243A}$ colony. Thus, $SPAK^{T243A/T243A}$ mice and their corresponding wild-type controls were infused with ANG II for 4 days following the same protocol used for $WNK4^{-/-}$ mice (4). Interestingly, as shown in Fig. 8, ANG II infusion resulted in a significant increase in NCC phosphorylation in wild-type mice, but this effect was not observed in $SPAK^{T243A/T243A}$ mice. Additionally, ANG II infusion increased the phosphorylation of KS-SPAK in wild-type mice. This increase was not observed in $SPAK^{T243A/T243A}$ mice, most likely because KS-SPAK phosphorylation was already increased in vehicle-treated animals. $SPAK^{T243A/T243A}$ mice have been shown to develop a Gitleman's-like syndrome, with salt-remediable hypotension (25). These mice exhibit increased activity of the renin-angiotensin system, which causes increased KS-SPAK phosphorylation in vehicle-treated animals. However, the increase in KS-SPAK phosphorylation did not result in increased NCC phosphorylation, indicating that ANG II signaling through NCC in the DCT requires integrity of the WNK4-SPAK pathway (4, 29).

High-K^+-citrate diet stimulates SPAK phosphorylation in both $WNK4^{+/+}$ and $WNK4^{-/-}$ mice. $WNK4^{-/-}$ mice maintained on a high-K^+ diet for 4 days exhibited results similar to $WNK^{+/+}$ mice. The high K^+ content in the diet did not affect food consumption (Table 1). As expected, the plasma aldosterone concentration and urinary K^+ excretion were significantly increased. The plasma K^+ concentration remained within the physiological range, and, as previously reported, the difference in plasma K^+ between $WNK4^{+/+}$ and $WNK4^{-/-}$ mice was no longer observed (4). In addition, as previously observed with high-K^+ diets (7), the urinary volume and water consumption were significantly increased in both genotypes.

With regard to the expression and phosphorylation of renal proteins, similar to what is shown in Fig. 1, in this new set of animals, we also observed that the high-K^+-citrate diet promoted increased NCC phosphorylation in $WNK4^{+/+}$ mice (Fig. 9). No pNCC was detected in $WNK4^{-/-}$ mice, even when they were fed the high-K^+ diet. Total NCC expression was unchanged by the high-K^+ diet in $WNK4^{+/+}$ and $WNK4^{-/-}$ mice. SPAK and OSR1 total expression were also unchanged, but phosphorylation of the SPAK S-motif was significantly increased in both genotypes.

Activation of NCC by the high-K^+ diet is aldosterone dependent. It has been previously described that aldosterone stimulates NCC expression, phosphorylation, and, thus, activation (16, 35). Because the plasma aldosterone concentration is greatly increased in mice fed a high-K^+ diet, we hypothesized that the increased NCC phosphorylation could be an aldosterone-induced effect. To investigate this hypothesis, we treated mice fed with either normal- or high-K^+-citrate diet with the mineralocorticoid receptor blocker spironolactone. Interestingly, in spironolactone-treated mice, the high-K^+ diet-induced increase in NCC and SPAK phosphorylation was not observed (Fig. 10), suggesting that these effects were indeed mediated by aldosterone. In contrast, NCC phosphorylation levels were reduced with the high-K^+ diet in spironolactone-treated mice.

DISCUSSION

Modulation of NCC expression/phosphorylation by changes in dietary K^+ content has been previously reported (10, 34). In this study, we began to investigate the signaling pathways involved in this modulation, which are completely unknown, and we challenged certain previously made observations. Here, we showed that a low-K^+ diet not only induced an increase in NCC NH_2-terminal phosphorylation but also in NCC total expression. In addition, we showed that a low-K^+ diet also increases the phosphorylation of SPAK-2 and KS-SPAK, the shorter isoforms of SPAK that are known to be expressed in the kidney (20). As previously described (25), in our blots, the full-length form of SPAK was not detected due to the low expression of this isoform in the kidney. However, it is accepted that, although undetectable by Western blot analysis, T-loop phosphorylation (Thr^{243}) of this isoform occurs and is

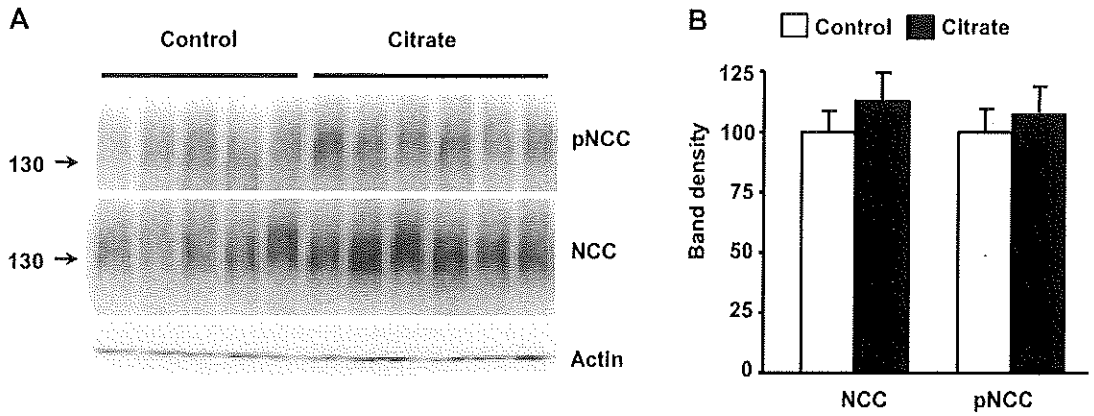

Fig. 4. Effect of high citrate intake on NCC phosphorylation. *A*: Western blot analysis of renal proteins of wild-type mice fed with a high-citrate diet. Citrate was administered in the drinking water. Both groups consumed a similar amount of water (5.16 ± 1.6 ml for the control group vs. 5 ± 1.4 ml for the citrate group). *B*: results of the densitometric analysis expressed as mean percentages ± SE of control (100%). No significant difference was observed. Three blots per assay were included in the analysis.

essential for SPAK activity within the kidney (25). In addition, although the relevance of S-motif phosphorylation is currently not as clear as that of T-loop phosphorylation, it has been shown to occur in conditions in which SPAK is expected to be activated to promote NCC function (4, 9, 35). The relevance of SPAK-2 and KS-SPAK phosphorylation in this and other situations remains unclear, given that these isoforms are predicted to be catalytically inactive (20). Anyhow, their S-motif is likely to become phosphorylated under the same stimuli that promote phosphorylation of this site in full-length SPAK. Thus, changes in the phosphorylation levels of these isoforms may be indicative of the activation state of the pathway, and here we are foreseeing that they are probably paralleled by changes in full-length SPAK phosphorylation. Thus, our data suggest that full-length SPAK is activated by low K^+ intake and is responsible for NCC phosphorylation. This, however,

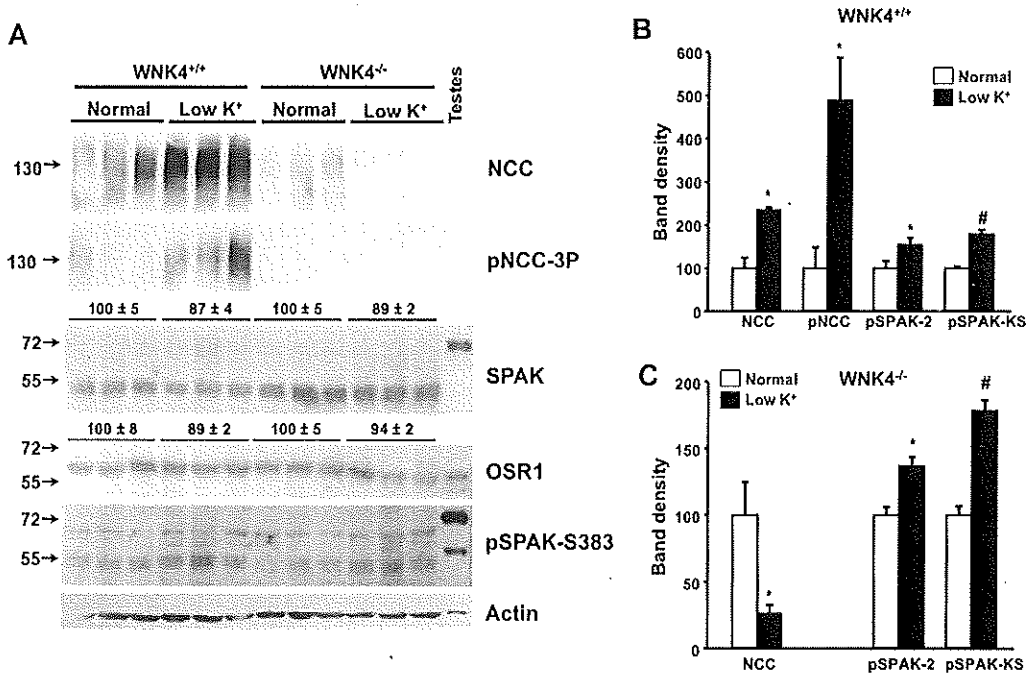

Fig. 5. Effects of low-K^+ diet on the expression and phosphorylation of Ste20-related proline-alanine-rich kinase (SPAK) and NCC in the renal cortex of with-no-lysine kinase 4 $(WNK4)^{+/+}$ and $WNK4^{-/-}$ mice. *A*: Western blot analysis of renal cortex protein samples of $WNK4^{+/+}$ or $WNK4^{-/-}$ mice kept on normal- or low-K^+ diets. Representative blots are shown. Densitometric analyses were performed on at least two blots per assay, including samples of 6 mice/group. For NCC, pNCC, pSPAK2, and the kidney-specific form of pSPAK (pKS-SPAK), the results of these analyses are shown for $WNK4^{+/+}$ mice (*B*) and $WNK4^{-/-}$ mice (*C*). For the total SPAK blot, the top lines show densitometric results for KS-SPAK bands. OSR1, oxidative stress response 1 kinase. Results are expressed as mean percentages ± SE of the normal diet (100%). $*P < 0.05$; $\#P < 0.005$ vs. the normal diet.

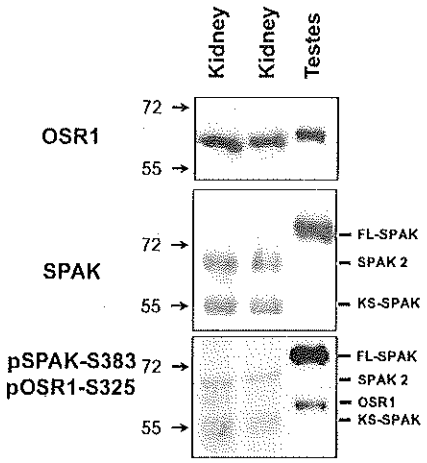

Fig. 6. Characterization of the SPAK Ser^{383}/OSR1 Ser^{325} phospho-antibody. Proteins extracted from mouse kidneys or testes were subjected to Western blot analysis with SPAK- and OSR1-specific antibodies and with a SPAK/OSR1 $pSer^{375}/Ser^{325}$ (S-motif) antibody. Labels on the right indicate to which protein each band corresponds according to the size and, in the case of the pSPAK/OSR1 blot, according to comparison with the other blots. FL-SPAK, full-length SPAK.

should be carefully considered until further investigations clarify the roles of SPAK short isoforms' phosphorylation.

OSR1 expression was indeed detected in the kidney. However, as previously reported (20), OSR1 could not be detected with pSPAK/OSR1 S-motif antibody, which was capable of detecting phosphorylated OSR1 in the testes (Fig. 6). Thus, OSR1 S-motif phosphorylation levels appear to be very low in the kidney. Nevertheless, the low-K^+-induced increase in NCC phosphorylation in $SPAK^{T243A/T243A}$ mice, which lack SPAK catalytic activity due to impaired T-loop phosphorylation, suggests that another kinase is responsible for this phosphorylation. A strong candidate for this phosphorylation is OSR1. It has been recently shown that in SPAK-deficient ($SPAK^{-/-}$) mice, OSR1 is upregulated in the DCT (30) and that OSR1 T-loop phosphorylation induced by vasopressin infusion occurs, suggesting that in $SPAK^{-/-}$ mice, OSR1 is responsible for the vasopressin-induced increase in NCC phosphorylation. Another piece of evidence suggesting that, in the absence of SPAK in the DCT, OSR1 can mediate NCC phosphorylation is that crossing $WNK4^{D561A/+}$ mice with $SPAK^{-/-}$ mice does not completely prevent the pseudohypoaldosteronism type II phenotype, whereas crossing $WNK4^{D561A/+}$ mice with $SPAK^{-/-}$ mice and $OSR1^{+/-}$ mice more efficiently reverts the phenotype (8). Thus, it is likely that NCC activation by a low-K^+ diet in $SPAK^{T243A/T243A}$ mice is due to a compensatory activity of OSR1.

The signaling pathway by which ANG II and a low-K^+ diet increased NCC phosphorylation seems to be different. We have previously shown that SPAK-NCC phosphorylation induced by ANG II does not occur in $WNK4^{-/-}$ mice (4). In this study, we show that the effect of ANG II on NCC phosphorylation is also lost in $SPAK^{T243A/T243A}$ mice, confirming that WNK4-SPAK pathway integrity is required for ANG II signaling to NCC. Additionally, we observed that phosphorylation of SPAK in $SPAK^{T243A/T243A}$ mice was already increased under basal conditions and that it was not further increased by ANG II. This could be due to the fact that in these mice, which are known to be salt depleted, a higher basal activity of the renin-angiotensin system is expected, which would probably promote higher basal levels of SPAK phosphorylation. Indeed, we have previously shown that SPAK S-motif phosphorylation is stimulated by a low-salt diet or ANG II infusion (4). This phosphorylation, however, cannot be translated into NCC activation because the T-loop site Thr^{243} cannot be phosphorylated and, thus, SPAK remains inactive (25). The observation that in $SPAK^{T243A/T243A}$ mice ANG II infusion is not translated into NCC phosphorylation suggests that OSR1 activity cannot compensate for the loss of SPAK activity under this circumstance. In contrast, the positive effect of the low-K^+ diet on SPAK phosphorylation persisted in $WNK4^{-/-}$ and $SPAK^{T243A/T243A}$ mice. Thus, WNK4 is not essential for SPAK phosphorylation under a low-K^+ diet, as opposed to what was observed for activation by ANG II, and the absence of SPAK activity in $SPAK^{T243A/T243A}$ mice is most likely compensated by OSR1 activity. It is possible that another WNK (namely, WNK1 or WNK3) may be responsible for SPAK/OSR1 activation when mice are fed a low-K^+ diet. In this regard, it is worth noting that WNK3 has been shown to induce both NCC activation (12, 27, 39) and ROMK inhibition (18), making it a suitable kinase to promote increased salt reabsorption and reduced K^+ secretion. Interestingly, although low-K^+ diet-induced SPAK phosphorylation was observed in $WNK4^{-/-}$ mice, NCC phosphorylation was not observed. These results suggest that, in the absence of WNK4, SPAK and

Table 2. *Physiological parameters of $SPAK^{+/+}$ and $SPAK^{T243A/T234A}$ mice on NKD or LKD*

	$SPAK^{+/+}$ Mice		$SPAK^{T243A/T234A}$ Mice	
	Means ± SE	*n*	Means ± SE	*n*
Food intake, g				
NKD	3.2 ± 0.3	6	2.6 ± 0.3	6
LKD	3.0 ± 0.2	6	2.4 ± 0.5	6
Water intake, ml				
NKD	7.3 ± 0.5	6	7.7 ± 0.9	6
LKD	8.8 ± 1.2	6	6.7 ± 1.0	6
Urinary volume, μl				
NKD	1.2 ± 0.2	6	1.4 ± 0.4	6
LKD	1.7 ± 0.6	6	1.3 ± 0.6	6
Weight, g				
NKD	27.0 ± 0.5	6	26.6 ± 0.6	6
LKD	26.8 ± 0.6	6	26.2 ± 1.2	6
	Urine data			
Urinary Na^+, mmol/24 h				
NKD	0.14 ± 0.03	5	0.15 ± 0.01	5
LKD	0.15 ± 0.03	4	0.10 ± 0.02	6
Urinary K^+, mmol/24 h				
NKD	0.56 ± 0.10	5	0.51 ± 0.05	5
LKD	0.02 ± 0.003†	4	0.02 ± 0.004†	6
	Plasma data			
Na^+, mM				
NKD	156.50 ± 1.87	6	157.33 ± 1.86	6
LKD	156.17 ± 2.40	6	156.17 ± 1.47	6
K^+, mM				
NKD	3.08 ± 0.56	6	3.79 ± 0.55	6
LKD	2.47 ± 0.43	6	2.60 ± 0.56†	6

Values are presented as means ± SE; the number of animals per group (*n*) is also shown. SPAK, Ste20-related proline-alanine-rich kinase. Urine collected on *day 4* of the treatment period was analyzed. †$P < 0.05$ vs. NKD (same genotype).

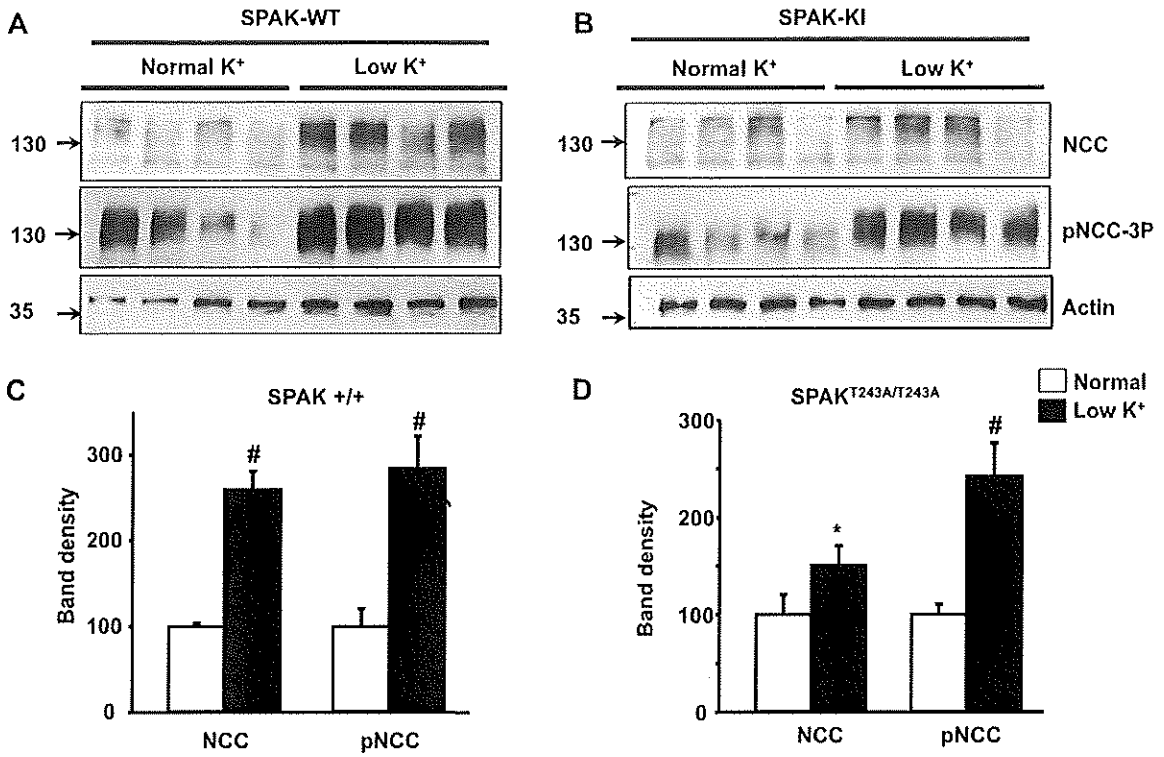

Fig. 7. Effects of low-K^+ diet on the expression and phosphorylation of NCC in $SPAK^{T243A/T243A}$ mice. *A* and *B*: Western blot analyses of total kidney protein samples of $SPAK^{+/+}$ [wild type (WT); *A*] and $SPAK^{T243A/T243A}$ [knockin (KI); *B*] mice kept on normal- or low-K^+ diets. Representative blots are shown. *C* and *D*: densitometric analyses of $SPAK^{+/+}$ (*C*) and $SPAK^{T243A/T243A}$ (*D*) mice for NCC and pNCC were performed on at least two blots per assay, including samples of seven mice for the normal-K^+ groups and six mice for the low-K^+ groups. Results are expressed as mean percentages ± SE of the normal diet (100%). $*P < 0.05$; $\#P < 0.005$ vs. the normal diet.

NCC phosphorylation are uncoupled. The reason for this phenomenon is currently unknown. In addition, in the absence of WNK4, a decrease in total NCC expression in mice fed a low-K^+ diet was observed, in contrast to the increase observed in wild-type mice. This finding may be due to the low aldosterone levels in these mice. Aldosterone is a well-defined positive modulator of NCC expression, and, in the absence of the positive stimulus (due to uncoupled SPAK-NCC phosphorylation), the aldosterone effect may be dominant.

