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FISIOLOGÍA RENAL

LA INVESTIGACIÓN BÁSICA Y TRASLACIONAL SOBRE EL COTRANSPORTADOR RENAL DE NACL. I. REGULACIÓN POR HORMONAS FEMENINAS. II. PAPEL EN LA HIPERTENSIÓN ARTERIAL INDUCIDA POR TACROLIMUS EN PACIENTES CON TRASPLANTE RENAL.

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El presente trabajo se realizó en la Unidad de Fisiología Molecular perteneciente al IIB y localizado en el INCMNSZ bajo la asesoría del Dr. Gerardo Gamba.

Mis estudios de doctorado fueron apoyados por la beca otorgada por el CONACyT con número de registro: 288537/262372. Los proyectos realizados durante el mismo fueron financiados por CONACyT: SEP-Conacyt 165815 y CONACyT-ANR 188712.

Resumen

Durante la realización de mi doctorado bajo la tutoría del Dr. Gerardo Gamba en el laboratorio periférico del IBB en el INNCMSZ, pude desarrollar dos trabajos paralelos que si bien tuvieron hipótesis y objetivos diferentes entre ellos, ambos coinciden en profundizar el conocimiento de los estímulos que activan al cotransportador renal NaCl (NCC) sensible a tiazidas. Ambos artículos han sido publicados, por lo que los resultados son mostrados dentro de los artículos anexados a esta tesis.

El primer trabajo publicado trata de la regulación dimórfica sexual de NCC por parte de las hormonas femeninas: Estrógenos, Progesterona y Prolactina, mientras que el segundo trata de la relación del aumento de la fosforilación de NCC y la presencia de hipertensión en pacientes con trasplante renal tratados con tacrolimus.

Regulación por hormonas femeninas

La fisiología femenina tiene fenómenos únicos como la menstruación, el embarazo y la lactancia que para su función requieren de la retención de volumen. El contransportador Na^{+}*:*Cl^{-} *(NCC), es una proteína que ya ha sido reportada por ser regulada de manera distinta en ambos géneros, sin embargo, dicho reporte, a pesar de ser una excelente observación, no fue estudiado a más profundidad.*

En este trabajo, en un principio, confirmamos a través del análisis de la expresión/actividad de NCC en muestras de rata, ratón y humanos que la regulación dimórfica permanece conservada en distintas especies. En la rata, en particular, la diferencia solo es en el grado de fosforilación/activación del transportador presente en hembras comparadas con ratas macho; mientras que en ratón y humano las diferencias son tanto en expresión como en fosforilación/activación de NCC. Dichas diferencias estuvieron relacionadas con una mayor expresión y fosforilación/activación de la cinasa maestra encargada de activar a NCC, STE-20 proline-alanine-rich kinase (SPAK), de igual manera en hembras en comparación con los machos.

Un objetivo en este trabajo fue poder analizar el estado del contransportador NCC en humanos, para ello se montó la técnica de extracción de exosomas de muestras de orina de humanos.

Para confirmar las observaciones del efecto del género sobre el estado del NCC, se practicaron gonadectomías en ratas y sólo la ovariectomía tuvo un efecto. Las hembras castradas presentaron una disminución en la expresión y fosforilación del transportador y de SPAK.

Utilizando diversos protocolos y modelos animales se analizó el efecto de las hormonas femeninas: estradiol (E2), progesterona (P4) y prolactina (PRL). Se encontró que las tres hormonas tienen la capacidad de fosforilar al cotransportador siendo más poderoso el estradiol.

Por lo resultados anteriores, se llegó a la conclusión que las hormonas ováricas y la prolactina son las responsables de la regulación dimórfica sexual.

Papel en la hipertensión arterial inducida por tacrolimus en pacientes con trasplante renal

Datos encontrados en modelos murinos sugieren que la hipertensión post-trasplante renal inducida por tacrolimus se puede deber a una sobreactivación de NCC. En este proyecto se analizó si mecanismos similares suceden en humanos. De Enero del 2013 a Junio del 2014 todos los receptores adultos de riñón del INNCMSZ fueron enlistados en un estudio de cohorte prospectiva. Todos los pacientes recibieron tacrolimus como parte de la terapia inmunosupresora. Seis meses después de la cirugía, se analizaron las variables clínicas y se hicieron estudios de rutina a las muestras obtenidas, entre ellos se analizaron las concentraciones de tacrolimus en sangre y se monitoreo la presión arterial de manera ambulatoria por 24 horas. Para analizar el estado de activación del NCC, se recolectaron muestras de orina seis meses post-trasplante renal. Después de purificar los exosomas se analizó el estado de fosforilación del NCC. Se estudiaron 52 pacientes, 17 mujeres y 35 hombres. Transcurridos seis meses, de los 35 hombres, 17 desarrollaron hipertensión y los 18 restantes permanecieron normotensos, mientras que de las 17 mujeres sólo 3 desarrollaron hipertensión. Los pacientes hipertensos fueron significativamente mayores en edad al grupo de normotensos; sin embargo, no hubo cambios entre el peso corporal, eventos de rechazo agudo, función renal y concentraciones de tacrolimus. En los exosomas urinarios, los pacientes hipertensos mostraron una mayor expresión y fosforilación de NCC en comparación de los normotensos. Estos datos sugieren una correlación positiva entre la expresión/fosforilación del NCC y el desarrollo de hipertensión en pacientes con trasplante renal tratados con tacrolimus, en específico en hombres.