Regarding NCC modulation under high dietary K^+ intake, our results clearly contrast with those previously reported by others (10, 34). We observed that mice maintained on a high-K^+-citrate diet had increased levels of pNCC, whereas in previous works, Vallon et al. (34) observed no changes or moderate decreases in pNCC, and Frindt and Palmer (10) reported decreased NCC apical expression. In this last work, K^+ was added to the diet as KCl. Therefore, the high Cl^- intake might have induced the decrease in NCC surface expression, masking the effects of K^+. There is no mention of the type of K^+ supplementation used by Vallon et al. (34), but it was presumably also KCl. In the present study, K^+ was added to the diet as K^+-citrate while the Cl^- and Na^+ dietary contents were kept constant to avoid confounding the effects of the different NCC modulators. Only in a selected experiment was K^+ added to the diet as KCl to confirm the results shown by others. Indeed, we observed a decrease in the expression and phosphorylation of NCC with this diet. In addition, because the mice with the high-K^+-citrate diet developed metabolic alkalosis, we performed experimental maneuvers to rule out that activation of NCC was due to the metabolic alkalosis produced by the high-K^+ diet or by citrate itself. In wild-type mice, in which metabolic alkalosis was induced by HCO_3^- loading, or in mice that were exposed to high citrate intake in the absence of increased K^+ intake, no increase in NCC expression and phosphorylation was observed. Although rats exposed to citrate alone ingested, on average, 30% less amount of citrate compared with those exposed to K^+-citrate, they still ingested an excess of citrate, and pNCC was not affected. Thus, neither metabolic alkalosis nor high citrate ion intake affect NCC phosphorylation. Additionally, we observed that increased NCC phosphorylation induced by the high-K^+-citrate diet was prevented by spironolactone, indicating that it is dependent on mineralocorticoid receptor activation by aldosterone, which is known to be increased by K^+ ingestion. Furthermore, observations made by Vallon et al. (34) also support the hypothesis of NCC upregulation caused by aldosterone in mice fed a high-K^+ diet. Although these authors observed that the high-K^+ diet induced mild decreases in NCC and pNCC, these effects were exacerbated in SGK1 knockout mice, and, for some NCC phosphorylation sites, the decrease was only observed in SGK1 knockout mice and not in wild-type mice. Because SGK1 is the aldosterone-responsive kinase that mediates certain aldosterone-induced effects, including the modulation of NCC (1), the positive effect of aldosterone may have

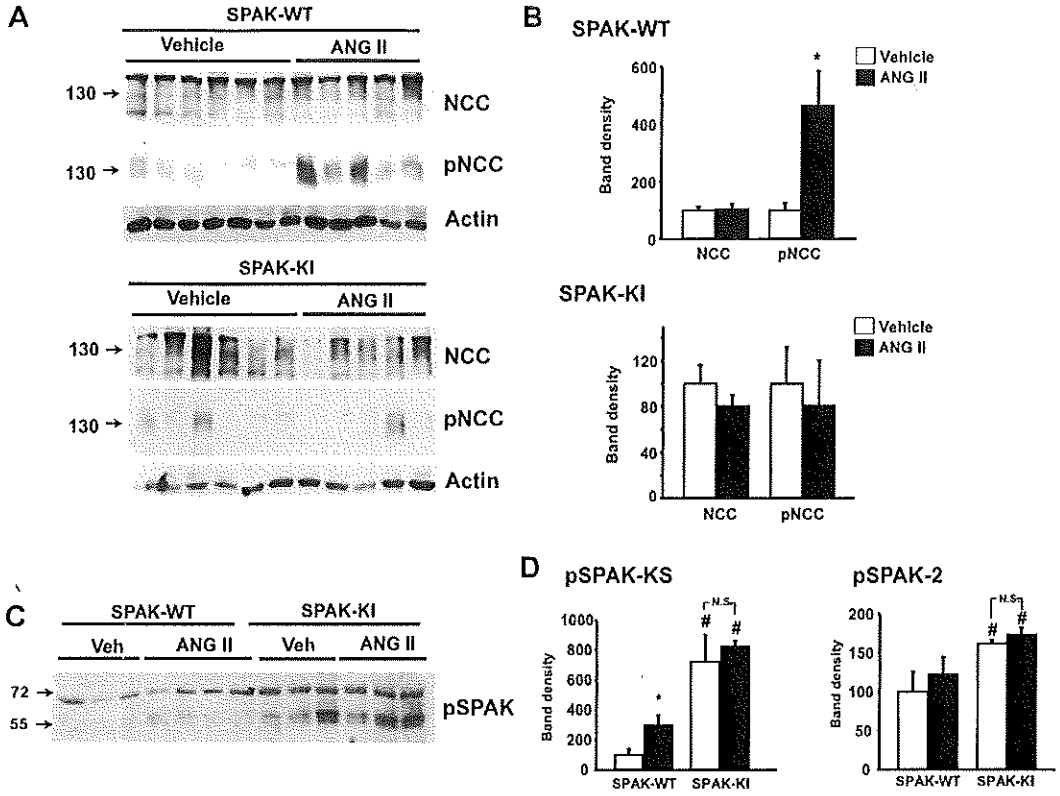

Fig. 8. Effects of ANG II infusion on the expression and phosphorylation of SPAK and NCC in SPAK$^{T243A/T243A}$ mice. *A*: Western blot analysis of NCC and pNCC in total kidney protein samples of wild-type SPAK and SPAK$^{T243A/T243A}$ mice infused with vehicle or ANG II. *B*: densitometric analyses for NCC and pNCC were performed on at least two blots per assay, including samples of six mice for the wild-type groups and seven mice for the SPAK$^{T243A/T243A}$ groups. *C*: Western blot analysis of pSPAK in total kidney protein samples of wild-type SPAK and SPAK$^{T243A/T243A}$ mice infused with vehicle or ANG II. *D*: densitometric analysis of pKS-SPAK and pSPAK-2. *$P < 0.05$ vs. vehicle; #$P < 0.05$ vs. the wild-type group.

been lost in SGK1 knockout mice, exacerbating the negative effect on NCC caused by that experimental protocol. More recently, Sornesen et al. (32) showed that acute K^+ loading in mice, achieved by administering K^+ through a gastric gavage or by allowing free access to food after a fasting period, induced a rapid decrease in NCC phosphorylation, which was related to the observed natriuresis. However, the K^+ effect was clearly independent of aldosterone, whereas in our study, the NCC phosphorylation induced by chronic administration of a high-K^+ diet was aldosterone dependent, indicating that the mechanisms mediating the acute and chronic effects are completely different. Thus, pNCC is increased when a high-K^+ diet is administered with citrate, whereas it is decreased when administered with Cl^-, suggesting that the inconsistencies between works are probably due to the anion coadministered with K^+ (24). These results suggest that high Cl^- intake may exert an inhibitory effect on NCC phosphorylation/activity. In this regard, it has been shown that NCC as well as Na^+-K^+-Cl^- cotransporter (NKCC)1 and NKCC2 (15a, 22a, 23) are modulated by intracellular Cl^- depletion. Thus, we speculate that changes in Cl^- intake may promote changes in extracellular Cl^- concentration that may eventually be translated in changes in intracellular Cl^- concentration. Although changes in plasma Cl^- concentration are not always observed in the face of changes in Cl^- intake, it is known that many physiological responses occur without an evident change in the blood levels of the physiological parameter to be modulated. Further investigation will be required to explore potential mechanisms by which high Cl^- intake is translated into a decreased pNCC-to-NCC ratio.

SPAK S-motif phosphorylation levels also increased with the high-K^+ diet in both WNK4$^{+/+}$ and WNK4$^{-/-}$ mice, and phosphorylation was prevented by spironolactone. Thus, the aldosterone-induced NH_2-terminal phosphorylation of NCC was most likely due to increased SPAK activity. WNK4 does not seem to be involved in this activation. Notably, KS-SPAK phosphorylation increased, but SPAK-2 phosphorylation did not, in contrast to what we observed with the low-K^+ diet. The meaning of this differential regulation of the phosphorylation of SPAK isoforms remains to be determined but could underlie the modulation of SPAK and NCC phosphorylation in mice fed the high-K^+ diet.

Although it has been previously proposed that decreased NCC activity under a high-K^+ diet is important to allow increased Na^+ and flow delivery to more distal nephron segments involved in K^+ secretion (2), our data suggest that this effect is achieved despite increased NCC activity because urinary volume was greatly increased in our mice on the high-K^+ diet, as previously reported (6). This observation suggests that inhibition of proximal nephron Na^+ and water reabsorption mechanisms

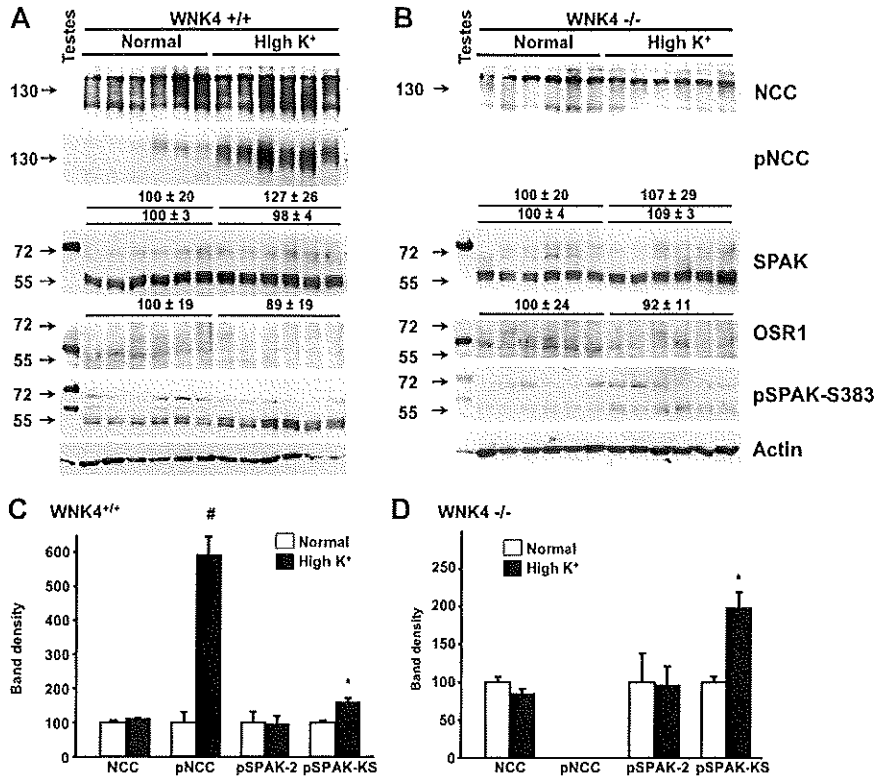

Fig. 9. Effects of high-K^+ diet on the expression and phosphorylation of SPAK and NCC in $WNK4^{-/-}$ mice. *A* and *B*: Western blot analyses of total kidney protein samples of $WNK4^{+/+}$ (*A*) or $WNK4^{-/-}$ mice (*B*) kept on normal- or high-K^+ diets. Representative blots are shown. *C* and *D*: densitometric analyses were performed on at least two blots per assay, including samples from six different mice per group, and are shown for $WNK4^{+/+}$ mice (*C*) and $WNK4^{-/-}$ mice (*D*). Results are expressed as mean percentages ± SE of the normal diet (100%). $*P <$ 0.005; $\#P < 0.00005$ vs. the normal diet.

may be more important during high K^+ intake. For instance, it has been previously reported that Na^+ reabsorption decreases under a high-K^+ diet in the proximal tubule (3) and in the thick ascending limb of Henle's loop (6, 33).

In conclusion, we propose that both low- and high-K^+-citrate diets promote NCC phosphorylation related to SPAK activation and that, under the high-K^+ diet, the effect is aldosterone dependent. On the low-K^+ diet, SPAK activity is

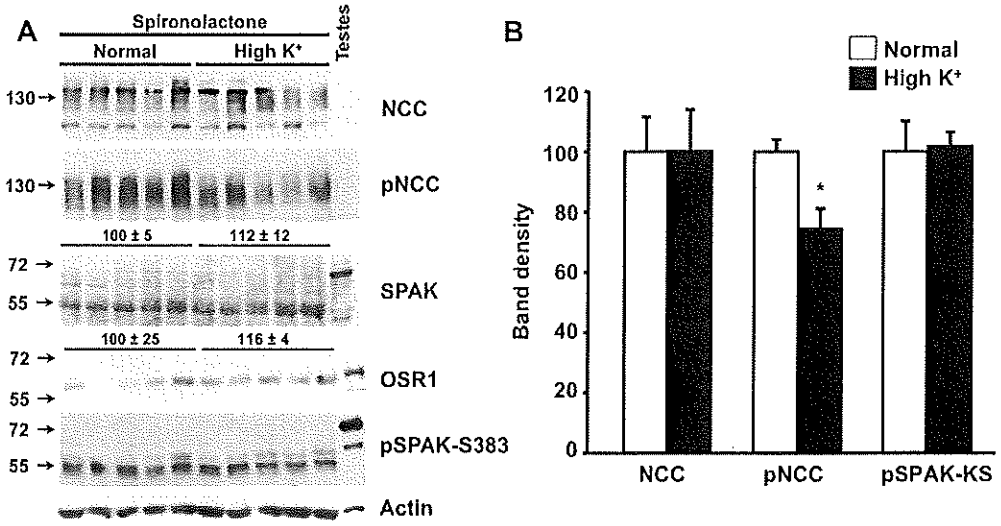

Fig. 10. Effects of high-K^+ diet on NCC and SPAK phosphorylation are blocked by spironolactone. Wild-type mice were kept on normal or high-K^+ diets. Ethanol-dissolved spironolactone or vehicle (ethanol) was added to the drinking water. The calculated dose was 40 $mg \cdot kg^{-1} \cdot day^{-1}$. *A*: Western blot analysis of total kidney protein samples of wild-type mice on normal- or high-K^+ diets treated with spironolactone. Representative blots are shown. *B*: Densitometric analyses were performed on at least two blots per assay, including samples from five different mice per group. Results are expressed as mean percentages ± SE of the normal diet (100%). $*P < 0.05$ vs. the normal diet.

apparently redundant for NCC activation, and OSR1 may be able to compensate for the loss of SPAK. The effects of low- and high-K^+ diets on SPAK phosphorylation were present in WNK4$^{-/-}$ mice, suggesting that these effects are not exclusively dependent on WNK4 and that other WNKs may be involved. This contrasts with the essential activity that WNK4 and SPAK play in the ANG II-induced activation of NCC.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.C.-B. and G.G. conception and design of research; M.C.-B., L.G.C.-P., L.R.-V., I.A.-G., N.V., and E.M. performed experiments; M.C.-B., L.G.C.-P., L.R.-V., I.A.-G., N.V., E.M., and G.G. analyzed data; M.C.-B., L.G.C.-P., L.R.-V., I.A.-G., E.M., and G.G. interpreted results of experiments; M.C.-B., L.R.-V., I.A.-G., E.M., and G.G. prepared figures; M.C.-B. and G.G. drafted manuscript; M.C.-B., L.G.C.-P., N.V., and G.G. edited and revised manuscript; M.C.-B., L.G.C.-P., L.R.-V., I.A.-G., N.V., E.M., and G.G. approved final version of manuscript.

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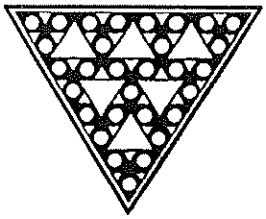

Original Article

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OPEN

Disruption of the with no lysine kinase–STE20-proline alanine-rich kinase pathway reduces the hypertension induced by angiotensin II

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Objective: The hypertensive effect of angiotensin II (AngII), a peptide hormone, is dependent on its intrarenal actions and the activation of the renal Na–Cl cotransporter (NCC), by AngII requires integrity of the with no lysine kinase/STE20-proline alanine-rich kinase (WNK/SPAK) signaling pathway. Here, we analyzed if the integrity of the WNK/SPAK pathway is required for AngII infusion to induce arterial hypertension.

Methods: We tested the effect of AngII or aldosterone administration on the blood pressure and on pNCC/NCC ratio in SPAK$^{T243A/243A}$ knock-in mice in which the kinase and thus NCC cannot be activated by WNK kinases. AngII or aldosterone was infused at 1440 or 700 μg/kg per day, respectively, for 14 days using osmotic minipumps. The aldosterone-treated mice were exposed to NaCl drinking water (1%) during the hormone administration. The arterial blood pressure was assessed using radiotelemetry.

Results: We observed that in the SPAK knock-in mice, the AngII-induced hypertensive effect was significantly reduced and associated with an absence of AngII-induced NCC phosphorylation. In contrast, the hypertensive effect of aldosterone was enhanced and was related with an increased response to amiloride, but not to thiazide-type diuretics, without a significant increase in NCC phosphorylation.

Conclusion: Our data suggest that AngII-induced hypertension requires, at least partly, NCC activation via the WNK/SPAK signaling pathway, whereas aldosterone-induced hypertension depends on epithelial sodium channel activation in a WNK/SPAK-independent manner. SPAK knock-in mice emerge as a useful model to distinguish between the effects of AngII and aldosterone on distal nephrons.

Keywords: aldosterone, distal convoluted tubule, salt transport, thiazide, with no lysine kinase 4

Abbreviations: CD, collecting duct; DCT, distal convoluted tubule; ENaC, epithelial sodium channel; NCC, Na–Cl cotransporter; SPAK, STE20-proline alanine-rich kinase; WNK, with no lysine kinase

INTRODUCTION

The arterial hypertension (AH) induced by angiotensin II (AngII) infusion depends on the intrarenal action of this hormone. The absence of AT1 exclusively in the kidney precludes the increase in blood pressure (BP) induced by AngII infusions using osmotic minipumps [1]. In the wild-type mice, this increase is associated with increased salt retention [2]. The absence of the intrarenal expression of angiotensin-converting enzyme also precludes the AngII-induced hypertension, suggesting that infused AngII induces the activation of the intrarenal renin angiotensin system; this activation then promotes salt retention and hypertension [3]. In addition, it has been shown that the salt retention and hypertension associated with AngII infusion is caused by increased salt reabsorption in the distal convoluted tubule (DCT) and the collecting duct [4,5].

The major salt transport pathway in the DCT is the thiazide-sensitive Na–Cl cotransporter (NCC). The NCC activation is associated with the phosphorylation of key threonine residues located in its amino terminus [6] by the Ste20-related proline–alanine-rich kinase (SPAK) [7]. In turn, this kinase is activated by the with no lysine kinases (WNKs) through WNK-induced phosphorylation of the threonine 243 and serine 383 of SPAK [8]. Mutation of threonine 243 in the SPAK$^{T243A/T243A}$ knock-in mice (SPAK-KI) precludes the activation of SPAK by WNKs and results in a decreased effect of SPAK on the NCC; these mice display Gitelman-like phenotype, which is

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associated with a basal reduction of NCC expression and phosphorylation levels [9]. In addition, the Mendelian disease known as familial hyperkalemic hypertension (FHH) or pseudohypoaldosteronism type II is caused by mutations in two different serine/threonine kinases, WNK1 and WNK4, and in two genes encoding the proteins KLHL3 and CUL3 that form a ring-type E3-ubiquitin ligase that targets WNK kinases for ubiquitylation and degradation [10–12]. Mutations in any of these genes result in increased expression of WNKs, which causes increased activity of the NCC via activation of the SPAK-induced phosphorylation of this cotransporter [13,14]. We have previously shown *in vitro* and *in vivo* that the presence of WNK4 is required to achieve the NCC activation by AngII [15,16], which modulates WNK4 activity and phosphorylation via a protein kinase C (PKC)-related mechanism [17]. These observations strongly suggest that the AngII effect on the NCC requires the integrity of the WNK4–SPAK–NCC pathway. Here, we present evidence that AngII-induced AH is, at least partly, dependent on the activation of the NCC by the WNK–SPAK pathway.

METHODS

Experimental protocol

SPAK-KI mice were previously generated and characterized [9]. The experimental protocols were conducted in 12–16-week old (approximately 25 g) male SPAK-KI mice and their wild-type littermates, which were bred and maintained in the animal facilities of our institution. All experiments were conducted according to the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Use Committee at our Institutions. Four separate experimental designs were followed: first, for telemetry studies SPAK-wild-type (SPAK-WT) and SPAK-KI mice were implanted and used as their own controls before and after AngII or Aldosterone infusion ($n = 5$). Second, for western blot analysis, SPAK-WT and SPAK-KI mice were infused with vehicle and compared with SPAK-WT and SPAK-KI mice infused with AngII or aldosterone ($n = 5$). BP was monitored by radiotelemetry to confirm the hypertensive effects of drugs. Third, for determining diuretic response, a single injection with hydrochlorothiazide or amiloride on SPAK-WT and SPAK-KI mice infused with AngII or aldosterone was administrated at 13th day of infusion ($n = 5$). Fourth, for studying diuretic effect on BP by radiotelemetry, a single doses of amiloride on SPAK-WT and SPAK-KI mice infused with AngII or aldosterone and with sensor implanted was injected at the 13th day of infusion ($n = 5$).

Radiotelemetry

The mice were anesthetized using LEI Medical Table Top Anesthesia Machine for isofluorane (4% for induction and 2.5% for maintenance) (LEI Medical, Portland, Oregon, USA). An incision was made in the front of the neck, and the carotid artery was separated from the jugular vein and the vagus nerve. After the artery was ligated near the head using 3–0 silk sutures, a small incision was made with a needle, and the tip of the catheter of the transmitter (model PA-C10; Data Science International, St. Paul, Minnesota, USA) was introduced into the vessel and pushed until it was close to the aortic arch. The catheter was fastened using the distal silk. To place the transmitter, a subcutaneous pocket was made. The neck incision was sutured, and the mice were allowed to recover completely.

Aldosterone and angiotensin II infusion

One week after the implantation of the radiotelemetry catheter and after 3-day 1-h of basal BP measurements, subcutaneous osmotic minipumps (model 1002; Alzet, Cupertino, California, USA) were implanted for the infusion of AngII (Sigma-Aldrich, St Louis, Missouri, USA) at 1440 μg/kg per day or aldosterone (Sigma-Aldrich) at 700 μg/kg per day for 14 days. The aldosterone-infused animals were provided with 1% saline solution as drinking water from days 0 to 14. The BP was measured using radiotelemetry (Dataquest A.R.T. system and PhysiolTel Receivers; Data Science International) every day at 1000 h for at least 1 h, with intervals of 5 s for each determination, giving 550 measures approximately for each mouse. During the 48-h period of continuous basal BP measurements, we determined the optimum time frame for reliable measurements, which was established for 1 h at 1000 h.

Na–Cl cotransporter expression and phosphorylation

At the end of the infusion period, the mice were sacrificed, and kidneys from each group were homogenized in lysis buffer containing the following: 50 mmol/l Tris–HCl (pH 7.5), 1 mmol/l ethylene-bis(oxyethylenenitrilo)tetraacetic acid, 1 mmol/l ethylenediaminetetraacetic acid, 50 mmol/l sodium fluoride, 5 mmol/l sodium pyrophosphate, 1 mmol/l sodium orthovanadate, 1% (wt/vol) Nonidet P-40 (Sigma-Aldrich), 0.27 mol/l sucrose, 0.1% (vol/vol) 2-β-mercaptoethanol and protease inhibitors (Complete tablets; Co-Ro Roche, Sigma-Aldrich). Sixty micrograms from each homogenate were resolved into 10% SDS–PAGE and transferred for 1 h to polyvinylidene difluoride membranes. Membranes were blocked in 10% skim milk and incubated overnight with sheep antipNCC (T60) antibody, and after stripping, they were incubated with anti-NCC antibody, both produced by Dario Alessi from Phosphorylation Research Unit (Dundee, Scotland, United Kingdom) that were previously used and characterized by our group [7,18] and anti β-actin (Santa Cruz Biotechnology Inc, Dallas, Texas, USA) antibodies. For densitometric analysis purpose, total NCC and pNCC were normalized with β-actin, then, total pNCC/NCC ratio was calculated.

Diuretic challenge

Another group of five SPAK-WT and five SPAK-KI mice were infused with AngII or aldosterone/NaCl 1% for 14 days. On ninth day, the mice were placed in metabolic cages for 3 days for acclimation. On 12th day, urine samples were collected for measuring basal sodium excretion. The next day (13th day), a single intraperitoneal (i.p.) injection of amiloride (Sigma-Aldrich) (5 mg/kg) or hydrochlorothiazide (Sigma-Aldrich) (50 mg/kg) was applied, and urine was collected 4 or 6 h, respectively, after diuretic injection and until 24 h for urinary sodium determination. Both collections were independent. Urine volume at 4 and 6 h

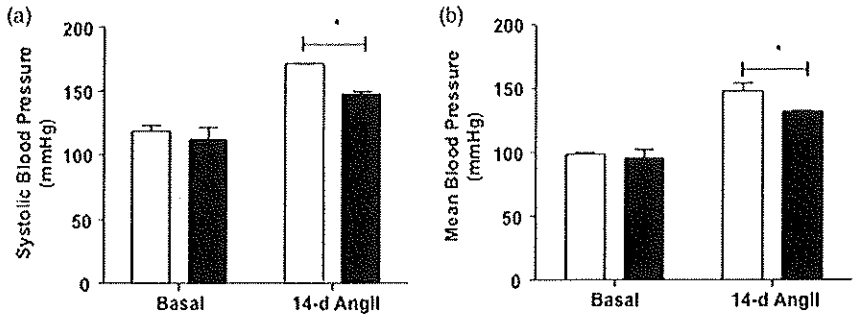

FIGURE 1 The arterial hypertension induced by angiotensin II infusion is blunted in Ste20-related proline–alanine-rich kinase-knock-in mice. (a) SBP and (b) mean blood pressure. Graphs represent SBP and mean blood pressure average of 3-day 1-h measures before angiotensin II infusion (basal) and the average of 12th, 13th and 14th-day 1-h after angiotensin II infusion (14-day) of wild-type (white bars) and Ste20-related proline–alanine-rich kinase-knock-in (black bars) mice. Blood pressure was assessed using radiotelemetry every day from 100 to 1100 h, during 17 days (before and after angiotensin II infusion). Each bar represents the mean ± standard error of approximately 550 measures of each mouse. $n = 5$ mice for each group. *P less than 0.05 wild-type vs. Ste20-related proline–alanine-rich kinase-knock-in.

was enough to measure electrolytes because we administrate a diuretic.

Diuretic effect on blood pressure in angiotensin II and aldosterone-infused-mice

Different groups of five SPAK-WT and five SPAK-KI mice were implanted with radiotelemetry sensors and infused with AngII or aldosterone and 1% NaCl in drinking water. After 13 days of infusion, the mice were challenged with a single injection of amiloride (5 mg/kg). BP was measured continuously for 2 h before and 12 h after amiloride injection.

Plasma and urinary determinations

Plasma and urinary samples were diluted 1 : 1 with distilled water and placed into an autoanalyzer (Technicon RA-1000; Bayer, Tarrytown, New York, USA) for sodium, potassium and creatinine determinations.

Statistics

All values represent the mean and the standard error for each experimental value. One-way or repeated measurements analysis of variance were performed to analyze the difference between the groups. The data were significant for P less than 0.05.

RESULTS

The hypertensive effect of angiotensin II is reduced in Ste20-related proline–alanine-rich kinase-knock-in mice

Figure 1 shows the average of SBP (Fig. 1a) and mean BP (Fig. 1b) values observed during the 3-day 1-h previous to AngII infusion at baseline and in the last 3 days of AngII administration by the osmotic minipump (days 12, 13 and 14). In the basal period, the SBP was 118 ± 4.6 and 112 ± 9.5 mmHg and the mean BP was 98 ± 1.6 and 95 ± 7.2 mmHg in the wild-type and SPAK-KI mice, respectively. The differences were NS. Thus, we did not observe the difference in BP in the SPAK-KI mice as reported previously by Rafiqi *et al.* [9]. In that work, however, the mean BP observed in the SPAK-KI mice was similar to our data, around 96 mmHg, while the mean BP of control mice was higher, around 105 mmHg.

As depicted in Fig. 1, at the end of the AngII infusion period, the SBP and mean BP were significantly higher in the wild type than in SPAK-KI animals. SBP were 171 ± 0.3 and 147 ± 2.0 mmHg and mean BP were 148 ± 6.1 and 131 ± 1.0 mmHg for wild-type and SPAK-KI mice, respectively. The increment of BP in wild-type animals was about 50 mmHg, whereas in the SPAK-KI mice was about 35 mmHg. Thus, BP increase in SPAK-KI mice was about 35% lower than in wild-type mice.

The Na–Cl cotransporter response to angiotensin II is abrogated in Ste20-related proline–alanine-rich kinase-knock-in mice

As previously shown [9], the NCC basal expression and phosphorylation levels are lower in SPAK-KI mice than in their corresponding littermates. We analyzed the effect of vehicle or AngII infusion on NCC expression and phosphorylation levels (Fig. 2). AngII induced a significant increase in the pNCC/NCC ratio in the wild-type mice. In contrast, no effect was observed in the SPAK-KI mice. These observations suggest that NCC phosphorylation and the hypertensive effect of AngII are blunted in the kidneys of the SPAK-KI mice.

The hypertensive effect of aldosterone is increased in Ste20-related proline–alanine-rich kinase-knock-in mice

We also analyzed the effect of aldosterone administration and a high-salt diet on the BP of wild-type and SPAK-KI mice. Figure 3 shows the mean for BP in the basal state and after 14 days of aldosterone administration, similar to the analysis showed in Fig. 1. SBP in wild-type mice increased from 109 ± 7.2 to 126 ± 3.8 mmHg, that is an increment of about 15 mmHg. In contrast, in the SPAK-KI mice, the observed increase was from 105 ± 2.7 to 143 ± 3.4 mmHg, for a difference of about 38 mmHg. Similar difference was observed for the mean BP. Thus, opposite to the observations for AngII, SPAK-KI mice were more sensitive than wild-type mice in response to aldosterone.

As expected, we observed an increase in the pNCC/total NCC ratio in the wild-type mice (Fig. 4a and b) that was likely due to the hypokalemia induced by aldosterone infusion (Fig. 4c). In contrast, the increased in pNCC did not occur in the SPAK-KI mice treated with aldosterone,

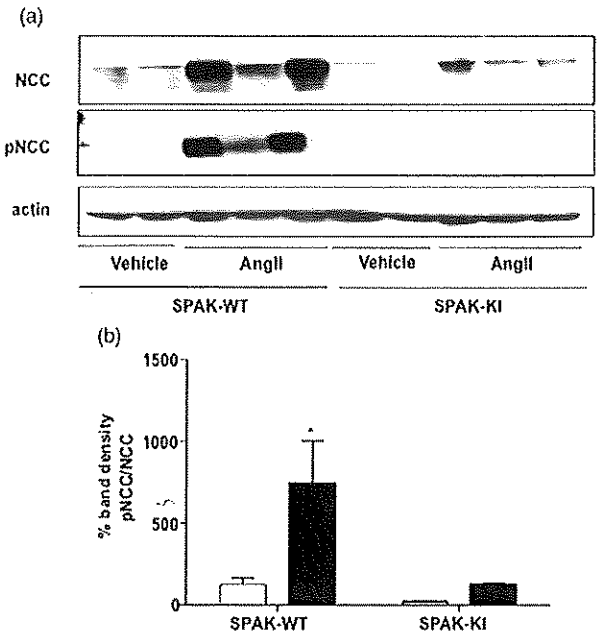

FIGURE 2 Angiotensin II infusion induced an increase in Na–Cl cotransporter expression and phosphorylation in wild-type, but not in Ste20-related proline–alanine-rich kinase-knock-in mice. (a) Representative western blot for total Na–Cl cotransporter, phosphor-Na–Cl cotransporter and β-Actin from proteins extracted from the kidney of wild-type or Ste20-related proline–alanine-rich kinase-knock-in mice infused with vehicle or angiotensin II, as stated. (b) Densitometric analysis of two independent western blot for total Na–Cl cotransporter and phosphor-Na–Cl cotransporter expressed as the pNa–Cl cotransporter/total Na–Cl cotransporter ratio after β-actin normalization. Open bars indicate vehicle infusion and closed bars indicate angiotensin II infusion. *P less than 0.0001 vs. all other groups.

despite a similar reduction in plasma potassium, supporting, as has been previously shown, that with no lysine kinase/STE20-proline alanine-rich kinase (WNK/SPAK) pathway is also required to achieve the NCC phosphorylation due to hypokalemia [19,20].

Na–Cl cotransporter and epithelial sodium channel involvement in the hypertensive effect of aldosterone in Ste20-related proline–alanine-rich kinase-knock-in

To analyze the activated pathway associated with the effect of aldosterone, we used the wild-type and SPAK-KI mice infused with aldosterone and measured the diuretic response to a single injection i.p. of hydrochlorothiazide or amiloride, which are well known blockers of the NCC and the epithelial Na^+ channel, epithelial sodium channel (ENaC), respectively. As shown in Fig. 5, the natriuretic response to amiloride was significantly higher in the SPAK-KI mice than in the wild-type mice. In contrast, the response to the thiazide diuretic was higher in the wild-type mice than in the SPAK-KI mice. These results suggest that the hypertensive effect of aldosterone in SPAK-KI mice was associated with ENaC activation, rather than NCC activation. We then proceeded to further analyze the role of ENaC in the development of hypertension in the SPAK-KI mice. We tested the effect of amiloride administration in BP after 10 days of aldosterone infusion in both SPAK-KI and wild-type mice. During the first 5 h after i.p. injection of amiloride, a marked reduction in SBP was observed in the SPAK-KI group (140–105 mmHg) but not in the wild-type mice (Fig. 6). This effect was attenuated in the following hours, consistent with the marked decrease in amiloride-induced natriuresis observed after the initial hours of dosage. Nevertheless, a lower SBP was maintained compared with the baseline measurements registered during aldosterone infusion without amiloride. This phenomenon was not observed in the wild-type group. The amiloride injection on SPAK-KI and wild-type mice infused with AngII did not show any effect on BP (Supplementary Fig. 1, http://links.lww.com/HJH/A843).

DISCUSSION

It is known that AngII-induced increase in arterial BP is due to the intrarenal effects of this peptide hormone [2] and several lines of evidence suggest that NCC activation could be responsible, at least partly, for the increased salt reabsorption that precedes the development of hypertension in mice infused with AngII [2]. We have proposed that the AngII effect on the NCC requires the integrity of the WNK-SPAK pathway. By using *Xenopus laevis* oocytes as an expression system, we observed that the activation of NCC by AngII requires the presence of WNK4 [15]. In mpkDCT cells, AngII induced an increase in the phosphorylation of both the SPAK and the NCC [15,21]. Then, we demonstrated that the SPAK phosphorylation induced by a low-salt diet or AngII in wild-type mice was not observed in the WNK4 knockout mice, suggesting that the presence of WNK4 is required for AngII to induce SPAK–NCC phosphorylation

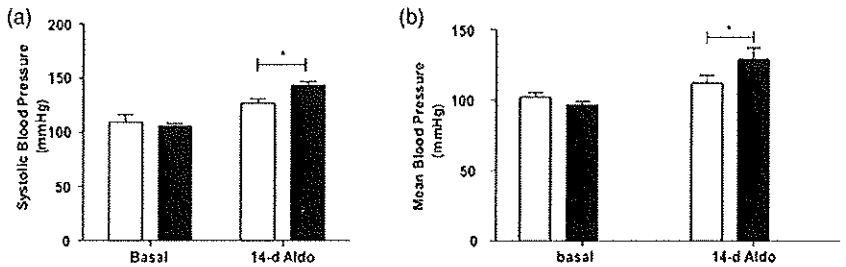

FIGURE 3 The effect in blood pressure induced by aldosterone infusion was higher in Ste20-related proline–alanine-rich kinase-knock-in mice. (a) SBP and (b) mean blood pressure. Graphs represent SBP and mean blood pressure average of 3-day 1-h measures before aldosterone infusion (basal) and the average of 12th, 13th and 14th-day 1-h after aldosterone infusion (14-day) of wild type (white bars) and Ste20-related proline–alanine-rich kinase-knock-in (black bars) mice. Blood pressure was assessed using radiotelemetry every day from 1000 to 1100 h, during 17 days (before and after aldosterone infusion). Each bar represents the mean ± standard error of approximately 550 measures of each mouse of each day. $n = 5$ mice for each group. *P less than 0.05 vs. Ste20-related proline–alanine-rich kinase-knock-in.

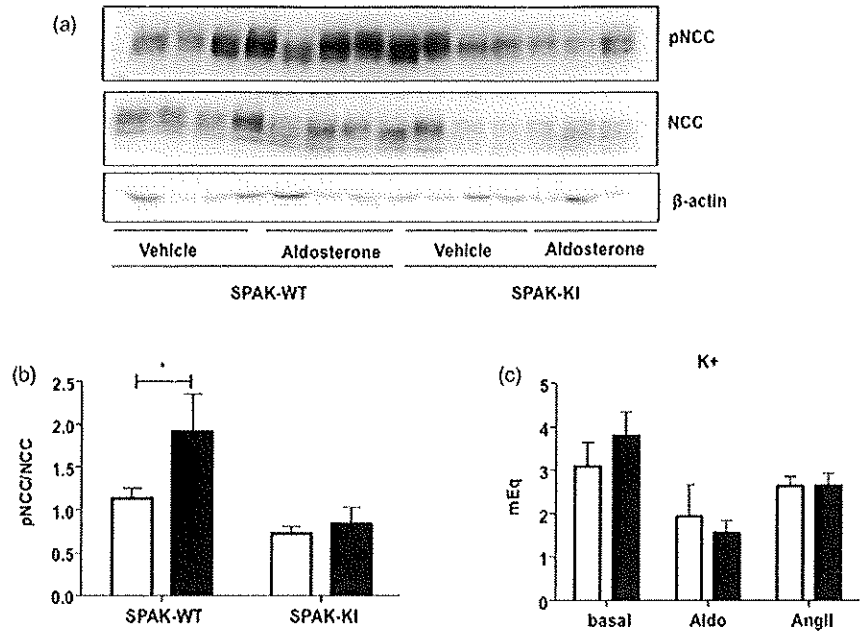

FIGURE 4 Aldosterone infusion increased the pNa–Cl cotransporter/Na–Cl cotransporter ratio in wild-type mice, but not in Ste20-related proline–alanine-rich kinase-knock-in mice. (a) Representative western blot for total Na–Cl cotransporter, phosphor-Na–Cl cotransporter and β-actin of proteins extracted from the kidney of wild-type or Ste20-related proline–alanine-rich kinase-knock-in mice infused with vehicle or Aldosterone, as stated. (b) Densitometric analysis of two independent western blot for total Na–Cl cotransporter and phosphor-Na–Cl cotransporter, expressed as the pNa–Cl cotransporter/total Na–Cl cotransporter ratio after β-actin normalization. Open bars indicate vehicle infusion, and closed bars indicate aldosterone infusion. (c) Plasma potassium levels after 13-day treatment with aldosterone or angiotensin II in STE20-proline alanine-rich kinase-WT and Ste20-related proline–alanine-rich kinase-knock-in mice. $n = 5$. Open bars show STE20-proline alanine-rich kinase-WT mice and close bars show Ste20-related proline–alanine-rich kinase-knock-in mice. *P less than 0.05 vs. Ste20-related proline–alanine-rich kinase-knock-in basal.

[16]. Supporting that AngII effects on DCT require the presence of WNK4, it was observed that AngII, via the PKC pathway, induces KLHL3 phosphorylation in the serine 433 precluding the effect of KLHL3–CUL3 complex on WNK4 and thus, preventing WNK4 ubiquitylation and its destruction [22]. Furthermore, the disruption of the SPAK-NCC cascade via crossing $WNK4^{D561A/-}$ mice (which recapitulate a FHH phenotype) with $SPAK^{-/-}$ mice has been proven to correct the hypertensive and hyperkalemic phenotype associated with NCC hyperactivity [23]. Most recently, we have shown that AngII via PKC phosphorylates WNK4 in key residues increasing the activity of the kinase toward SPAK and NCC [17]. Given these lines of evidence, we used SPAK-KI mice to assess the role of the WNK4–SPAK–NCC pathway in the AngII-induced increase in BP [8].

Our results suggest that, indeed, the AngII-induced hypertension is at least partly dependent on the NCC activation via the SPAK pathway. AngII infusion using a minipump with pressor dose was associated with increased phosphorylation of the NCC in wild-type mice. In contrast, the absence of SPAK activity precluded the phosphorylation of NCC by AngII. Radiotelemetry measurements of BP revealed that AngII-induced hypertension was partially blunted in SPAK-KI mice. The BP still increased, indicating that there are other pathways in the kidney through AngII induces hypertension in addition to the activation of SPAK-NCC. One possibility is that the activation of ENaC plays a

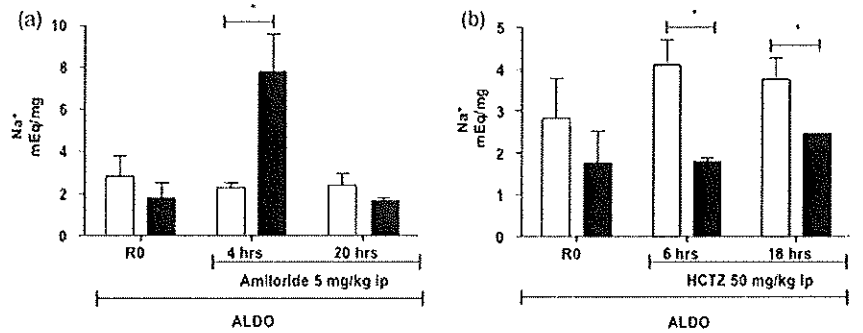

FIGURE 5 Diuretic challenge with hydrochlorothiazide or amiloride in STE20-proline alanine-rich kinase-WT and Ste20-related proline–alanine-rich kinase-knock-in mice infused with aldosterone and 1% NaCl in the drinking water. Mice were kept in a metabolic cage to determine the basal urinary sodium excretion (adjusted per volume as mEq sodium over mg of creatinine in urine) and the urinary sodium excretion after a single i.p. injection of hydrochlorothiazide (50 mg/kg) or amiloride (5 mg/kg). Bars represent mean ± standard error in wild-type (white) and Ste20-related proline–alanine-rich kinase-knock-in (black), before (R0), 4 and 20 h after amiloride administration, and 6 and 18 h after hydrochlorothiazide administration. $n = 5$. *P less than 0.05 vs. Ste20-related proline–alanine-rich kinase-knock-in at 4, 6 and 18 h.

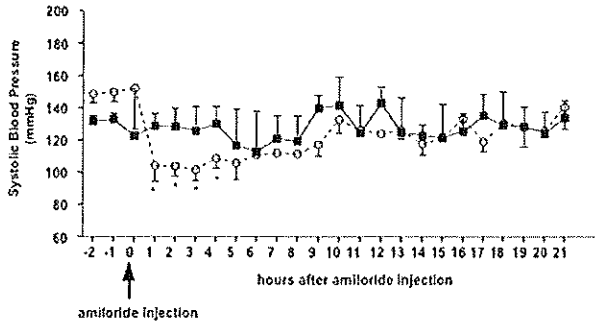

FIGURE 6 Effect of amiloride injection in blood pressure in aldosterone-infused and 1% Na–Cl Ste20-related proline–alanine-rich kinase-knock-in and STE20-proline alanine-rich kinase-WT mice. Closed squares indicate wild-type mice, and open circles indicate Ste20-related proline–alanine-rich kinase-knock-in mice. By telemetric measured, the response to a single doses of amiloride was tested on 1% Na–Cl-aldosterone-infused Ste20-related proline–alanine-rich kinase-knock-in and STE20-proline alanine-rich kinase. The blood pressure was recorded continuously 2 h before and 12 h after amiloride administration. Each point represents the mean ± standard error of approximately 550 measurements equivalent to 1 h of recording for each mouse. $n = 5$ mice from each group. *P less than 0.05 vs. Ste20-related proline–alanine-rich kinase-knock-in.

role in this response to AngII, because this hormone also increases the activity of this channel [5,24,25]. In addition, AngII also has positive effects on the proximal tubule $Na^+ : H^+$ exchanger, NHE3 [26], as well as in the expression and cleavage of ENaC, both in the cortex and in the medulla which might also be important for salt reabsorption in this setting [27].

Another potential mechanism could implicate blood vessels. It has been suggested that WNK–SPAK pathway is capable to regulate contractibility of the vessels. It is known that NKCC1, that is a target of SPAK, is expressed in vascular smooth muscle cells. Bergaya *et al.* [28], showed that reduction of WNK1 expression in the $WNK1^{+/-}$ prevented phenilephrine-induced vasocontraction of aorta rings and mesentery vessels. However, AngII-induced vasocontraction was similar between control and $WNK^{+/-}$ mice, suggesting a specific alpha-adrenergic activation when the pathway is incomplete [3,28]. Zeniya *et al.* [29] showed that KLHL2, homologue of KLHL3, is present in aorta and vascular smooth muscle cells and AngII diminished its expression and augmented WNK3 expression [29], also suggesting the participation of WNKs in vascular tone.. In addition, the SPAK null mice that express a Gitelman-like phenotype (hypotension, hypokalemia and alkalosis) exhibits a decrease in the phosphor-NKCC1 in blood vessels [30] and reduced response to phenylephrine, suggesting that activation of NKCC1 by SPAK may play a role on vasoconstriction. This would explain the differences in BP in the initial days of AngII treatment, during which the effect of AngII on BP might not be entirely attributed to NCC activation. The observation that AngII-induced hypertension is significantly blunted in the absence of SPAK activity supports the proposal that strategies preventing SPAK–NCC interactions could be a new therapeutic avenue for hypertension [31].