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Abreviaturas

a.a.	Aminoácidos
AAH	Asa ascendente de Henle
ADH	Asa descendente de Henle
AH	Asa de Henle, entiéndase a todo el segmento
Ang II	Angiotensina II
BFTZ	Bendroflumetiazida
CL	Cuerpo Lúteo
DMSO	Dimetilsulfóxido
ECA	Enzima convertidora de angiotensina
EHH	Eje hipotálamo-hipófisis
ELISA	Ensayo por inmunoabsorción ligado a enzimas
ENaC	Canal de Na^+ sensible a amilorida
ERC	Enfermedad renal crónica
ERCT	Enfermedad renal crónica terminal
E2	17ß-Estradiol
FSH	Hormona folículo estimulante
GnRH	Hormona liberadora de Gonadotrofina
Gdx	Gonadectomía
HAD	Hormona arginina vasopresina/antidiurética
HTA	Hipertensión arterial
ICN	Inhibidores de calcineurina
LIC	Líquido intracelular
LH	Hormona leutinizante
MR	Receptor de mineralocorticoides
MD	Mácula densa
NCC	Cotransportador Na^+:Cl^-
NKCC1	Cotransportador de Na^+:K^+:$2Cl^-$ tipo 1
NKCC2	Cotransportador de Na^+:K^+:$2Cl^-$ tipo 2
PA	Presión arterial
p.b.	Pares de bases
P4	Progesterona
PRL	Prolactina
PRLR	Receptor de prolactina
OSR1	*Oxidative Stress-Responsive 1 protein*
SLC12	Familia de co-transportadores electroneutros acoplados a cloruro
SNP	Polimórfismo de nucleótido individual
SPAK	*Ste20-related proline alanine-rich kinase*

TCD	Túbulo contorneado distal
TCN	Túbulo conector
TM	Transmembrana
TP	Túbulo próximal
TFG	Tasa de filtrado glomerular
UFG	Ultrafiltrado glomerular
Vhl	Vehículo de administraión de algún fármaco.
w.b.	Western blot
WNK	Cinasas sin Lisina (With no “K” lysine kinase)

Iones

Na^{+}	Sodio
Ca^{2+}	Calcio
K^{+}	Potasio
Cl^{-}	Cloro
H^{+}	Hidrogeniones
HCO^{-}_{3}	Bicarbonato

INTRODUCCIÓN

Riñón

El riñón es el órgano encargado del balance global de sal y agua en el organismo. Los riñones tienen tres funciones esenciales: 1) Filtros, que remueven los productos metabólicos y toxinas del torrente sanguíneo y las excretan en la orina; 2) Regulan el equilibrio de los fluidos y electrolitos corporales así como del balance ácido-base; y 3) Aparato endocrino, al producir hormonas activas involucradas en la eritrogénesis, el metabolismo del Ca^{2+}, la regulación de la presión sanguínea y el volumen sanguíneo (74).

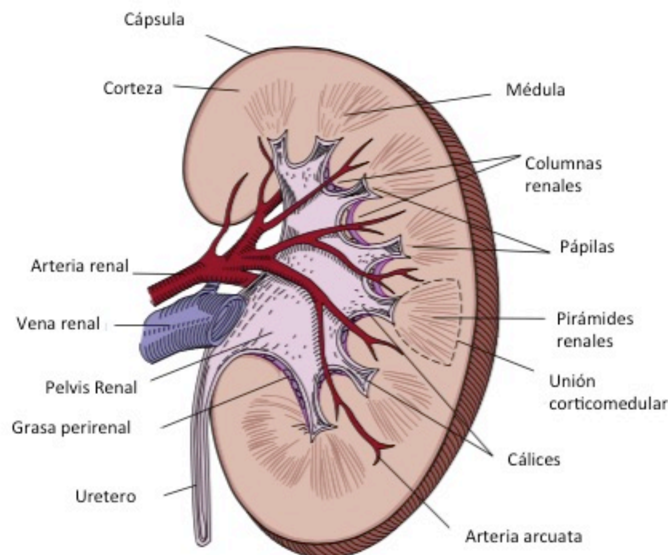

Figura 1. Esquema de la anatomía del riñón. Modificado de: (142).