In contrast to the observations made with the AngII infusion in the current study, we observed a significant increase in BP in response to the combination of aldosterone and high salt-diet in the SPAK-KI mice. The absence of aldosterone-induced hypertension in the wild-type mice might be explained by the C57BL/6J genetic background of the SPAK-WT and SPAK-KI mice, because C57BL/6J mice are often resistant to aldosterone/salt treatment [32]. The SPAK-KI mice, however, developed a significant increase in BP. After aldosterone infusion, we observed a significantly higher response to amiloride-induced natriuresis accompanied by marked decrease in arterial BP in SPAK-KI mice. In contrast, the response to hydrochlorothiazide was higher in the wild-type mice than in the SPAK-KI mice, consistent with the lower expression of NCC and pNCC in the SPAK-KI mice. In fact, the expected increase of pNCC during aldosterone infusion due to the development of hypokalemia was observed in the wild type, but not in the SPAK-KI mice, indicating that NCC response to changes in serum potassium also requires the integrity of the WNK/SPAK pathway.

Our observations thus suggest that aldosterone-induced hypertension in SPAK-KI mice is mostly associated with the activity of the ENaC in the collecting duct. This is supported by the observations of Rafiqi *et al.* [9], who demonstrated that the expression of all three ENaC subunits was increased in SPAK-KI mice under normal serum aldosterone levels, both in regular and low-salt diet. Of note, due to the decreased expression of NCC and increased expression of ENaC, SPAK-KI mice are thus resistant to AngII-induced hypertension, but particularly sensitive to aldosterone-induced hypertension. As a conclusion, the disruption of the WNK–SPAK pathway attenuates the AH and NCC phosphorylation induced by AngII, while it enhances the aldosterone-induced hypertension mediated by ENaC.

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Conflicts of interest

There are no conflicts of interest.

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Reviewers' Summary Evaluations

Reviewer 2

The significance of the present study lies on the differential novel role of WNK-SPAK pathway in hypertension. The disruption of the WNKSPAK pathway attenuates the arterial hypertension and NCC phosphorylation induced by angiotensin II, while it enhances the aldosterone-induced hypertension mediated by ENaC. The weaknesses of the study lie in the lack of exploring deep mechanistic and see whether the reduction in blood pressure is associated with vascular and/or heart structure and function improvement.

Reviewer 3

The authors investigate whether the WNK/SPAK (with no lysine kinases/Ste20-related proline-alanine-rich kinase) is required for angiotensin II induced arterial hypertension using SPAK knock-in with mutation of threonine 243. This mutation prevents activation of SPAK (SPAK and SPAK-2) by WNKs, decreasing the effect of SPAK on the thiazide-sensitive Na–Cl cotransporter. They provide convincing evidence that angiotensin II dependent hypertension is partially dependent on this pathway whereas aldosterone-induced hypertension depends on epithelial sodium channel activation. The study provides novel mechanistic insights as to how angiotensin II-mediated signaling contributes arterial hypertension but clinical translation may be limited given the availability of thiazides.

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Review

Regulation of the renal Na^+-Cl^- cotransporter by phosphorylation and ubiquitylation

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Gamba G. Regulation of the renal Na^+-Cl^- cotransporter by phosphorylation and ubiquitylation. *Am J Physiol Renal Physiol* 303: F1573–F1583, 2012. First published October 3, 2012; doi:10.1152/ajprenal.00508.2012.—The activity of the renal thiazide-sensitive NaCl cotransporter (NCC) in the distal convoluted tubule plays a key role in defining arterial blood pressure levels. Increased or decreased activity of the NCC is associated with arterial hypertension or hypotension, respectively. Thus it is of major interest to understand the activity of NCC using in vivo models. Phosphorylation of certain residues of the amino-terminal domain of NCC has been shown to be associated with its activation. The development of phospho-specific antibodies against these sites provides a powerful tool that is helping to increase our understanding of the molecular physiology of NCC. Additionally, NCC expression in the plasma membrane is modulated by ubiquitylation, which represents another major mechanism for regulating protein activity. This work presents a review of our current knowledge of the regulation of NCC activity by phosphorylation and ubiquitylation.

WNK4; distal tubule; ion transport; hypertension; diuretics

THE RENAL THIAZIDE-SENSITIVE NaCl cotransporter (NCC) is the major salt transport and limiting step for salt reabsorption in the distal convoluted tubule (DCT) of mammalian kidneys. The role of NCC in the regulation of arterial blood pressure and in the renal ability for potassium, calcium, and proton excretion has been firmly established by the clinical effects of reduced or augmented activity of the cotransporter. Inactivating mutations in the *SLC12A3* gene encoding NCC results in Gitelman's disease, in which patients exhibit hypokalemic metabolic alkalosis, arterial hypotension, and hypocalciuria. In contrast, an increased activity of NCC, as a result of mutations in the with no lysine kinases WNK1 or WNK4 genes, produces the mirror image condition: hyperkalemic metabolic acidosis, accompanied by arterial hypertension, and hypercalciuria. This condition is known as Gordon syndrome, pseudohypoaldosteronism type II (PHAII), or familial hyperkalemic hypertension (FHHt) (27). The thiazide-type diuretics that specifically inhibit NCC have been used for years and are recommended as the first-line pharmacological therapy for arterial hypertension (16). Finally, in an open population, rare inactivating mutations in one allele of NCC, which reduce the activity of the cotransporter (3), are associated with reduced blood pressure, lower risk for arterial hypertension, and no cardiovascular mortality (3, 40). Thus the activity of NCC plays a fundamental role in cardiovascular physiology and pathophysiology.

The study of NCC was practically impossible for years because of the lack of a native cell model purified from the DCT that exhibited thiazide-sensitive Na^+ transport activity. The first tool for studying NCC using in vivo models was developed by Fanestill and coworkers (7) at the end of the 1980s: binding of the tracer [^{3}H]metolazone to crude plasma membranes extracted from rat or mouse renal cortex was assessed. The authors demonstrated that [^{3}H]metolazone binds to both a low- and a high-affinity site in the renal cortex. The high-affinity site showed several binding characteristics, demonstrating that the tracer was actually binding to NCC, then called the thiazide receptor. These characteristics included the absence of high-affinity sites in any other tissue, including the renal medulla; displacement of the tracer by different thiazide-type diuretics, but not by any other tested drug, with a similar potency to thiazide in clinical studies; and the localization of these sites only to the DCT as confirmed using autoradiography (22). A few years later, however, better tools were developed to study NCC in both in vitro and in vivo models. First, expression cloning was used to identify the cDNA encoding the NCC from winter flounder (*Pseudopleuronectes americanus*) urinary bladder (30). Next, the mammalian NCC cDNA was isolated using a homology approach, first from rat kidney (29) and later from human (53, 80), mouse (48), and rabbit (91) kidney. With the molecular identification of NCC, it became possible to generate antibodies against the cotransporter that allowed the immunolocalization of NCC specifically to the DCT, with more prominent expression in the early portion of the DCT (DCT1) than in the later portion (DCT2) (69). Together with probes and specific primers for assessing mRNA expression, the effects of several stimuli on NCC mRNA and protein expression levels (44) were studied using animal models to understand the role of NCC in physiological and pathophysiological conditions. NCC, and therefore DCT, was identified as a key site for modulation of NaCl transport and hence,

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for blood pressure regulation (21). However, it quickly became obvious that many stimuli do not affect NCC expression levels. Thus assessing the activity of the cotransporter, in addition to the expression level, would be required for a better understanding of the role of the cotransporter in many situations.

NCC Activity Correlates with Phosphorylation

The regulation of NCC activity by the phosphorylation of conserved threonine and serine residues in the amino-terminal domain was first demonstrated by Pacheco-Alvarez et al. (65). They first demonstrated in *Xenopus laevis* oocytes expressing NCC that the activity of NCC dramatically increased when the intracellular chloride concentration was lowered using two different techniques (Fig. 1*A*). One method involved low chloride hypotonic stress, in which incubation of oocytes in a light hypotonic medium (170 instead 210 mosmol/kgH_2O) without chloride (substituted by isothaionate) promotes the opening of endogenous chloride channels (2) that efficiently decrease the intracellular chloride concentration (42). The other was the coinjection of oocytes with NCC cRNA, together with the cRNA of K^+-Cl^- cotransporter KCC2. This isoform of KCC remains active in isotonic conditions

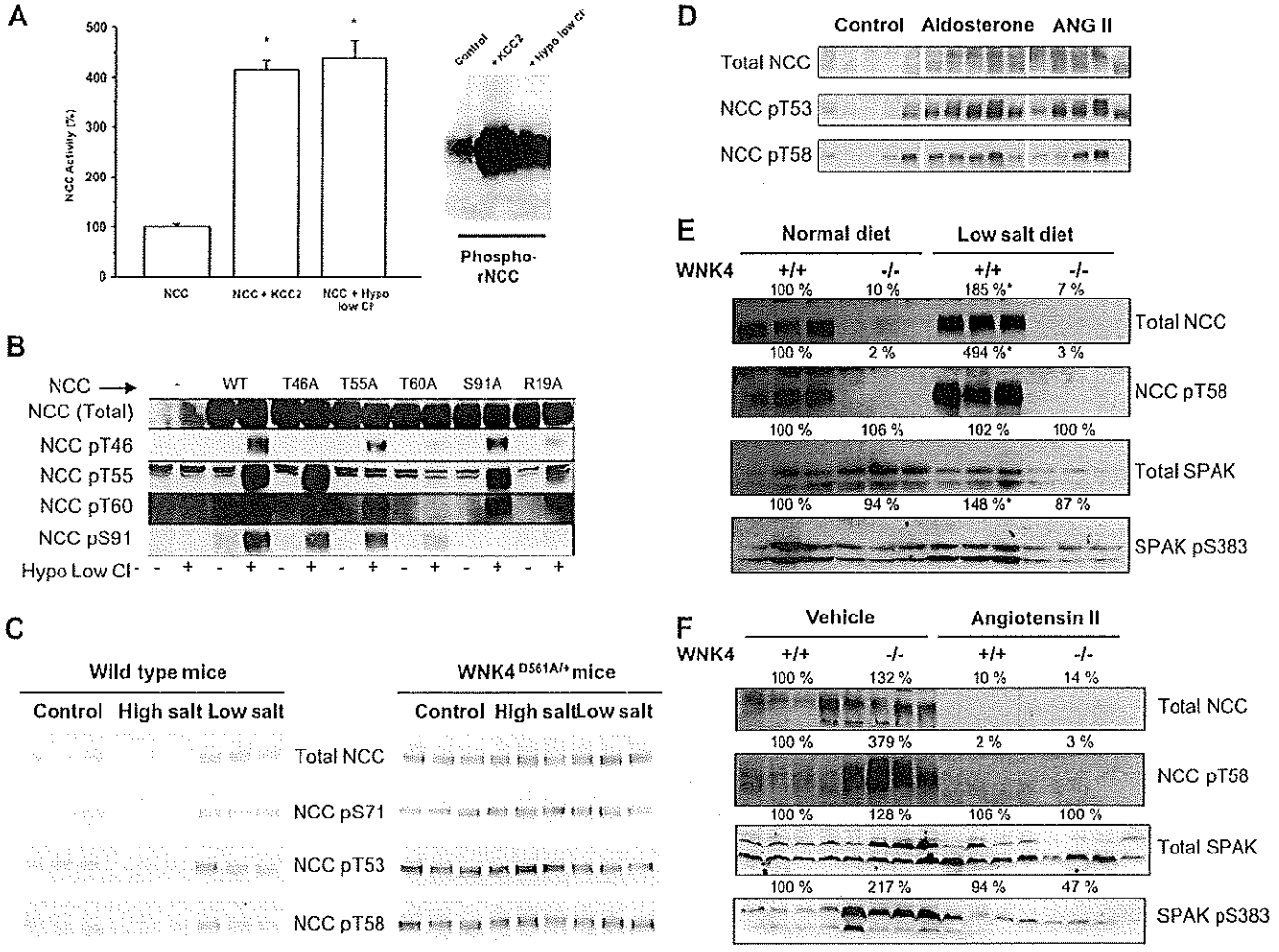

Fig. 1. Activity of the renal thiazide-sensitive NaCl cotransporter (NCC) is modulated by phosphorylation. *A*: percent NCC activity in the heterologous expression system of *Xenopus laevis* oocytes is increased by 2 different maneuvers inducing intracellular chloride depletion (*$P < 0.001$ vs. NCC alone). This activation is associated with increased phosphorylation of threonine residues 53 and 58, as detected with the phospho-specific antibody R5 (modified from Ref. 65). *B*: detection of total NCC or phosphorylated NCC in threonine residues 46, 55, 60 or serine residue 91 using specific phospho-antibodies rose against each site, in extracted proteins from HEK-293 cells transiently transfected with wild-type or mutant NCC, as stated. The blots are shown in control conditions or after exposing of the cells to low chloride hypotonic stress, as stated (modified from Ref. 72 with permission). *C*: Western blot analysis of renal proteins from wild-type or with no lysine kinase $WNK4^{D561A/+}$ mice exposed to control, a high-salt, or low-salt diet. Western blots were performed using the total anti-NCC antibody, or specific antibodies against phosphorylated threonine 53, threonine 58, or serine 71, as stated (modified from Ref. 15 with permission). *D*: NCC expression and phosphorylation at residues T53 or T58 in adrenalectomized rats treated with vehicle, aldosterone, or angiotensin II infusion (modified from Ref. 89 with permission). *E*: NCC expression and phosphorylation at T60 and Ste20-related proline/alanine-rich kinase (SPAK) expression and phosphorylation at S383, as stated, in wild-type or WNK4 total knockout mice exposed to a normal- or low-salt diet, as stated (modified from Ref. 12). *F*: NCC expression and phosphorylation at T60 and SPAK expression and phosphorylation at S383, as stated, in wild-type or WNK4 total knockout mice infused with vehicle or angiotensin II (modified from Ref. 12).

(83) and was therefore expected to promote a continuous extrusion of chloride ions from the oocytes during the incubation period. The efficiency of KCC2 in decreasing the intracellular chloride concentration was later demonstrated by Bertram et al. (8). At the time of these findings, there were no phospho-specific NCC antibodies. Therefore, the NKCC1 phospho-specific antibody (R5) developed by Forbush and coworkers (26) was used. However, for the R5 antibody to recognize phospho-NCC, a single amino acid change was made to rat NCC. A tyrosine residue in position 56 was changed to a histidine, which is the residue in the same position of Na^+-K^+-$2Cl^-$ cotransporters NKCC1 and NKCC2 that is recognized by R5 antibodies. It was observed that this single point mutation did not change the functional properties of NCC or its activation by intracellular chloride depletion.

As shown in Fig. 1*A*, the increased activity of NCC induced by intracellular chloride depletion was associated with increased phosphorylation of threonine residues 53 and 58, as detected with the R5 antibody (65). An increased signal in activated NCC was not observed in the presence of alkaline phosphatase, indicating that it was a result of phosphorylation. Eliminating threonine residues 53 and 58, as well as serine residue 71, was associated with decreased basal activity of NCC and the complete prevention of the activation by intracellular chloride depletion, particularly when T58 was eliminated. Interestingly, the decreased activity observed in the triple mutant of NCC (T53A, T58A, and S71A) was not associated with a change in the expression level at the cell surface. Thus this study demonstrated that increased phosphorylation of amino-terminal threonine/serine residues in NCC are correlated with increased activity of the cotransporter. Therefore, "NCC activity" could be indirectly assessed by analyzing NCC phosphorylation with in vivo models. Moreover, a subsequent study showed that, in *X. laevis* oocytes, NCC activity is significantly reduced by coexpression with protein phosphatase 4 and that T58 is the target threonine for dephosphorylation by this phosphatase (32).

After this observation, specific phospho-antibodies for detecting equivalent threonine/serine residues in human or mouse NCC were raised (72, 99). Richardson et al. (72) used mass spectrophotometric analysis of NCC protein extracted from NCC-transfected HEK-293 cells and observed that low chloride hypotonic stress resulted in the phosphorylation of threonine residues 46, 55, and 60 and serine residue 91 of human NCC. This finding corroborated the importance of T53 and T58 of rat NCC (equivalent to 55 and 60 in human NCC) and added two new sites. Specific phospho-antibodies were raised for each site and used in transfected HEK-293 cells and in mpkDCT cells endogenously expressing NCC. Thus it was confirmed that the low chloride hypotonic stress increased the phosphorylation of these sites (Fig. 1*B*). There were two additional important observations from this study. One was that the R19A mutation, which eliminated a Ste20-related proline/alanine-rich kinase (SPAK) binding site in NCC, prevented the phosphorylation of these sites, implicating SPAK as the responsible kinase (Fig. 1*B*). The other was that eliminating threonine 60 (NCC-T60A) prevented or reduced the phosphorylation of the other sites, supporting a previous suggestion (65) that this threonine residue was key in the regulation of NCC activity.

Yang et al. (99) raised specific phospho-antibodies against serine 71. These antibodies were used to show in a knockin model of PHAII that, indeed, the mutation D561A in one allele of WNK4 resulted in increased phosphorylation of NCC in this residue (Fig. 1*C*). $WNK4^{(D561A/+)}$ mice exhibited increased expression of NCC in the apical membrane of DCT cells and increased phosphorylation of SPAK in renal tissue. These findings suggested that WNK4 mutations causing PHAII produce an increase in SPAK phosphorylation, which in turn induces an increase in NCC phosphorylation at serine 71 and in the surface expression of the cotransporter.

Two additional phosphorylation sites have been observed in NCC by using large-scale proteomics from human urine or rat renal cortex. In humans exosomes from urine, phosphorylation of NCC at serine 811 was observed (34). This serine is part of an exon that is present in humans, but not in the rat or mouse. Its significance is not known. In rat renal cortex, phosphorylation of serine 124 of NCC was also detected by this methodology (24). A recent study shows that this serine phosphorylation is increased by vasopressin and a low-salt diet (74).

Activation of NCC by a Low-Salt Diet

The studies discussed above revealed the close correlation between NCC activity and phosphorylation (65) and generated the tools for detecting NCC phosphorylation (72, 99). Thus it became possible to measure NCC activity using in vivo models. Table 1 shows a compilation of the studies in which the phosphorylation of NCC has been assessed. One of the first issues for researchers in the field was to analyze the role of NCC in salt handling when the salt content of a diet changed. Thus NCC phosphorylation was assessed in animals subjected to low- or high-salt diets. The first such study was performed by Chiga et al. (15), who observed that high- and low-salt diets were associated with decreased and increased phosphorylation of NCC, respectively, in threonine residues 53 and 58, as well as in serine residue 71 (Fig. 1*C*). The phosphorylation of serine 71 during a low-salt diet was partially prevented by spironolactone, suggesting that aldosterone modulates the phosphorylation of NCC. However, the ratio between phosphorylated NCC and total NCC was not presented. Thus the effect observed with spironolactone could be solely a consequence of NCC expression levels, which are affected by aldosterone (44). In another study, Vallon et al. (88) observed that the increased phosphorylation of NCC at the same residues during a low-salt diet was attenuated in serum glucocorticoid 1 (SGK1)-knockout mice. Because SGK1 is involved in the effects of aldosterone in the distal nephron, this observation supported the idea that increased phosphorylation of NCC during a low-salt diet is partially a result of aldosterone's effects. It was subsequently observed that NCC phosphorylation could be achieved independently by both angiotensin II and aldosterone. Angiotensin II modulates NCC trafficking in DCT cells from rats (78, 79) and increases the activity and phosphorylation of NCC in oocytes (77). Therefore, to distinguish between the effects of angiotensin II and aldosterone, Van der Lubbe et al. (89) performed a study in rats in which the adrenal glands were removed and subsequently treated with vehicle, aldosterone, low nonpressor doses of angiotensin II, or pressor doses of this hormone. Without adrenal glands, NCC expression and phosphorylation and SPAK phosphorylation in the rats were in-

Table 1. *Phosphorylation of NCC in different sites and conditions*

Experimental System/Model	Stimulus	Effect	Phospho-Site*	Kinase Involved	Reference No.
Xenopus oocytes	Intracellular Cl^- depletion	↑ ↑	T^{55}, T^{60}, S^{73}	?	65
Mouse	$WNK4^{(D561A/+)}$ knockin	↑ ↑	S^{73}	WNK4	99
HEK-293 cells	Intracellular Cl^- depletion	↑ ↑	T^{46}, T^{55}, T^{60}, S^{91}	SPAK	72
Rat	Low-salt diet	↑ ↑	T^{55},T^{60}, S^{73}	?	15
Human urine	None	↑	S^{811}	?	34
Xenopus oocytes/mpkDCT cells	Angiotensin II	↑ ↑	T^{55}, T^{60}	WNK4-SPAK	77
Mouse	Low-K^+ diet	↑	T^{55}, T^{60}, S^{73}	SGK1	88
Mouse	WNK4 hypomorphic	↓	T^{55}, T^{60}, S^{73}	WNK4	64
Brattleboro rats	dDAVP	↑ ↑	T^{55}, S^{73}	?	60
Brattleboro rats/Wistar rats	dDAVP	↑ ↑	T^{55}, T^{60}	SPAK	67
Mouse	SPAK knockin	↓ ↓	T^{55}, T^{60}, S^{91}	SPAK	71
mpkDCT cells/mouse	Angiotensin II/aldosterone	↑ ↑	T^{55}, T^{60}, S^{73}	SPAK	86
Adrenalectomized rats	Angiotensin II/aldosterone	↑ ↑	T^{55}, T^{60}	WNK4-SPAK	89
Mouse	KS-WNK1 knockout	↑	T^{45}, T^{55}, T^{60}, S^{73}	WNK1/WNK4	36
Mouse	KS-WNK1 knockout	↑	T^{60}	WNK1/WNK4	51
In tube	Incubation with MO25	↑	T^{45}, T^{55}, T^{60}, S^{91}	SPAK/OSR1	25
Mouse	SPAK knockout	↓ ↓	T^{55}, T^{60}, S^{73}	SPAK	98
Mouse	SPAK knockout	↓ ↓	T^{55}, S^{73}	SPAK	54
mDCT cells/rats	Cyclosporine	↑	T^{55}, T^{60}, S^{73}	?	56
Mouse	Tacrolimus	↑	T^{55}	WNK3/WNK4	38
Mouse	NCC transgenic	⇆	T^{55}	?	55
Mouse	WNK4 Knockout	↓ ↓	T^{60}	WNK4/SPAK	12
mpkDCT cells/mouse	Insulin	↑	T^{55}, T^{60}, S^{73}	WNK4/SPAK	81
Xenopus oocytes Ex vivo kidney	Insulin	↑	T^{60}	WNK3	14
ZO obese Zucker rats	Hyperinsulinism	↑	T^{55}	WNK4	47
Mouse	KS-OSR1 knockout	↑	T^{55}	OSR1/SPAK	50
Mouse	Isoproterenol-salt-sensitive hypertension	↑	T^{55}, S^{73}	WNK4	58
In tube	$WNK4/Ca^{2+}$	↑ ↑		WNK4	61
Xenopus oocytes	WNK3	↑ ↑	T^{60}	WNK3/SPAK	66
Rats	Large-scale proteomics	↑	S^{124}	?	24
Brattleboro rats	dDAVP	↑	S^{124}	?	74

*Numbers correspond to human Na-Cl cotransport (NCC) sequence. DCT, distal convoluted tubule; WNK, with no lysine kinase; SPAK, Ste20-related proline/alanine-rich kinase.

creased by either angiotensin II or aldosterone, indicating that angiotensin II affects NCC independently of aldosterone. Thus the increased phosphorylation of NCC during a low-salt diet is most likely a result of the combined effects of angiotensin II and aldosterone on DCT cells (Fig. 2). The aldosterone-sensitive distal nephron begins at DCT2 because 11-β-hydroxysteroid dehydrogenase type II enzyme is absent in DCT1, where NCC is more heavily expressed. This enzyme eliminates cortisol, preventing the promiscuous effect of the glucocorticoid on the aldosterone receptor (6). Thus the angiotensin II effects on NCC phosphorylation might occur in both DCT1 and DCT2, while the effect of aldosterone occurs only in the NCC of DCT2. No study thus far has specifically address this issue.