La unidad funcional del riñón es la nefrona. Ésta representa a un grupo de células epiteliales especializadas en el transporte de solutos y del agua que se encuentran dispuestas dentro del parénquima renal de tal manera que llevan a cabo la filtración, reabsorción y secreción, procesos centrales de la función renal. Cada riñón está compuesto de casi un millón de nefronas.

La nefrona se divide en dos porciones: a) el glomérulo y b) los túbulos. El glomérulo es la primera porción de la nefrona y está compuesto por la arteriola aferente,

los capilares glomerulares, la arteriola eferente y la cápsula de Bowman; es en el glomérulo donde ocurre el proceso de filtración. Los capilares glomerulares se encuentran rodeados por células epiteliales especializadas de la cápsula de Bowman conocidas como podocitos. La cápsula de Bowman se integra de células epiteliales parietales que engloban a los capilares glomerulares de tal manera que el filtrado se recolecta dentro de la cápsula de Bowman. Los capilares glomerulares están fijados al polo arterial de la cápsula de Bowman mediante un grupo de células mesangiales que se encuentran en contacto con las arteriolas y los capilares glomerulares.

Componentes de la nefrona

El líquido filtrado desde los capilares glomerulares hacia la cápsula de Bowman se conoce como ultrafiltrado glomerular (UFG). Una vez depositado en la cápsula de Bowman, el UFG se desplaza a través de los túbulos renales donde se somete a procesos de reabsorción y secreción que convierten una gran cantidad de ultrafiltrado glomerular en una pequeña cantidad de orina. A los túbulos renales se los puede subdividir como sigue (Figura 2) (167):

1. Túbulo proximal (TP): es la porción de la nefrona inmediatamente después de la cápsula de Bowman. En esta región se reabsorbe agua y solutos. Las células del TP se caracterizan por ser metabólicamente activas ya que realizan cerca del 65 al 70% de la reabsorción del filtrado glomerular; tienen borde en cepillo apical, lo cual aumenta el área de superficie de manera significativa. En esta sección de la nefrona se reabsorbe el 100% de sustancias como glucosa, aminoácidos (a.a.) y se secretan fármacos que se excretan por la orina (113).
2. Asa de Henle: Sigue de manera inmediata al TP y desempeña una función de suma importancia en la concentración y dilución urinaria; se compone de varios segmentos:
    a. Asa descendente de Henle (ADH): El primer segmento se conoce como asa descendente de Henle. En las nefronas corticales, ésta posee tan sólo una porción delgada, mientras que en las nefronas yuxtaglomedulares se divide en porciones gruesa y delgada. En el ADH ocurre la reabsorción

pasiva de agua, dado que esta porción de la nefrona es permeable al agua, pero es impermeable a solutos (142).

b. Asa ascendente de Henle (AAH): el segundo segmento del AH es la porción ascendente. Tanto en nefronas corticales como en nefronas yuxtamedulares, el AAH consta de dos porciones, la delgada y a continuación la gruesa. A diferencia de lo observado con el ADH, el AAH es permeable a solutos, pero no al agua (18).

3. Mácula densa (MD): Inmediatamente distal a la porción gruesa del AAH se encuentra una región histológicamente similar al asa ascendente gruesa, pero cuya disposición anatómica hace posible que entre en contacto con las arteriolas aferente y eferente del glómerulo, así como con las células mesangiales. A este segmento del AH se le conoce como MD y, en conjunto con las arteriolas, recibe el nombre de aparato yuxtaglomerular. La MD funciona como sensor y en ella la cantidad de Na^+ y Cl^- se utiliza como determinante de la regulación de la filtración glomerular (7).
4. Túbulo contorneado distal (TCD): Es la porción inmediata que se localiza después de la MD. En esta porción de la nefrona se reabsorben agua y solutos, y es el sitio en donde comienza la regulación fina de la excreción de Na^+, Cl^- y Ca^{2+} (186).
5. Túbulo conector/colector: Justo después del TCD se localiza la siguiente porción tubular de la nefrona. En algunas especies se puede subdividir en túbulo conector (ya que se conecta al TCD con el sistema colector) y en túbulo colector. En esta porción de la nefrona es donde ocurre la regulación fina de la excreción urinaria de Na^+, K^+, H^+, HCO^-_3 y la reabsorción facultativa de agua. El túbulo colector desemboca en los cálices renales que al final drenan a los uréteres (128).