Regulation of NCC by the WNKs-SPAK Complex

A decade ago, it was revealed that PHAII is a result of mutations in two genes encoding WNK1 and WNK4 (94). The PHAII clinical picture is the mirror image of Gitelman's disease and is reversed by low doses of thiazide-type diuretics. Therefore, it was suggested that WNK1 and WNK4 regulate NCC and that the pathophysiology of PHAII may be caused by impaired regulation of NCC by mutant WNK kinases. PHAII resulting from mutations in the *PRKWNK1* gene is caused by intronic deletions that apparently increase the expression of wild-type WNK1. In contrast, PHAII associated with mutations in the *PRKWNK4* gene is caused by missense mutations in a highly conserved acidic region of WNKs. Initial observations suggested that wild-type WNK4 reduces NCC activity and that this effect did not occur in WNK4 harboring PHAII-type missense mutations (11, 95, 96). Subsequently, it was demonstrated using in vivo models that extra-wild-type WNK4 activity (transgenic mice with 4 wild-type WNK4 alleles) is associated with reduced activity of NCC (Gitelman-like phenotype) (49). In contrast, mice with WNK4-PHAII-type mutant alleles (99), even in the presence of two normal WNK4 alleles (49), developed the PHAII phenotype associated with hypertrophy of the DCT and increased phosphorylation of NCC at serine 71. WNKs were found to lie upstream of other serine/threonine kinases of the STE-20 type known as SPAK and OSR1 (92), which appear to be the kinases that actually phosphorylate NCC (Fig. 2) (72). Supporting this hypothesis, WNKs induce phosphorylation of SPAK at threonine residue 243 (93). Additionally, the knockin mice in which this site in SPAK was substituted with alanine, $SPAK^{T243A/T243A}$ mice, developed a Gitelman-like syndrome, with arterial hypotension and a decreased phosphorylation of NCC at residues T53, T58, and S71 (71). Similar observations were obtained with the complete SPAK knockout colony (54, 98).

Experimental evidence in several models including *X. laevis* oocytes, mammalian epithelial cells, and genetically altered mice consistently shows that under certain circumstances, WNK4 behaves as an inhibitor of NCC. However, in the presence of PHAII-type mutations, WNK4 acts as an activator (13). This can be explained, at least in part, by the observation that the WNK4 effect on SPAK/NCC is modulated by angiotensin II (Fig. 2). This peptide hormone produces a positive

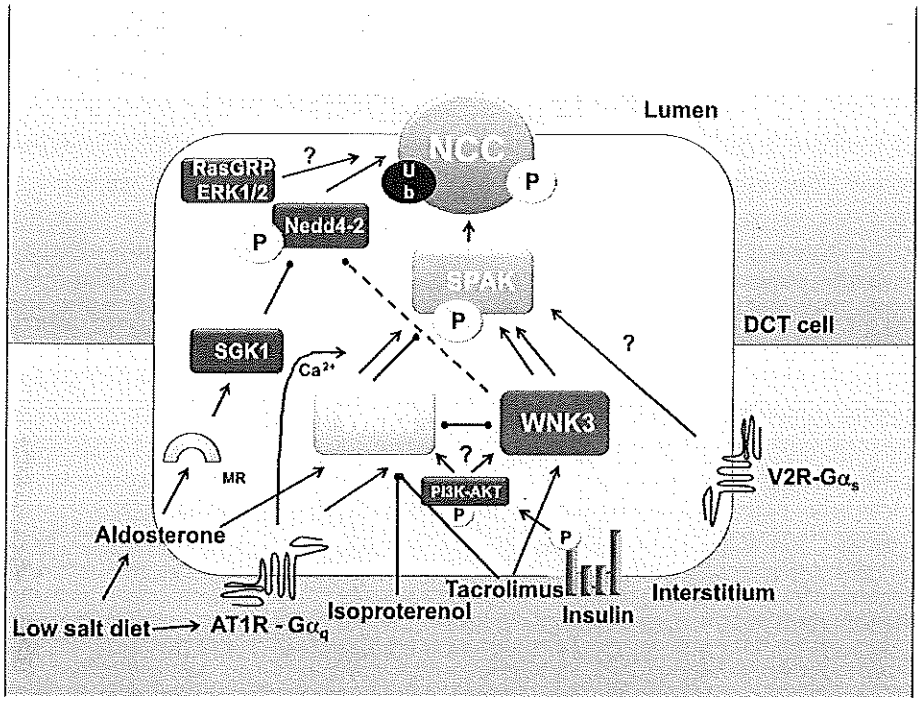

Fig. 2. Regulation of NCC by ubiquitylation and phosphorylation. See text for explanations. MR, mineralocorticoid receptor; red arrows, activation; black lines, inhibition. To simplify the figure, WNK1 effects were not included.

effect on NCC trafficking toward the apical membrane of DCT cells (79) and induces increased thiazide-sensitive salt reabsorption in the distal nephron (101). In the *X. laevis* oocyte expression system, coexpression of WNK4 and NCC results in reduced activity of NCC (95, 96). This effect is reversed in the presence of angiotensin II and can be prevented by losartan, a specific AT_1 receptor blocker (77). Interestingly, angiotensin II increased the activity of NCC when oocytes were coinjected with WNK4, but not in the absence of WNK4. This finding suggested that the effect of angiotensin II on NCC is WNK4 dependent. The activation of NCC by angiotensin II in the presence of WNK4 was also SPAK dependent and associated with increased phosphorylation of both SPAK and NCC, not only in oocytes but also in the mpkDCT cell line (77, 86). The requirement of WNK4 for the angiotensin II-induced activation of NCC has been confirmed in vivo. The NCC-induced phosphorylation in threonine 60 and SPAK at serine 383 by a low-salt diet or by chronic angiotensin II administration in wild-type animals was not observed in the $WNK4^{-/-}$ knockout mice (12) (Fig. 1, *E* and *F*). A potential biochemical explanation has been proposed. The WNK4-induced SPAK/OSR1 and NCC phosphorylation appears to be modulated by the calcium ion concentration. The NCC phosphorylation is higher when incubated in high (1 μM) than in low (10 nM) calcium concentrations. Interestingly, this difference does not occur when WNK4 constructs with PHAII-type mutations are used (61). Because the AT_1 receptor is coupled to a $G\alpha_q$ type of G protein, angiotensin II binding induces an increase in calcium concentration in the cytoplasm by promoting the production of IP_3 that stimulates the release of calcium by the smooth endoplasmic reticulum.

Considering all of these studies together, we have suggested that PHAII-type mutations in WNK4 are gain-of-function mutations, mimicking the effect of angiotensin II on the WNK4-SPAK-NCC (6, 12, 13, 77) and providing a pathophysiological explanation for PHAII. Because PHAII is a dominant disease, having one constitutively active allele of WNK4, the DCT behaves as if angiotensin II is present and explains the salt retention and hence arterial hypertension. This explanation also applies to hyperkalemia because increased salt reabsorption in the DCT reduces the salt delivery to the collecting duct, decreasing the possibility for Na^+/K^+ exchange via the epithelial sodium channel (ENaC)/renal outer medullary potassium channel (ROMK). Additionally, angiotensin II reduces the activity of ROMK by a WNK4-dependent mechanism (100).

Phosphorylation of NCC by WNK4/SPAK may be involved in at least two known models of arterial hypertension. Mu et al. (58) presented evidence supporting the hypothesis that salt-sensitive hypertension induced by isoproterenol administration is caused by an epigenetic-induced decrease in WNK4 expression, leading to an increased phosphorylation of NCC, thus increasing salt reabsorption in the DCT (Fig. 2). Similarly, two independent groups have shown that the arterial hypertension associated with calcineurin inhibitors, such as cyclosporine or tacrolimus, that are extensively used to prevent allograft rejection in organ transplantation, is associated with changes in WNK3 and WNK4 expression, and consequently in NCC phosphorylation (Fig. 2). This finding suggests that the arterial hypertension induced by these compounds is a result of increased salt reabsorption in the DCT (38, 56).

The effect of WNK1 on NCC activity remains elusive. No evidence of WNK1 effect on NCC has been reported. Instead, it was observed in *X. laevis* oocytes that WNK1 prevented the WNK4-induced inhibition of NCC activity (96), thus suggesting that increased WNK1 expression could exaggeratedly prevent the WNK4 inhibition of NCC, thus increasing the activity of the cotransporter. Later on it was observed that WNK1

produces a specific variant that is exclusively expressed in the kidney, particularly in DCT. This variant, named KS-WNK1 for kidney-specific WNK1, lacks the entire kinase domain and prevents the WNK1-induced inhibition of WNK4's negative effect on NCC (18, 64, 85). Thus it was proposed that under normal circumstances, KS-WNK1 modulates the effect of the longer WNK1 on WNK4-NCC. In this case, PHAII could be explained by deletion of intron 1 of WNK1 that results in increased expression of the longer form of WNK1, surpassing the inhibitory effect of KS-WNK1 and thus preventing the inhibitory effect of WNK4 on NCC. This proposal, however, awaits confirmation in animal models. On the one hand, deletion of intron 1 in mouse results in overexpression of both the long and the short WNK1 variants (17). On the hand, however, KS-WNK1 knockout mice exhibit increased expression and phosphorylation of NCC (Table 1) (36, 51).

WNK3 kinase is a powerful activator of NCC and consequently also phosphorylates threonine residue 58 in NCC (Fig. 2) (66). The positive effect is also associated with increased expression of NCC in the plasma membrane (33, 73). One group suggested that this effect is SPAK independent because coexpression of WNK3 and NCC with a dominant negative version of SPAK did not prevent the activation of NCC by WNK3 (33). However, the elimination of the SPAK binding site located at phenylalanine 242 of WNK3 completely prevents the activation of NCC, suggesting that a WNK3-SPAK interaction is required (66). Moreover, the requirement of SPAK for WNK3 modulation of other members of the SLC12 family, including NKCC1, NKCC2, and KCC4, has also been observed (66, 70).

Vasopressin Modulates NCC Activity

Vasopressin is a nanopeptide hormone produced in the hypothalamus and released in the posterior pituitary gland in response to increased plasma osmolarity or decreased blood pressure. Consequently, vasopressin promotes the formation of concentrated urine and increases blood pressure. Similarly to many other hormones (catecholamines, atrial natriuretic peptide, angiotensin II, aldosterone, etc.), vasopressin modulates blood pressure by modifying both vascular smooth muscle contraction and urinary salt and water reabsorption. For vasopressin, these effects are transduced in the smooth muscle and renal tubular cells through the Gα-coupled receptors V1 ($G\alpha_q$) and V2 ($G\alpha_s$), respectively. The role of antidiuretic hormone in modulating the activity of NKCC2, urea transporters, and aquaporin 2 in the kidney is very well known. However, these actions modulate extracellular osmolarity rather than arterial pressure. Early micropuncture studies from Elalouf et al. (20) in homozygous Brattleboro rats suggested that vasopressin increases salt reabsorption in the DCT. In addition, the expression of the V2 receptor in the DCT has been documented using in situ hybridization and immunohistochemistry of human, mouse, and rat kidney (59). Furthermore, by analyzing the phosphorylation of NCC, two groups have independently confirmed the effect of vasopressin on NCC (60, 67). Administering dDAVP to Brattleboro rats resulted in increased phosphorylation of NCC at residues T53, T58, and S71. Both groups showed that the effect occurs in the DCT, independently of other potential hormones such as angiotensin II or aldosterone. Pedersen et al. (67) suggested that vasopressin's effect could be through SPAK activation (Fig. 2). An interesting discrepancy regarding the effect of phosphorylation on NCC membrane trafficking is discussed below.

Insulin Modulates NCC Activity

There is a clear association between blood pressure levels and body weight. The higher the body mass index, the higher the prevalence of arterial hypertension. Metabolic syndrome, obesity, and diabetes mellitus type II are risk factors for the development of hypertension. One possibility is that hyperinsulinism that is often seen in these syndromes plays a critical role in the development of hypertension. It is known that obesity and hyperinsulinemic states are associated with increased expression of the distal nephron salt transporters (9, 43, 82, 87). In this regard, it has recently been shown by three independent groups that insulin or hyperinsulinemic states are associated with increased activity and phosphorylation of NCC (Table 1). Shoara et al. (81) observed in mpkDCT cells that insulin induces phosphorylation of SPAK and NCC by a phosphoinositol 3-kinase (PI3K)-dependent pathway and that this is reduced by knocking down WNK4, suggesting that it is WNK4 dependent. They also observed that intraperitoneal injection of insulin is associated with increased phosphorylation of SPAK and NCC in the kidney of wild-type mice but not in a WNK4 hypomorphic mouse. Komers et al. (47) demonstrated in obese Zucker rats that response to hydrochlorthiazide is increased, together with the phosphorylation of NCC at threonine 53. They also observed in vitro that insulin's effect on phosphorylating NCC can be prevented by PI3K blockers and suggested that WNK4 could be implicated in the insulin effect on NCC. Finally, the observation of Chavez-Canales et al. (14) in *X. laevis* oocytes showed that insulin increases the activity of NCC, assessed by thiazide-sensitive $^{22}Na^+$ uptake and that this is associated with increased phosphorylation of threonine 60. Inhibitors of the PI3K, akt1, and mammalian target of rapamycin (mTOR)2 pathway prevented the insulin effect on NCC. It was also observed by using a kidney ex vivo perfusion technique that insulin perfusion into the kidney induces phosphorylation of the cotransporter. Thus insulin is an activator of NCC by inducing its phosphorylation in the amino-terminal domain acceptor sites (Fig. 2).

Do NCC Phosphorylation and Activity Correlate with Surface Expression?

A subject of debate is the location at which the phosphorylation that activates NCC occurs. Does it occur in the vesicles containing NCC copies, promoting their trafficking toward the plasma membrane, thus increasing surface expression of the cotransporter? Alternatively, does it occur at the NCC molecules that are already present in the plasma membrane, increasing their transport capacity as a consequence of the phosphorylation? As shown in Table 2, a few studies have addressed this issue. The data obtained from NCC cRNA-transfected *X. laevis* oocytes, a robust NCC expression system used to clone and study NCC properties (28), suggest that amino-terminal threonine phosphorylation occurs in the proteins that are already in the membrane. Intracellular chloride depletion increases the activity and phosphorylation of NCC, without changing the level of expression at the cell surface. Eliminating threonine 53 and 58, as well as the serine residue 71, dramatically reduced the basal activity of the cotransporter without affecting its

Table 2. *Phosphorylation and cell surface expression of NCC*

Experimental Model	Stimulus	Phosphorylation/Activity	Surface Expression	Reference No(s).
Xenopus oocytes	Intracellular chloride depletion	↑ ↑	⇆	65
Xenopus oocytes	Protein phosphatase 4	↓ ↓	⇆	32
Xenopus oocytes	WNK3	↑ ↑ ↑	↑ ↑ ↑	66, 73, 97
Xenopus oocytes	Mutant NCC-T58D	↑ ↑	⇆	32
Xenopus oocytes	Mutant NCC-T58A	↓ ↓	⇆	32, 65
Mouse	WNK4$^{(D561A/+)}$ knockin	↑ ↑	↑ ↑	99
Brattleboro rats	dDAVP	↑ ↑	↑ ↑ (no shift)	67
Brattleboro rats/Wistar rats	dDAVP	↑ ↑	↑ ↑ (shift)	60
Mouse	SPAK knockout	↓ ↓	?	98
Mouse	SPAK knockout	↓ ↓	?	54

cell surface expression (65). Supporting these observations, another study demonstrated that protein phosphatase 4 reduces NCC activity, presumably by dephosphorylation, without affecting NCC surface expression. In the same study, phosphorylation that mimicked NCC mutation T58D increased the basal activity of the cotransporter without affecting its expression levels at the cell surface (32).

The data from in vivo models are less clear regarding the relationship between NCC plasma membrane expression and phosphorylation. Using immunogold electron microscopy, Pedersen et al. (67) showed in rats that the vasopressin-induced increase in NCC phosphorylation apparently occurs in the NCC that is already present in the plasma membrane. In contrast, Mutig et al. (60) proposed that vasopressin-induced NCC phosphorylation occurs in the intracellular vesicles that then move toward the plasma membrane. This last possibility is supported by a previous study showing that vasopressin activation and phosphorylation of NKCC2 in the thick ascending limb of Henle's loop in mice is associated with increased expression of the cotransporter in the apical membrane (31). Thus in the heterologous expression system it appears as if phosphorylation occurs in NCCs that are already at the cell surface. However, the in vivo data do not yet allow a definitive conclusion.

With regard to the regulation of NCC by the WNKs-SPAK pathway, it appears as if both phosphorylation and trafficking occur, most likely because WNKs modulate either independently of each other. In oocytes, the activation of NCC by WNK3 is associated with both increased phosphorylation and cell surface expression (66, 73). Preliminary evidence suggests that WNK3, in addition to phosphorylating NCC (most likely via SPAK), also reduces the Nedd4–2-induced ubiquitylation of the cotransporter. Therefore, the increase in NCC expression in the plasma membrane could be explained by decreased NCC ubiquitylation (see below) rather than by increased NCC phosphorylation (5). In the WNK4$^{(D561A/+)}$ knockin mouse resembling PHAII, there is a clear increase in phosphorylation of NCC and in the expression at the apical surface of DCT cells (99). The elimination of the kinase SPAK, either by a knockout (54, 98) or by a knockin model in which SPAK is present but is unable to be activated by WNKs (71), is associated with a reduction in total NCC expression. Thus interpreting the decrease in both cell surface expression and phosphorylation of NCC in these models is difficult.

Modulation of NCC by Trafficking

In the cell, NCC resides within intracellular vesicles that undergo exocytosis and endocytosis. In this regard, one proposed mechanism is that WNK4, which is known to reduce NCC activity in basal conditions (95), reduces the delivery of NCC to the plasma membrane by redirecting NCC molecules from the Golgi complex to lysosomes, in which NCC is then degraded. Supporting this view, WNK4 stimulates the interaction between NCC and the AP3 protein complex that promotes the switching of proteins from endosomes to lysosomes (84). Another study showed that WNK4 also promotes the interaction between NCC and sortilin, a DCT protein that redirects peptides from the Golgi and other compartments to lysosomes (102). This degradation-promoting effect of WNK4 could explain, at least in part, the Gitelman-like effect in which wild-type WNK4 is overexpressed in a BAC transgenic model (49) that exhibits remarkable DCT hypotrophy. In addition, accelerated degradation in the endoplasmic reticulum appears to be the major mechanism for reducing the expression of NCC in the plasma membrane in Gitelman's disease (48, 62, 76).

Protein Ubiquitylation: A Novel Regulatory Mechanism for NCC

Adding the 76-amino acid protein ubiquitin to lysine residues in target proteins is a powerful way to modulate the activity of proteins because ubiquitylation marks the protein for destruction, modulating its half-life (90). There are two major types of ubiquitin ligases: HECT (for homologous to the E6-AP carboxyl terminus) and RING (for really interesting new genes). These ligases contain several members that, in conjunction with a variety of interacting proteins, constitute hundreds of possibilities for highly specific ubiquitylation of proteins at lysine residues, just as kinases induce phosphorylation of proteins at specific threonine, serine, or tyrosine residues. Several membrane proteins, including transporters and channels in the kidney, are regulated by ubiquitylation (75).

A well-known example of ubiquitylation-mediated regulation occurs with ENaC. This channel in the collecting duct is ubiquitylated by a HECT-type ubiquitin ligase known as Nedd4–2. This protein directly interacts with ENaC via a specific PY motif in the channel, inducing ubiquitylation of the channel. This ubiquitylation marks the channel to be removed from the apical plasma membrane and thus reduces sodium reabsorption. The mineralocorticoid hormone aldosterone increases ENaC activity, at least in part, by stimulating the expression of SGK1. SGK1 phosphorylates Nedd4–2 at serine residues 222 and 328, preventing Nedd4–2-ENaC interaction and thus ENaC ubiquitylation. As a consequence, the expression of the channel in the apical membrane is increased, as is the sodium reabsorption. The loss of the PY motif of the β- or γ-subunit of ENaC by specific mutations that occur in Liddle's

syndrome provides a pathophysiological explanation for the disease. The absence of the PY motif in just one of the ENaC subunits is sufficient to preclude the Nedd4–2-induced ubiquitylation of the channel and thus chronically increase the expression/activity of the channel (41).

Modulation of NCC activity by ubiquitylation is an emerging field that is attracting the interest of researchers. The first observation suggesting that NCC is modulated by ubiquitylation was performed by Ko et al. (46) by searching for the mechanism by which phorbol esters reduce NCC activity via RasGRP1 and ERK1/2 activation (Fig. 2) (45). In that study, mDCT cells endogenously expressing NCC and Madin-Darby canine kidney cells transiently transfected with NCC cDNA were used. Exposing the cells to TPA resulted in increased ubiquitylation of NCC that could be prevented with UBEI-41, a compound that inhibits the activity of the E1 ligase, thus preventing TPA-induced ubiquitylation and reduction of NCC activity. The ubiquitin ligase responsible for NCC ubiquitylation was not elucidated. Subsequently, the SGK1-Nedd4–2 pathway that modulates ENaC activity was found to also regulate NCC (Fig. 2) (4). Aldosterone promotes increased NCC protein expression (44) by a mechanism that does not affect the NCC mRNA levels (1, 57), strongly suggesting that aldosterone increases NCC by a posttranslational effect. Supporting this hypothesis, Arroyo et al. (4) recently showed that Nedd4–2 induces an increase in NCC ubiquitylation, reducing the cotransporter in the plasma membrane, and thus its activity. Consistent with this observation, Nedd4–2 conditional knockout mice developed a remarkable increase in NCC expression a few days after the administration of doxycycline to induce the loss of Nedd4–2 in the kidney. Similarly to ENaC regulation, SGK1 prevented the Nedd4–2 effect in NCC. Supporting this, Faresse et al. (23) recently showed that inducible renal tubule-specific SGK1 knockout mice exhibit impaired Na^+ reabsorption on a low-NaCl diet, and this was associated with decreased NCC abundance. Thus it is probable that the aldosterone activation of NCC is caused, at least in part, by decreasing the Nedd4–2-induced ubiquitylation of NCC in an SGK1-dependent mechanism. A recent study suggests that phosphorylation and ubiquitylation of NCC could interact with each other in such a way that phosphorylating NCC prevents ubiquitylation (39), and it has been shown in vitro that WNK1 and WNK4 are able to phosphorylate Nedd4–2 at sites overlapping with SGK1 (37). The significance of this finding is not known but could represent a possibility for regulating phosphorylation and ubiquitylation of NCC simultaneously.

Evidence suggests that NCC could be a substrate for other types of ubiquitin ligases. Degradation of NCC in the endoplasmic reticulum has been shown to be associated with the E3-ubiquitin ligase Hrd1 (62) and, as discussed below, NCC could be a target for the RING-type ubiquitin ligases.

Ubiquitylation is Also Involved in the Pathophysiology of PHAII

After the discovery of WNK1 and WNK4 as the causative genes of PHAII, it became clear that in several families this disease could occur in the absence of WNK1 or WNK4 mutations. Therefore, one or more unknown genes were capable of producing the same clinical outcome (19, 35). The causative genes have now been uncovered. Boyden et al. (10) observed that two genes encoding proteins that form a RING-type complex of ubiquitin ligase produce PHAII. These genes are known as Kelch-like 3 and Cullin 3. The human genome contains at least seven different cullin proteins; each constitutes the backbone of several multisubunit ubiquitin ligase complexes. In forming the complex, Cullin 3 specifically binds proteins, for example Kelch 3, that contain BTB domains. These two proteins, Kelch-like 3 and Cullin 3, form a complex that associates with a RING-type ubiquitin ligase (68). Kelch 3 or any of the BTB-containing domain proteins serves as the specific substrate recognition site of the complex, and the RING-type protein promotes the transfer of ubiquitin residues from E2 ligases onto the substrate recognized by the BTB protein. Using exome sequencing, mutations in Kelch 3 or Cullin 3 in 41 unrelated families with PHAII were identified (10). Although Kelch 3 mutations were either recessive or dominant, the Cullin 3 mutations were predominantly de novo. Interestingly, the PHAII patients with Cullin 3 mutations exhibited a more severe disease. As shown in Table 3, the severity of PHAII syndrome in terms of the mutated gene is Cullin 3 > Recessive Kelch 3 > Dominant Kelch 3 > WNK4 > WNK1. This ranking accounts for the age of diagnosis, the percentage of affected members under 18 yr of age exhibiting hypertension, the level of hyperkalemia, and the level of metabolic acidosis at the time of diagnosis.

For the reasons discussed above, it is generally accepted that the level of activity of NCC plays a key role in the development of PHAII. Thus these observations suggest that Cullin 3 and Kelch 3 must be regulators of NCC, by ubiquitylation of the cotransporter or of proteins that in turn modulate NCC activity (such as WNKs, SPAK, and phosphatases). Louis-Dit-Picard et al. (52) also reported on PHAII families resulting from mutations in Kelch 3 and presented preliminary evidence suggesting that Kelch 3 may modulate NCC. They showed that Kelch 3 in the nephron is mostly expressed in the DCT. Additionally, in HEK-293 cells transiently transfected with NCC cDNA, treatment with Kelch 3 siRNA increased the presence of NCC protein in the plasma membrane. These observations do not necessarily probe the direct effect of Kelch 3 on NCC. However, these findings do support the hypothesis that by a direct or indirect pathway, NCC expression is most likely affected by the Cullin 3/Kelch 3 complex. Therefore, the disruption of NCC regulation by Cullin 3/Kelch 3 would most

Table 3. *Severity of PHAII stratified by genotype*[1]

Mutated Gene	No. of Families	No. of People Affected	HTA[4] <age 18 yr, %	Age at Diagnosis, yr	K^+,[5] mM	HCO_3^-,[6] mM
Cullin 3	17	21	94	9 ± 6	7.5 ± 0.9	15.5 ± 2.0
Kelch Rec[2]	8	14	14	26 ± 14	6.8 ± 0.5	17.6 ± 1.5
Kelch Dom[3]	16	40	17	24 ± 18	6.2 ± 0.6	17.2 ± 2.5
WNK4	5	15	10	28 ± 18	6.4 ± 0.7	20.8 ± 2.3
WNK1	2	23	13	36 ± 20	5.8 ± 0.8	22.4 ± 4.6

PHAII, pseudohypoaldosteronism type II; 1, modified from Boyden et al. (10); 2, recessive; 3, dominant; 4, hypertension; 5, serum potassium; 6, serum bicarbonate.

likely provide a pathophysiological explanation for PHAII. Because Cullin 3/Kelch 3 patients exhibit a more severe phenotype than WNK4 or WNK1 patients, it is possible that the effect of these ubiquitin ligases on NCC is more pronounced than that of WNKs or, alternatively, that in addition to NCC, ubiquitin ligases may affect other unknown players in the distal nephron for the development of PHAII, which has not become evident from studying WNKs. A decade has elapsed since the discovery of WNKs as causative genes for PHAII, but there are still several important unanswered questions about the mechanisms by which WNKs produces PHAII. Thus exciting times are ahead of us in defining the molecular mechanisms by which mutations in Cullin 3/Kelch 3 produce the disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author.

AUTHOR CONTRIBUTIONS

Author contributions: G.G. provided conception and design of research; G.G. performed experiments; G.G. analyzed data; G.G. interpreted results of experiments; G.G. prepared figures; G.G. drafted manuscript; G.G. edited and revised manuscript; G.G. approved final version of manuscript.

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Mechanisms of sodium–chloride cotransporter modulation by angiotensin II

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Purpose of review
The renin–angiotensin–aldosterone system is an important modulator of renal salt excretion and arterial pressure. An important body of evidence now supports that angiotensin II (AngII) modulates the function of the renal sodium–chloride cotransporter (NCC), independently of aldosterone. Here we summarize these data, as well as recent knowledge regarding the intracellular mechanisms underlying this effect.

Recent findings
AngII has the ability to modulate NCC total expression, apical localization, and phosphorylation by aldosterone-independent mechanisms. Recent evidence suggests that these effects are achieved through modulation of the With No Lysine kinase 4 (WNK4) and Ste20-related, proline–alanine-rich kinase (SPAK) pathway. Missense mutations in the acidic domain of WNK4, which are the cause of one of the subtypes of pseudohypoaldosteronism type II (PHAII), could be mimicking the effect produced by AngII on NCC through the WNK4–SPAK pathway. WNK4 activity has been shown to vary in response to changes in calcium concentration and PHAII-WNK4 mutants apparently lose this ability. Thus, AngII may regulate WNK4 activity through the modulation of intracellular calcium concentration.

Summary
Modulation of WNK4 activity by AngII underlies the effects of AngII on NCC activity and this is probably important for the stimulation of renal sodium retention, as well as for the prevention of potassium loss, during hypovolemia.

Keywords
distal convoluted tubule, hypertension, pseudohypoaldosteronism type II, SPAK, spironolactone, WNK

INTRODUCTION

The renin–angiotensin–aldosterone system is fundamental for the regulation of the extracellular fluid volume, and thus arterial blood pressure (BP). The octapeptide hormone angiotensin II (AngII) performs its actions by interacting with its Gαq-coupled, AT1 receptor. It was thought for many years that the increase in salt reabsorption induced by AngII was primarily due to its effect on the adrenal gland, increasing aldosterone secretion. However, it is now clear that AngII exerts several direct effects on the kidney including modulation of renal hemodynamics and proximal and distal sodium reabsorption [1,2]. Recent works have shown that one of the direct effects of AngII in the kidney is to increase the activity of the thiazide-sensitive sodium–chloride cotransporter (NCC) by mechanisms that are clearly independent of those of aldosterone, another known activator of NCC. The purpose of this work is to review the recent evidence demonstrating that NCC is a target for AngII regulation and to discuss insights into the intracellular mechanisms by which this regulation takes place.

INTRACELLULAR MECHANISMS FOR MODULATION OF SODIUM–CHLORIDE COTRANSPORTER ACTIVITY

The activity of NCC is fundamental for BP regulation. NCC inhibition with thiazides is recommended as the first-line pharmacologic treatment for uncomplicated arterial hypertension [3]. Inactivating mutations in the *SLC12A3* gene, encoding

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KEY POINTS

- AngII modulates NCC activity independently of aldosterone, through a WNK4–SPAK-dependent pathway.
- Modulation by calcium concentration of the WNK4 kinase activity toward SPAK–NCC may be involved in the NCC regulation by AngII.
- Mutations in the kinase WNK4 causing PHAII are probably mimicking the AngII effect on NCC, hyperactivating the cotransporter.

NCC, are the cause of Gitelman's disease featuring arterial hypotension with hypokalemic metabolic alkalosis [4]. Rare mutant variants of *SLC12A3* that reduce NCC activity [5▪] protect against hypertension and cardiovascular mortality when present in a heterozygous fashion [6]. The pseudohypoaldosteronism type II (PHAII) or familial hyperkalemic hypertension is the mirror image of Gitelman syndrome and presents with arterial hypertension and hyperkalemic metabolic acidosis, and is highly sensitive to thiazide treatment [7,8]. This rare disease is due to mutations in With No Lysine kinase 1 (WNK1) or WNK4, or in the proteins kelch-like 3 or cullin 3, which are components of the cullin-RING (Really Interesting New Genes) ubiquitin ligase 3 [9▪,10▪]. Because in patients with PHAII, thiazide treatment not only corrects hypertension but also all other metabolic alterations observed [8,7], and because in WNK4-PHAII mouse models, pharmacological or genetic ablation of NCC also has this effect [11,12], it is believed that increased activity of NCC is key in the pathophysiology of PHAII.

Several groups have shown, using *Xenopus laevis* oocytes, Cos-7 cells, and HEK-293 cells, that wild-type WNK4 inhibits NCC activity and expression at the plasma membrane, whereas mutant WNK4 carrying PHAII mutations does not exert, or only partially exerts, this inhibitory effect [13–17]. Observations in mice support this view. Overexpression of wild-type WNK4 produces inhibition of NCC and a Gitelman-like phenotype, whereas the PHAII-mutant WNK4 recapitulates the PHAII-like phenotype [11,12]. It has been demonstrated that WNKs lie upstream of the kinases Ste20-related, proline–alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1), phosphorylating SPAK/OSR1within the T-loop (T-233/T-185) and the S-motif (S-373/S-325) [18,19], and resulting in SPAK/OSR1 activation, which in turn phosphorylate NCC (and other members of the SLC12A family) [19–22].

The observation that rat NCC activation by intracellular chloride depletion is associated with phosphorylation of the threonine residues 53 and 58, and serine 71, in the N-terminal domain [23], opened the possibility of raising specific phosphoantibodies to detect these sites, providing a powerful tool to indirectly assess NCC 'activity' in in-vivo models [12,24–27]. Supporting the association between NCC activation and phosphorylation, it was observed that NCC activity is negatively correlated to that of protein phosphatase 4 [28]. It is still a matter of debate whether phosphorylation is involved in activation of NCC that is already in the membrane, in NCC trafficking, or both [29]. NCC is activated by WNK3 in a kinase-dependent manner [30]. This effect is dependent on WNK3–SPAK interaction [20] and is probably the pathway through which intracellular chloride depletion signals to activate the cotransporter [21,31,32]. It has been proposed that WNK3–WNK4 interactions could be an important mechanism for NCC regulation by these kinases [33].

Another important mechanism for regulation of NCC activity is modulation of the cotransporter degradation. It has been suggested that WNK4 promotes NCC inhibition by diverting forward trafficking of NCC to the lysosome, and thus promoting degradation [17,34]. Additionally, NCC has been shown to be a substrate of the E3-HECT ubiquitin ligase Nedd4-2, which promotes NCC ubiquitylation and internalization [35▪▪]. Finally, it has been recently shown that mutations in cullin 3 [9▪] and kelch-like 3 [9▪,10▪] produce PHAII in some kindreds. It is possible that these proteins modulate NCC stability and that this is affected by the mutations [10▪].

SODIUM–CHLORIDE COTRANSPORTER MODULATION BY LOW-SALT DIET

The activity of NCC is modulated in response to changes in salt intake. NCC is activated under low-salt diet and inhibited with high-salt diet, contributing to decreased and increased levels of renal salt excretion, respectively. This was initially reported by Ellison *et al.* [36], who showed that low-salt diet increased the thiazide-sensitive reabsorption in the kidney. Low-salt diet has been shown to increase NCC protein levels [37,24], abundance at the plasma membrane [38,39], and N-terminal phosphorylation levels [24]. There is a controversy regarding the effect of low-salt diet on NCC mRNA levels. It was initially reported that low-salt diet did not change NCC mRNA [37,40], but in a more recent work, in which rats were maintained on this diet for 14 days, a 2.5-fold increase in NCC mRNA was observed using real-time PCR [41], with a significant decrease in WNK4 mRNA. However, in our hands,

mice exposed to low-salt diet (0.02%) for 9 days exhibited no significant change in NCC or WNK4 mRNA levels, also using real-time PCR (data not shown), although the expected increase in NCC protein expression was observed [42▪▪]. Thus, it is clear that NCC protein expression is increased by low-salt diet due to posttranslational mechanisms, but it is still a matter of debate whether this includes modulation of *SLC12A3* transcription rate and/or mRNA stability.

Sodium–chloride cotransporter modulation by aldosterone

Aldosterone infusion in adrenalectomized rats increased thiazide-sensitive sodium reabsorption and metolazone binding sites [43,44]. NCC expression observed through western blot was increased by aldosterone in normal or furosemide-treated rats [45,46]. Additionally, aldosterone administration increased NCC N-terminal phosphorylation [47▪▪,24], and this was prevented with spironolactone. However, although NCC protein expression and phosphorylation were increased by aldosterone, this hormone apparently did not affect NCC apical localization [39].

The increased levels of NCC phosphorylation observed with low-salt diet and aldosterone infusion were accompanied by increased levels of SPAK phosphorylation, and these effects were blocked with spironolactone, suggesting that stimulation of NCC function under low-salt diet is at least partially mediated by aldosterone and involves regulation of the WNK–SPAK–NCC pathway (Fig. 1) [24]. Because regulation of NCC phosphorylation, in the face of dietary salt changes, was attenuated in serum and glucocorticoid-induced kinase 1 (SGK1) knockout mice, it was proposed that SGK is implicated in this response [48]. *SGK1* is a well known aldosterone-responsive gene. The key pathway for the modulation by aldosterone of the epithelial sodium channel (ENaC) involves the phosphorylation of Nedd4-2 by SGK1, preventing the Nedd4-2-mediated ubiquitylation and degradation of ENaC [49]. As mentioned above, Arroyo *et al.* [35▪▪] reported that NCC is regulated by the ubiquitin ligase Nedd4-2 (Fig. 1). *In vitro,* Nedd4-2 activity induced a reduction in NCC surface expression, which was associated with a reduction in cotransporter activity. It was also observed that SGK1 disrupted the Nedd4-2–NCC interaction, preventing the Nedd4-2 negative effect on NCC, and that this effect of SGK1 was presumably due to Nedd4-2 phosphorylation. Additionally, in an inducible knockout model of Nedd4-2, knockdown of the ubiquitin ligase provoked a dramatic increase in

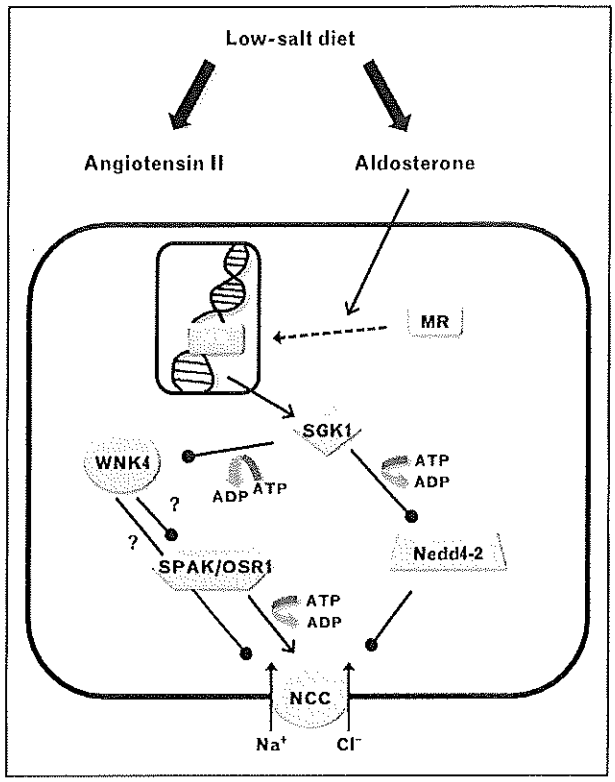

FIGURE 1. Sodium–chloride cotransporter modulation by aldosterone. Aldosterone binds to the mineralocorticoid receptor, which translocates to the nucleus to increase SGK1 transcription. SGK1 phosphorylates Nedd4-2, preventing Nedd4-2-mediated ubiquitylation and thus reduction of NCC activity. Another potential mechanism for NCC modulation by SGK1 is the phosphorylation of WNK4 to prevent the inhibitory effect exerted by this kinase on NCC. It is presently unclear whether the inhibitory effect of WNK4 on NCC does or does not involve SPAK/OSR1 inhibition (that may be direct or indirect, through the modulation of another upstream kinase). SPAK and OSR1 kinases, however, are activated by aldosterone. Thus, the release of WNK4 inhibition by aldosterone may be involved in their activation. MR, mineralocorticoid receptor; NCC, sodium–chloride cotransporter; SGK1, serum and glucocorticoid-induced kinase 1; SPAK/OSR1, Ste20-related, proline–alanine-rich kinase/oxidative stress response 1; WNK4, With No Lysine kinase 4.

NCC total expression [35▪▪]. Another potential mechanism for SGK1 modulation of NCC was suggested by Rozansky *et al.* [50], who showed that inhibition of NCC activity by WNK4 in *X. laevis* oocytes was attenuated by SGK1 activity and that this effect was dependent on the phosphorylation of two sites within the C-terminal domain of WNK4 (Fig. 1). Thus, aldosterone, acting through SGK1, modulates expression and phosphorylation of NCC.

Despite the fact that the mineralocorticoid receptor is expressed all along the distal convoluted

tubule (DCT) [51], aldosterone actions are thought to occur only in DCT2, because the early portion (DCT1) lacks the expression of 11β-hydroxysteroid dehydrogenase [52]. This enzyme converts cortisol into cortisone, preventing displacement of aldosterone by cortisol from mineralocorticoid receptors in aldosterone-sensitive cells [52].

Sodium–chloride cotransporter modulation by angiotensin II

Several lines of evidence suggest that AngII has a direct effect on NCC activity (Fig. 2). Acute captopril infusion in rats promoted redistribution of NCC from the apical membrane to subapical vesicles. This effect was reverted by coinfusion of AngII with captopril and occurred in a time course too short to be attributed to aldosterone [53]. Low-salt diet administration to rats promoted an increase in NCC apical expression and this effect could not be reproduced with aldosterone infusion [39]. Chronic AngII infusion increased thiazide-sensitive distal sodium and chloride reabsorption and this increase was not blocked with spironolactone, suggesting that it was AngII dependent [54]. Finally, aldosterone-independent NCC N-terminal phosphorylation was induced by chronic AngII infusion in adrenalectomized rats [47▪▪] and in spironolactone-treated mice [42▪▪], especially when nonpressor doses were used. These findings were corroborated in mpkDCT cells in which the AT1 blockers losartan or valsartan prevented the AngII-induced increase in SPAK and NCC phosphorylation [55,56].

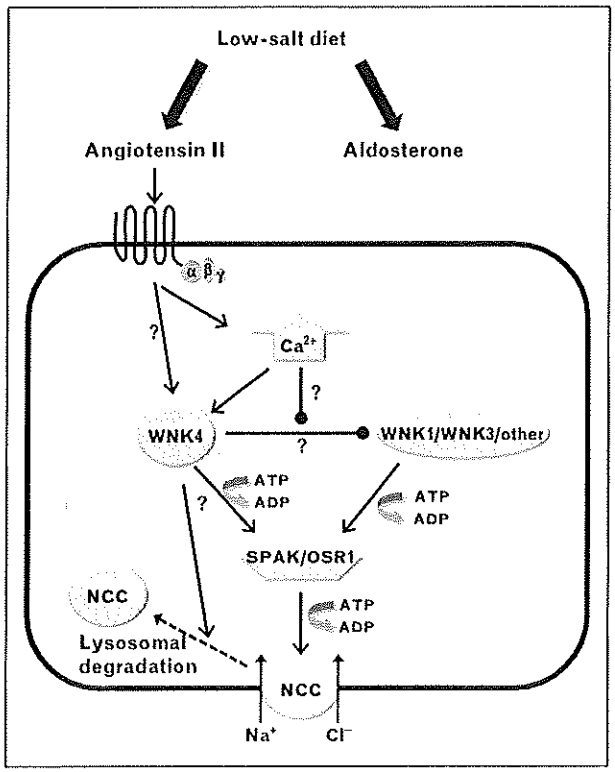

FIGURE 2. Sodium–chloride cotransporter modulation by angiotensin II. Angiotensin II binds to the AT1 receptor in the plasma membrane, eliciting the release of calcium from the endoplasmic reticulum. It has been proposed that the increase in intracellular calcium might modulate the effect of WNK4 towards SPAK–NCC. *in vitro*, WNK4 has the ability to phosphorylate and activate SPAK/OSR1–NCC and this activity can be modulated by calcium. This observation awaits confirmation in a cellular context. The effect of WNK4 toward SPAK/OSR1–NCC could be direct or by modulating other kinases/phosphatases that are known to regulate NCC, such as WNK1, WNK3, or PP4. WNK4 also promotes lysosomal degradation of NCC. It remains to be determined whether this effect of WNK4 is also modulated by angiotensin II. NCC, sodium–chloride cotransporter; SPAK/OSR1, Ste20-related, proline–alanine-rich kinase/oxidative stress response 1; WNK4, With No Lysine kinase 4.

The effects of PHAII-mutant WNK4 in the ion transport systems of the distal nephron set the activity of this segment in a functional state similar to that observed during hypovolemia; that is, with high salt reabsorption and low potassium secretion capacity [57]. Hence, the activity of NCC and ENaC is increased [13,58] (because PHAII-WNK4, in contrast to WNK4, does not exert an inhibitory effect on these proteins), whereas the renal outer medullary potassium channel (ROMK) is inhibited [59] (because PHAII mutations increase WNK4 ability to inhibit this channel). In addition to NCC, AngII also activates ENaC [60], while this hormone inhibits ROMK, by a mechanism that is partially WNK4 dependent [61]. Thus, AngII could be the signal to switch WNK4 from its inhibitory to the stimulatory effect on NCC (Fig. 2). Supporting this proposal, in oocytes expressing NCC and WNK4, incubation with AngII was shown to revert WNK4 inhibition of NCC activity, and thus promoted an increase in NCC-mediated uptake [55]. This effect was AT1 dependent because it was blocked with losartan. The PHAII-mutant version of WNK4 did not exert a negative effect on NCC activity and no longer was activated by AngII. Thus, a similar level of NCC activity was observed in NCC–WNK4-injected oocytes stimulated with AngII and in NCC–PHAII-WNK4-injected oocytes in the absence or presence of AngII [55]. It was also observed that a mutant version of NCC, carrying a mutation that affects a SPAK binding site (NCC-R18A), which prevents direct interaction with this kinase [62], had lower basal activity and could not be activated by AngII in the presence of wild-type WNK4 [55]. Thus, in oocytes, NCC

stimulation by AngII was WNK4–SPAK dependent. These observations have been extended to an *in-vivo* model. In WNK4 knockout mice with total absence of WNK4, the low-salt diet or AngII infusion-induced increase in phosphorylation of SPAK and NCC was lost [42▪▪]. Whether WNK4 mediates direct phosphorylation of SPAK–NCC or modulates the activity of another kinase or protein phosphatase (e.g., L-WNK1, WNK3, PP4), which modulates this phosphorylation, remains obscure.

Because PHAII due to missense mutations in WNK4 follows a dominant pattern of inheritance, it is likely that PHAII-type mutations are of the 'gain of function' type, which means that a physiological property of a protein is mimicked by the mutant version. We have hypothesized that the PHAII-type mutations on WNK4 mimic the effect produced by an upstream modulator on WNK4, which could be the functional state of the kinase induced by AngII, rather than by aldosterone. Patients with PHAII represent the pathophysiological version of a chronic increase in sodium reabsorption, with potassium retention, which is similar to the picture desired during hypovolemia in which plasma AngII and aldosterone levels are elevated. Both hormones participate in promoting renal sodium retention, but aldosterone has also the ability of stimulating renal potassium excretion when it is secreted in response to plasma potassium concentration elevations. It is well known that NCC activity is inversely related to the level of distal tubular potassium secretion [11,48,63,64], mainly because NCC-mediated electroneutral sodium reabsorption competes with ENaC-mediated electrogenic reabsorption, which is important for generating the electrochemical gradient that drives potassium secretion [65]. Thus, activation of NCC during hypovolemia is important not only to enhance sodium retention but also to prevent potassium loss in the context of high aldosterone. Although aldosterone has also been shown to promote NCC activation, this effect is not observed when aldosterone increases in response to a rise in plasma potassium concentration [48,63]. In fact, high potassium diet reduces the phosphorylation and expression of NCC in the apical membrane [48,63]. The mechanisms preventing NCC activation by aldosterone under hyperkalemia are presently unknown, but must be due, at least in part, to the fact that DCT1, in which NCC is heavily expressed, is not affected by aldosterone. In contrast, in the face of hypovolemia, in addition to the effect of aldosterone on NCC in DCT2, AngII promotes activation of NCC also in DCT1.

Finally, supporting the hypothesis that PHAII mutations mimic the effect of AngII on WNK4, a recent study demonstrated that, *in vitro,* kinase activity of WNK4 toward SPAK/OSR1 and NCC is affected by the calcium concentration of the media, whereas the kinase activity of PHAII-mutant versions of WNK4 remains constant under different calcium concentrations (Fig. 2) [66▪▪]. The sensitivity to calcium was only observed with wild-type WNK4 constructs containing the acidic motif. Thus, it was proposed that calcium binds to this motif to modulate the activity of WNK4 toward SPAK–NCC and that PHAII mutations yield a similar effect to that produced by calcium binding. It is worth nothing that PHAII mutations in the acidic domain are in charged amino acid residues. This mechanism could be involved in the AngII modulation of WNK4 activity because AT1 receptor activation raises intracellular calcium through the Gαq–phospholipase C–inositol triphosphate pathway. It has been shown that WNK4–SPAK–NCC form a protein complex [13,11,62,67]. Thus, it is possible that in the absence of AngII (low intracellular calcium), the complex is formed, but SPAK and/or NCC do not become phosphorylated, hijacking NCC from activation. In the presence of AngII (high intracellular calcium; WNK4 with PHAII mutation) the formed complex successfully phosphorylates SPAK and NCC, activating the cotransporter.

CONCLUSION

AngII is an activator of NCC in the distal convoluted tubule. The existence of PHAII, a syndrome that likely is due to the pathophysiological activation of NCC, has revealed several players involved in the intracellular mechanisms for NCC regulation. Recent data suggest that one of these players, WNK4, is involved in the modulation of NCC by AngII. The precise roles of other players, such as WNK1 [14,67], WNK3 [30], kelch-like 3 and cullin 3 [9▪,10▪], remain to be established.

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Conflicts of interest

There are no conflicts of interest.

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■■ phorylation of the thiazide-sensitive sodium chloride cotransporter independent of aldosterone. Kidney Int 2011; 79:66–76.

This is an elegant study clearly showing that in the complete absence of aldosterone, AngII infusion increases the NCC expression and N-terminal phosphorylation, supporting the proposal that AngII modulates NCC by an aldosterone-independent mechanism.

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This study shows that WNK4 kinase activity toward SPAK/OSR1 and NCC is modulated by calcium concentration. This effect is lost when WNK4 harbors PHAII mutations in the acidic domain, suggesting that these mutations are mimicking the effect of calcium.

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México Cd.Mx., a 20 de Marzo del 2018

Dra. Norma Bobadilla Sandoval
Coordinadora de la CINVA
Presente

Estimada Dra. Bobadilla:

Por este conducto me permito solicitar el cierre del protocolo: "Mecanismo de la regulación del cotransportador renal de NaCl por angiotensina II" con registro **CINVA NMM-25** debido a que el protocolo ha concluido.

Sin otro particular por el momento, quedo de usted.

Atentamente,

Gerardo Gamba Ayala

Gerardo Gamba, MD, PhD
gamba@biomedicas.unam.mx
Gerardo.gambaa@incmnsz.mx

Vasco de Quiroga No. 15
Tlalpan 14080, Ciudad de México.

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

Prot. 25

Ciudad de México a 24 de Mayo de 2017.

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la Comisión de Investigación en Animales
INCMNSZ.

Estimada Dra. Bobadilla:

En relación al Protocolo: "MECANISMOS DE REGULACIÓN DEL COTRANSPORTADOR RENAL DE NACI POR ANGIOTENSINA II", me permito solicitar una prórroga al 31 de Diciembre del año en curso, debido a que se encuentra en la fase final y requerimos de estos últimos meses para concluir con todos los trabajos derivados del Proyecto.

Sin más por el momento, aprovecho la oportunidad para enviarle un cordial saludo.

Atentamente

Dr. Gerardo Gamba Ayala
Director de Investigación

INS. NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN"
24 MAR 2017
Comité de Investigación en Animales

Avenida Vasco de Quiroga No. 15
Colonia Belisario Domínguez Sección XVI
Delegación Tlalpan
Código Postal 14080
México, Distrito Federal
Tel. (52)54870900
www.incmnsz.mx

acuse

"2017, Año del Centenario de La Promulgación de la Constitución Política de los Estados Unidos Mexicanos"

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

México, D. F., a 24 de abril de 2017.

No. Oficio CINVA 025-17

DR. GERARDO GAMBA AYALA
Depto. Nefrología y Metabolismo Mineral
P r e s e n t e

INVEST. EXPERIMENTAL Y BIOTERIO
24 [illegible] 7
1391
INST. NAL. CIENCIAS MEDICAS Y NUTRUCIÓN INCMYN. S.Z.

Estimado Dr. Gamba:

De la Auditoría al Desempeño 01/2017 realizada por el Órgano Interno de Control a la Dirección de Investigación derivo la recomendación preventiva:

> **"3. Establecer un mecanismo, para promover con los investigadores la importancia de la integración de los documentos que soporten o documenten cada una de las etapas del desarrollo de sus proyectos en los expedientes correspondientes a la aprobación de un proyecto, desarrollo, conclusión y cierre del mismo"**

Para subsanar esta recomendación se revisaron los expedientes de los protocolos autorizados por la Comisión de Investigación de Animales y se detectó que al expediente con **REF: CINVA-025** con título "MECANISMOS DE REGULACIÓN DEL COTRANSPORTADOR RENAL DE NACI POR ANGIOTENSINA II" le falta la siguiente información:

1. La forma única de registro completa con firmas
2. El protocolo en extenso
3. Carta de cierre
4. Informe final
5. Productos de investigación derivados del proyecto (artículo, libro, cartel o capítulo de algún libro entre otros

Por lo que pido de su apoyo para que me proporcione la información descrita antes del 25 de abril. Lo anterior para realizar los trámites administrativos correspondientes.

Sin más por el momento quedo de usted.

Atentamente,

PD.

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la Comisión
de Investigación en Animales
EN AUSENCIA DE LA COORDINADORA DE LA CINVA
DR. JORGE ALBERTO BARRIOS PAYAN
QUIÉN FUNGE COMO SECRETARIO

Vo, Bo.

Dr. Gerardo Gamba Ayala
Director de Investigación
y Presidente de la CINVA

Avenida Vasco de Quiroga No. 15
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México, Distrito Federal
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c.c.p. M.V.Z. Mariela Contreras Escamilla, Jefa del DIEB

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

"2015, Año del Generalísimo José María Morelos y Pavón"

México, D. F., a 16 de Febrero del 2015.

DR. GERARDO GAMBA AYALA
Depto. de Nefrología y Metabolismo Mineral
P r e s e n t e .

REF.: CINVA 025, Clave: NMM-25-10/12-1

Estimado Dr. Gamba:

Habiendo analizado detalladamente el Protocolo de Investigación Experimental titulado:

"MECANISMOS DE REGULACIÓN DEL COTRANSPORTADOR RENAL DE NACI POR ANGIOTENSINA II."

Este comité ha dictaminado **aprobar** la prórroga solicitada hasta el 31 de Diciembre del 2016.

Sin más por el momento quedo de usted.

Atentamente,

M.V.Z. Mariela Contreras Escamilla
17 FEB 2015

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la Comisión de Investigación en Animales

INS. NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN"
17 FEB 2015
DIRECCIÓN DE INVESTIGACIÓN

c.c.p. M.V.Z. Mariela Contreras Escamilla, Jefa del Bioterio

Vasco de Quiroga No. 15
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INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

México, D.F. a 20 de Enero del 2015

Dra. Norma A. Bobadilla Sandoval
Investigador Principal
Depto. de Nefrología y Metabolismo Mineral
P R E S E N T E

REF.: CINVA 25, Clave NMM-25-10/12-1

Estimada Dra. Bobadilla:

Por medio de la presente solicito a usted prórroga al 31 de Diciembre del 2017 para mi proyecto titulado

"MECANISMOS DE REGULACIÓN DEL COTRANSPORTADOR RENAL DE NACI POR ANGIOTENSINA II"

Sin más por el momento quedo de usted.

Atentamente,

Dr. Gerardo Gamba Ayala
Director de Investigación

c.c.p. Dra. Ma. Elena Flores – Encargada del Bioterio

Vasco de Quiroga No. 15
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INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

"2014, Año de Octavio Paz"

Ausse

México, D.F. a 28 de Octubre del 2014

Dr. Gerardo Gamba A.
Depto. de Nefrología y
Metabolismo Mineral
Presente

Estimado Dr. Gamba:

Por este conducto le informo que su proyecto: **"MECANISMOS DE REGULACIÓN DEL COTRANSPORTADOR RENAL DE NACI POR ANGIOTENSINA II"**, con registro **CINVA 25** finalizó en el año 2013. Por lo que le solicito de la manera más atenta me haga saber si el proyecto requerirá una prórroga. En caso afirmativo, favor de enviar a la **CINVA** el periodo de extensión que solicita y de requerir un mayor número de animales especificar y justificar como se utilizarán y los procedimientos experimentales que se llevarán a cabo con los mismos. En caso de no requerir una prórroga favor de llenar el formato de cierre del protocolo que se anexa a la presente.

Sin otro particular por el momento, quedo de usted.

INS. NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN"
31 OCT 2014
DIRECCIÓN DE INVESTIGACIÓN

Atentamente,

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la CINVA

c.c.p. Dra. María Elena Flores Carrasco, Encargada del Departamento de Investigación Experimental y Bioterio.

DEPARTAMENTO DE INVESTIGACIÓN EXPERIMENTAL Y BIOTERIO
04-11-14

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Delegación Tlalpan
México, D. F. 14000
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INNSZ

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

INST. NACIONAL DE CIENCIAS MEDICAS Y NUTRICION "SALVADOR ZUBIRAN"
20 FEB 2012
DEPARTAMENTO DE INVESTIGACION EXPERIMENTAL Y BIOTERIO

17 de febrero de 2012.

Dr. Rafael Hernández González
Coordinador de la CINVA
Instituto.

En relación con su comunicado del día 13 del presente en donde me solicito si el proyecto intitulado *"Mecanismos de regulación del Contransportador Renal de NaCl por Angiotensina II "* requerirá prórroga, le comento lo siguiente: este proyecto es financiado por la Fundación Welcome Trust. Inicialmente, por error, se registró en CADI con la Forma Unica para Registro de Proyectos con fecha de terminación en abril del 2012. Sin embargo, posteriormente se corrigió la fecha al mes de junio de 2013. Asimismo le anexo copia de la FORMA UNICA en donde ya aparece con la fecha corregida y que fue recibida por CADI en marzo del 2011.

Por lo anterior, solicito de la manera más atenta se realice la corrección al Proyecto con registro CINVA 25.

Sin otro particular por el momento, quedo a sus órdenes para cualquier aclaración.

Atentamente,

Dr. Gerardo Gamba A.
Investigador en Ciencias Médicas "F"
Depto. de Nefrología y Metabolismo Mineral.

• Vasco de Quiroga 15,
• Delegación Tlalpan
• C. P. 14000 México, D. F.
• Tel. 54-87-09-00

INNSZ

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

13 de febrero del 2012

DR. GERARDO GAMBA AYALA
DEPARTAMENTO DE NEFROLOGÍA Y METABOLISMO MINERAL
P R E S E N T E

Estimado Dr. Gamba.

Por este conducto le informo que el proyecto que lleva por nombre: **Mecanismos de Regulación del Cotransportador Renal de NaCl por Angiotensina** con registro CINVA 25 finalizará el mes de abril del año en curso. Le solicito de la manera más atenta me haga saber si el proyecto requerirá una prorroga. En caso afirmativo le solicitaré envié a la CINVA la justificación correspondiente.

Sin otro particular por el momento.

Atentamente.

Dr. Rafael Hernández González
Coordinador de la CINVA

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN"
17 FEB 2012
DEPTO. DE NEFROLOGÍA Y METABOLISMO MINERAL

• Vasco de Quiroga 15,
• Delegación Tlalpan
• C. P. 14000 México, D. F.
• Tel. 54-87-09-00

"2011, Año del Turismo en México"

INNSZ

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

24 de marzo de 2011

Dr. Gerardo Gamba Ayala
Investigador Principal
Departamento de Nefrología y
Metabolismo Mineral del INCMNSZ

Con referencia a su comunicado de fecha 18 de marzo del presente, informo a usted que hemos tomado nota de la llegada de los 15 ratones de la cepa C57BL/6 WNK4 KO.

Sin otro particular reciba usted un cordial saludo.

Atentamente

MVZ. M.Sc. Rafael Hernández González
Jefe del Departamento de Investigación
Experimental y Bioterio

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN"
24 MAR 2011
DEPTO. DE NEFROLOGÍA Y METABOLISMO MINERAL

Investigación
Tradición Servicio
Asistencia Docencia

• Vasco de Quiroga 15,
• Delegación Tlalpan
• C. P. 14000 México, D. F.
• Tel. 54-87-09-00

INCMNSZ

INSTITUTO NACIONAL DE CARDIOLOGÍA IGNACIO CHÁVEZ

Unidad de Fisiología Molecular

Instituto de Investigaciones Biomédicas

Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán

Instituto Nacional de Cardiología Ignacio Chávez

Vasco de Quiroga No. 15, Tlalpan
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(5255) 5573 6902

gamba@biomedicas.unam.mx
gerardo.gambaa@quetzal.innsz.mx
gerardo.gamba@cardiologia.org.mx

México, D.F. a 18 de Marzo de 2011.

MVZ., M. Sc. Rafael Hernández González.
Coordinador del Comité de Investigación en Animales (CINVA) del INCMN-SZ.
P r e s e n t e.

En relación con el proyecto de Investigación titulado: "**Mecanismos de regulación del cotransportador renal de NaCl por Angiotensina II**", registro CINVA: #25, Clave: NMM-25-10-12-1, me permito solicitar la introducción al bioterio del INCMN-SZ de 15 ratones de la cepa C57BL/6 WNK4 KO, provenientes del bioterio del Instituto de Investigaciones Biomédicas-UNAM. A la vez de reportar el uso de los animales, única y exclusivamente con la finalidad de continuar con los objetivos descritos en el proyecto CINVA #25.

El periodo de permanencia de los animales será de 3 semanas posteriores al día 18 de marzo del año en curso.

Dichos animales serán mantenidos, manejados y sacrificados de acuerdo al reglamento, a los manuales y guías de procedimientos del Depto. de Investigación experimental y Bioterio, mismos que contiene la Norma Oficial Mexicana NOM-062-ZOO-1999, especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio publicada por la SAGARPA en el Diario Oficial del día 22 de Agosto del 2001, y los lineamientos nacionales e internacionales para el buen uso de los animales de experimentación.

A t e n t a m e n t e,

Dr. Gerardo Gamba Ayala
Investigador Principal
Departamento de Nefrología y Metabolismo Mineral del INCMNSZ.

ccp. MVZ. Mónica Guevara Canizal. Coord. Área de producción de roedores del Bioterio. INCMNSZ.

INNSZ

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

México, D.F. a 27 de Julio de 2010.

MVZ., M. Sc. Rafael Hernández González.
Coordinador del Comité de Investigación en Animales (CINVA) del INCMN-SZ.
P r e s e n t e.

En relación con el proyecto de Investigación titulado: "**Mecanismos de regulación del cotransportador renal de NaCl por Angiotensina II**", registro CINVA: #25, Clave: NMM-25-10-12-1, me permito solicitar la introducción al bioterio del INCMN-SZ de 15 ratones de la cepa C57BL/6 provenientes del bioterio del Instituto de Investigaciones Biomédicas-UNAM. A la vez de reportar el uso de los animales, única y exclusivamente con la finalidad de continuar con los objetivos descritos en el proyecto CINVA #25.

El periodo de permanencia de los animales será de 4 semanas posteriores al día 27 de Julio del año en curso.

Al final de su estudio, dichos animales serán mantenidos, manejados y sacrificados de acuerdo al reglamento, a los manuales y guías de procedimientos del Depto. de Investigación experimental y Bioterio, mismos que contiene la Norma Oficial Mexicana NOM-062-ZOO-1999, especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio publicada por la SAGARPA en el Diario Oficial del día 22 de Agosto del 2001, y los lineamientos nacionales e internacionales para el buen uso de los animales de experimentación.

A t e n t a m e n t e,

Dr. Gerardo Gamba Ayala
Investigador Principal
Departamento de Nefrología y Metabolismo Mineral del INCMNSZ.

ccp. MVZ. Mónica Guevara Canizal. Coord. Área de producción de roedores del Bioterio. INCMNSZ.

Instituto Nacional de Ciencias Médicas y Nutrición
Salvador Zubirán

COMITÉ INSTITUCIONAL DE INVESTIGACIÓN BIOMÉDICA EN HUMANOS

FORMATO DE EVALUACIÓN DE PROYECTO DE INVESTIGACIÓN

No. de registro CIIBH: NMM-25-10-12-1

1. Título del proyecto

Mecanismos de regulación del cotransportador renal de NaCl por angiotensina II

2. Investigadores

2a. Identificación

INVESTIGADOR	Posición institucional	Posición en el proyecto	Teléfono (ext.)	Correo-E
GAMBA AYALA GERARDO	INVESTIGADOR EN CIENCIAS MED F	Investigador responsable		gamba@quetzal.inns

2b. Pertinencia del grupo de investigadores con respecto del proyecto

3. Instituciones participantes

4. Patrocinio

4a. Organismos patrocinadores

4b. Especificar si los investigadores reciben pago (monetario o en especie) por su participación es investigación.

5. Marco teórico

6a. Hipótesis

La angII modula la actividad del NCC a traves de una vía de señalización intracelular que involucra a la cinasa WNK4

6b. Objetivos.

General:

Específicos:

7. Metodología: Diseño general.

Tenemos acceso a los dos tipos de ratones mencionados en objetivos: el ratón knockout para WNK4 (Wnk4 KO) y ratón c silvestre (Wnk4 +/+ +/+). El fondo genético es C57BL/6.\Mediante técnica bioquímicas (western blot y PCR en tiempo real y fosforilación de proteínas de interés (WNK4, SPAK, NCC). También se estudiarán parámetros fisiológicos en condicion arterial y la concentración plasmática de electrolitos. \Luego se someterán los ratones a dos maniobras experimentales. \I baja en sal. Se administrará una dieta al 0.01% de NaCl por 10 días y durante este periodo se mantendrán los ratones monitorear la excreción urinaria de sal. También se va a monitorear el peso corporal y el consumo de agua y aliment analizará la expresión y fosforilación de NCC, WNK4 y SPAK.\2. Administración crónica de AngII. Se administrará

bombas miniosmóticas implantadas en el dorso de los animales de manera subcutánea. A lo largo del la presión arterial med las mediciones por telemetría se insertará un cateter en la arteria carótida izquierda conectado a el transmisor que enviará arterial al sistema detector. El transmisor se implantará de manera subcutánea aprovechando la misma incisión hecha para l \Los animales se anestesiarán con ketamina (90 mg/Kg i.p.) y acepromazina (1.8 mg/Kg i.p). \Obtención de muestras muestra de sangre al inicio a través de seno ocular usando capilar.\El tamaño de la muestra aproximado será de 20 ratones experimental y un número equivalente de ratones como controles.\

8. Metodología: Criterios de selección

No aplica

9. Metodología: Desenlaces y variables

Desenlaces esperados:\1. Estudios de la integridad de la vía de señalización y de parámetros fisiológicos en condiciones l KO. Se espera encontrar a la vía de señalización suprimida ante la deficiencia de un componente de ésta. Los parámetros fis hipofunción del NCC.\b. Wnk4 +/+ +/+. Se espera encontrar una capacidad de respuesta aumentada ante el estím sobreexpresado un componente de la vía. \2. Administración de dieta baja en sal. \a. Ratones Wnk4 KO. Se espera observ balance entre ingesta y excreción si efectivamente la activación de NCC por AngII mediada por WNK4 es impor fisiológica.\b. Wnk4 +/+ +/+. Se espera encontrar que los ratones transgénicos alcanzan el balance más rápido que los contr cónica de AngII. \a. Ratones Wnk4 KO. Se espera observar cierta protección contra el desarrollo de hipertensión arterial.\b observar desarrollo de niveles mayores de presión arterial que en los ratones control.\Equipos:\El equipo de radiotelemet presión arterial en tiempo real que se usará es el modelo PA-C10 de Data Sciences International. Las determinaciones de plasma y orina se realizarán en la unidad metabólica del depto. de nefrología y metabolismo mineral del instituto, en el equi

10. Riesgos y beneficios del estudio

BENEFICIOS INDIRECTOS:

RIESGOS:

11. Costos

	COSTOS TOTALES DE LA INVESTIGACIÓN
Admon. Gastos pacientes	
Animales	
Equipos	
Estudios	
Materiales	
Personal	

No. de registro CIIBH: NMM-25-10-1

1. Título del proyecto

Mecanismos de regulación del cotransportador renal de NaCl por angiotensina II

2. Investigadores

2a. Identificación

INVESTIGADOR	Posición institucional	Posición en el proyecto	Teléfono (ext.)	Correo-E
GAMBA AYALA GERARDO	INVESTIGADOR EN CIENCIAS MED F	Investigador responsable		gamba@quetzal.inn

2b. Pertinencia del grupo de investigadores con respecto del proyecto

3. Instituciones participantes

4. Patrocinio

4a. Organismos patrocinadores

4b. Especificar si los investigadores reciben pago (monetario o en especie) por su participación es investigación.

5. Marco teórico

6a. Hipótesis

La angII modula la actividad del NCC a traves de una vía de señalización intracelular que involucra a la cinasa WNK4

6b. Objetivos.

General:

Específicos:

7. Metodología: Diseño general.

Tenemos acceso a los dos tipos de ratones mencionados en objetivos: el ratón knockout para WNK4 (Wnk4 KO) y ratón WNK4 silvestre (Wnk4 +/+ +/+). El fondo genético es C57BL/6.\Mediante técnica bioquímicas (western blot y PCR en tie la expresión y fosforilación de proteínas de interés (WNK4, SPAK, NCC). También se estudiarán parámetros fisiológicos e como la presión arterial y la concentración plasmática de electrolitos. \Luego se someterán los ratones a dos maniobra Administración de dieta baja en sal. Se administrará una dieta al 0.01% de NaCl por 10 días y durante este periodo se mant jaulas metabólicas para monitorear la excreción urinaria de sal. También se va a monitorear el peso corporal y el consum Al final del periodo se analizará la expresión y fosforilación de NCC, WNK4 y SPAK.\2. Administración crónica de A durante 21 días mediante bombas miniosmóticas implantadas en el dorso de los animales de manera subcutánea. A lo largo mediante radiotelemetría. Para las mediciones por telemetría se insertará un cateter en la arteria carótida izquierda conectac enviará las mediciones de presión arterial al sistema detector. El transmisor se implantará de manera subcutánea aprovecha hecha para la implantación del catéter. \Los animales se anestesiarán con ketamina (90 mg/Kg i.p.) y acepromazina (1.8 m de muestras de sangre. Se recolectara muestra de sangre al inicio a través de seno ocular usando capilar.\El tamaño de la será de 20 ratones de cada cepa por maniobra experimental y un número equivalente de ratones como controles.\

8. Metodología: Criterios de selección

No aplica

9. Metodología: Desenlaces y variables

Desenlaces esperados:\1. Estudios de la integridad de la vía de señalización y de parámetros fisiológicos en condicione Wnk4 KO. Se espera encontrar a la vía de señalización suprimida ante la deficiencia de un componente de ésta. Los pa deben reflejar la hipofunción del NCC.\b. Wnk4 +/+ +/+. Se espera encontrar una capacidad de respuesta aumentada ante el al estar sobreexpresado un componente de la vía. \2. Administración de dieta baja en sal. \a. Ratones Wnk4 KO. Se espera alcanzar el balance entre ingesta y excreción si efectivamente la activación de NCC por AngII mediada por WNK4 es i función fisiológica.\b. Wnk4 +/+ +/+. Se espera encontrar que los ratones transgénicos alcanzan el balance más rápi silvestres. \3. Infusión cónica de AngII. \a. Ratones Wnk4 KO. Se espera observar cierta protección contra el desar arterial.\b. Wnk4 +/+ +/+. Se espera observar desarrollo de niveles mayores de presión arterial que en los ratones control.\I radiotelemetría para las mediciones de presión arterial en tiempo real que se usará es el modelo PA-C10 de Data Scien determinaciones de electrolitos y creatinina en plasma y orina se realizarán en la unidad metabólica del depto. de nefr

mineral del instituto, en el equipo Synchron CX5.\

10. Riesgos y beneficios del estudio

BENEFICIOS INDIRECTOS:

RIESGOS:

11. Costos

COSTOS TOTALES DE LA INVESTIGACIÓN	
Admon. Gastos pacientes	$ 0.00
Animales	$ 0.00
Equipos	$ 0.00
Estudios	$ 0.00
Materiales	$ 0.00
Personal	$ 0.00
Publicaciones	$ 0.00
Suscripciones	$ 0.00
Varios	$ 0.00
Viaticos	$ 0.00

12. Citas bibliográficas.

1. Gamba, G. (2005) Physiol Rev. 85, 423-493\ 2. Simon D B, et al. (1996) Nature Genetics 12, 24-30\ 3. Mastroi
Am.J.Hum.Genet. 59, 1019-1026\ 4. Gordon, R. D. (1986) Hypertension 8, 93-102\ 5. Lalioti, M. D., et al. (2006) Nat.Ge
Wilson, F. H., et al. (2003) Proc.Natl.Acad.Sci.U.S.A 100, 680-684\ 7. San Cristobal, P., et al. (2008) Am.J.Nephrol. 28, 8
et al. (2007) Pharmacol.Rev. 59, 251-287\ 9. Sandberg, M. B., et al. (2007) Am J Physiol Renal Physiol 293, F662-F669\
et al. (2009) Proc.Natl.Acad.Sci.U.S.A 106,\ 11. Richardson, C., et al. (2008) J.Cell Sci. 121, 675-684\

GI (2276)

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRAN

08 MAR 2011

DIRECCIÓN DE INVESTIGACIÓN
DEPTO. DE CONTROL DE FONDOS
ESPECIALES A LA INVESTIGACIÓN

Dirección de Investigación

INCMNSZ

FORMA ÚNICA PARA REGISTRO DE PROYECTOS

No invada las zonas sombreadas

CLAVE: NMM - 25 - 10 / 12 - 1

FECHA DE RECEPCIÓN: 2011-02-15

TÍTULO: Mecanismos de regulación del cotransportador renal de NaCl por angiotensina II

INVESTIGADOR RESPONSABLE: GERARDO GAMBA

DEPARTAMENTO O SERVICIO: NEFROLOGIA Y METABOLISMO MINERAL

TIPO DE INVESTIGACIÓN:

	1. Investigación Clínica	(incluye seres humanos o sus productos biológicos)
X	2. Investigación Experimental	(incluye animales de investigación o sus productos biológicos)
	3. Investigación Documental	(revisión de expedientes, revisión bibliográfica, informe de casos, etc.)
	4. Desarrollo Tecnológico	(instrumental, equipo, metodos diagnósticos, drogas nuevas, etc)
	5. Investigación Epidemiológica	(estudios en poblaciones, en comunidad o en hospital)
	6. Otros	(organización de eventos, asistencia a reuniones, donativos, etc)

PATROCINADORES:

	Cantidad:	
Fundacion Welcome Trust, Universidad de Dundee	0	2800064
	0	TOTAL
	0	210004 Fondo de Apoyo

PERÍODO DE UTILIZACIÓN DE LOS RECURSOS: de mes: 07 año: 2010 a mes: 06 año: 2013

FORMA EN LA QUE SE RECIBIRÁN LOS FONDOS:

	primer trimestre	segundo trimestre	tercer trimestre	cuarto trimestre
Primer año:	933354.66	0	0	0
Segundo año:	933354.66	0	0	0
Tercer año:	933354.66	0	0	0
Cuarto año:	0	0	0	0
Quinto año:	0	0	0	0

COSTOS TOTALES DE LA INVESTIGACIÓN
(ver instrucciones al reverso)

1. Personal: (sueldos y sobresueldos al personal)	800000
2. Equipos: (de laboratorio, cómputo, transporte, etc.)	0
3. Materiales: (reactivos, consumibles, desechables, etc.)	1500000
4. Animales: (adquisición, cuidado, procedimientos, etc.)	190060
5. Estudios: (de laboratorio, gabinete, especiales, etc.)	0
6. Viáticos: (reuniones científicas y trabajo de campo)	100000
7. Publicaciones: (costos directos de publicación, sobreliros)	0
8. Suscripción: (libros, revistas, software, periódico, etc.)	0
9. Varios: (teléfono, fax, fotocopias, mensajería, etc.)	0
10. Fondo de Apoyo: (15% de la cantidad total del proyecto)	7.5 % 210004
TOTAL:	2800064

INSTITUCIONES PARTICIPANTES
Instituto Nacional de Nutrición Salvador Zubirán

FIRMAS

Investigador Responsable

Jefe del Departamento

Comité de Investigación en Humanos

Comité de Investigación en Animales

Director de Investigación

Director General

7-marzo-2011
Fecha de Resolución

LATIS Sistema Integral

Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán

Folio del registro: NMM-25-10-12-1

Notice: Undefined index: 2006_comites_idComite in C:\Program Files (x86)\Apache Software Foundation\Apache2.2\htdocs\latis\include\latis\utiles.php on line 2776 Notice: Undefined index: 2006_comites_idComite in C:\Program Files (x86)\Apache Software Foundation\Apache2.2\htdocs\latis\include\latis\utiles.php on line 2776 Notice: Undefined index: 2006_comites_idComite in C:\Program Files (x86)\Apache Software Foundation\Apache2.2\htdocs\latis\include\latis\utiles.php on line 2776 Notice: Undefined index: 2006_comites_idComite in C:\Program Files (x86)\Apache Software Foundation\Apache2.2\htdocs\latis\include\latis\utiles.php on line 2776 Notice: Undefined index: 2006_comites_idComite in C:\Program Files (x86)\Apache Software Foundation\Apache2.2\htdocs\latis\include\latis\utiles.php on line 2776 Notice: Undefined index: 2006_comites_idComite in C:\Program Files (x86)\Apache Software Foundation\Apache2.2\htdocs\latis\include\latis\utiles.php on line 2776

Formato Único de Registro

(0) Comentarios

Título del proyecto: Mecanismos de regulación del cotransportador renal de NaCl por angiotensina II

Tipo de proyecto:

Antecedentes:

El cotransportador renal de Na+:Cl- (NCC) es la principal vía absorción de sal en la membrana apical del túbulo contorneado distal (TCD) del riñón de mamífero. Es la proteína blanco de los diuréticos de tipo tiazida, utilizados en el tratamiento de la hipertensión arterial. Mutaciones inactivantes en el gen de NCC causan síndrome de Gitelman, enfermedad hereditaria que cursa con hipotensión arterial, alcalosis metabólica hipocalémica e hipocalciuria (2;3). El síndrome de Gordon es una enfermedad autosómica dominante que cursa con hipertensión arterial, acidosis metabólica, hipercalemia e hipercalciuria (4), es decir, es una imagen en espejo del síndrome de Gitelman. Éste se debe a mutaciones en la cinasa WNK4. Diversos estudios han establecido que WNK4 es un inhibidor del NCC y que las mutaciones de tipo Gordon resultan en pérdida de la inhibición, por lo que el síndrome es consecuencia de hiperactividad de NCC (5-7). El sistema renina-angiotensina es un eje hormonal para la regulación de la presión arterial y el balance hidroelectrolítico. El efecto de AngII sobre NCC ha sido recientemente estudiado. La AngII promueve el tráfico de vesículas con NCC hacia la membrana apical de las células del TCD, lo que aumenta la reabsorción de sal en esta región (9). Se desconoce el mecanismo por el cual la AngII logra este efecto.Recientemente demostramos, en un modelo in vitro, que AngII modula el efecto de WNK4 sobre NCC (10). En ausencia de AngII la WNK4 inhibe al NCC, al disminuír la cantidad de NCC en la membrana plasmática (6). En presencia de AngII se pierde la inhibición de WNK4 sobre NCC. Este efecto es dependiente de la presencia del receptor AT1 y requiere de la presencia de la cinasa SPAK, otra cinasa que forma parte de la vía de señalización y que fosforila al NCC (11). Finalmente, la WNK4 con mutaciones de Gordon no responde al estímulo de AngII, por lo que propusimos que mutaciones en WNK4 imitan el efecto de la AngII y que los efectos observados de AngII sobre NCC se logran a través de una vía de señalización en la que participa WNK4. Sin embargo, es necesaria la utilización de un modelo in vivo para verificar las observaciones hechas in vitro.

Definición del problema:

Justificación:

Hipótesis: La angII modula la actividad del NCC a traves de una vía de señalización intracelular que involucra a la cinasa WNK4

Fecha estimada de inicio:

Fecha estimada de término:

Comisión a la que somete

¿Incluye documentos anexos?:

Investigadores participantes

(0) Comentarios

Investigador	Participación	Orden de participación	Investigador responsable
Gamba Ayala, Gerardo	Investigador responsable	1	Sí

Objetivos

(0) Comentarios

Objetivo:

Objetivo general: Demostrar, en un modelo in vivo, la participación de la cinasa WNK4 en la vía de señalización mediante la cual AngII modula la actividad de NCC. Objetivos específicos: Determinar el efecto de la administración de dieta baja en sal (que estimulará la activación del eje renina-AngII-aldosterona, RAA) en ratones deficientes de Wnk4 (knockout; Wnk4 KO) y en ratones que sobreexpresan a la proteína (ratones transgénicos que presentan dos copias extras del gen, se abreviarán: Wnk4 +/+ +/+). Determinar el efecto de la infusión crónica de AngII en los mismos ratones. Determinar el papel que juega la vía de WNK4-NCC en el desarrollo de hipertensión arterial inducido por AngII.

Tipo de objetivo:

Objetivo:

Tipo de objetivo:

Metodología: Diseño general

(0) Comentarios

Metodología gral:

Tenemos acceso a los dos tipos de ratones mencionados en objetivos: el ratón knockout para WNK4 (Wnk4 KO) y ratón con cuatro alelos de WNK4 silvestre (Wnk4 +/+ +/+). El fondo genético es C57BL/6.\

Mediante técnica bioquímicas (western blot y PCR en tiempo real) se analizará la expresión y fosforilación de proteínas de interés (WNK4, SPAK, NCC). También se estudiarán parámetros fisiológicos en condiciones basales como la presión arterial y la concentración plasmática de electrolitos. \

Luego se someterán los ratones a dos maniobras experimentales. \

1. Administración de dieta baja en sal. Se administrará una dieta al 0.01% de NaCl por 10 días y durante este periodo se mantendrán los ratones en jaulas metabólicas para monitorear la excreción urinaria de sal. También se va a monitorear el peso corporal y el consumo de agua y alimento. Al final del periodo se analizará la expresión y fosforilación de NCC, WNK4 y SPAK.\

2. Administración crónica de AngII. Se administrará durante 21 días mediante bombas miniosmóticas implantadas en el dorso de los

animales de manera subcutánea. A lo largo del la presión arterial mediante radiotelemetría. Para las mediciones por telemetría se insertará un cateter en la arteria carótida izquierda conectado a el transmisor que enviará las mediciones de presión arterial al sistema detector. El transmisor se implantará de manera subcutánea aprovechando la misma incisión hecha para la implantación del catéter. \
Los animales se anestesiarán con ketamina (90 mg/Kg i.p.) y acepromazina (1.8 mg/Kg i.p). \
Obtención de muestras de sangre. Se recolectara muestra de sangre al inicio a través de seno ocular usando capilar.\
El tamaño de la muestra aproximado será de 20 ratones de cada cepa por maniobra experimental y un número equivalente de ratones como controles.\

Metodología: Criterios de selección

(0) Comentarios

Criterios de selección del protocolo: No aplica

Metodología: Desenlace y variables

(0) Comentarios

Metodología de desenlace y variables:

Desenlaces esperados:\
1.Estudios de la integridad de la vía de señalización y de parámetros fisiológicos en condiciones basales. \
a.Ratones Wnk4 KO. Se espera encontrar a la vía de señalización suprimida ante la deficiencia de un componente de ésta. Los parámetros fisiológicos deben reflejar la hipofunción del NCC.\
b.Wnk4 +/+ +/+. Se espera encontrar una capacidad de respuesta aumentada ante el estímulo de la AngII al estar sobreexpresado un componente de la vía. \
2.Administración de dieta baja en sal. \
a.Ratones Wnk4 KO. Se espera observar retraso para alcanzar el balance entre ingesta y excreción si efectivamente la activación de NCC por AngII mediada por WNK4 es importante para dicha función fisiológica.\
b.Wnk4 +/+ +/+. Se espera encontrar que los ratones transgénicos alcanzan el balance más rápido que los controles silvestres. \
3.Infusión cónica de AngII. \
a.Ratones Wnk4 KO. Se espera observar cierta protección contra el desarrollo de hipertensión arterial.\
b.Wnk4 +/+ +/+. Se espera observar desarrollo de niveles mayores de presión arterial que en los ratones control.\
Equipos:\
El equipo de radiotelemetría para las mediciones de presión arterial en tiempo real que se usará es el modelo PA-C10 de Data Sciences International. Las determinaciones de electrolitos y creatinina en plasma y orina se realizarán en la unidad metabólica del depto. de nefrología y metabolismo mineral del instituto, en el equipo Synchron CX5.\

Riesgo (s) del estudio

(0) Comentarios

Molestias generadas por el estudio:

No hay riesgos potenciales.\
En cuanto a beneficios, este estudio permitirá obtener un conocimiento más profundo sobre los mecanismos de regulación de la reabsorción de sal a nivel del túbulo contorneado distal de la nefrona, que se ha implicado en el mantenimiento de la presión arterial

y de la homeostasis electrolítica. Además, podría también generarse conocimiento valiosos sobre los mecanismos fisiopatológicos de la hipertensión arterial inducida por AngII, lo cual podría ser de utilidad en un futuro para el diseño de nuevos tratamientos.\

Complicaciones del procedimiento:

Efectos adversos reportados de medicamentos o sustancias utilizadas:
Métodos de seguridad para el diagnóstico oportuno y prevención de los riesgos:
Procedimientos a seguir para resolver los riesgos en caso de que se presenten:
Otro tipo de riesgo: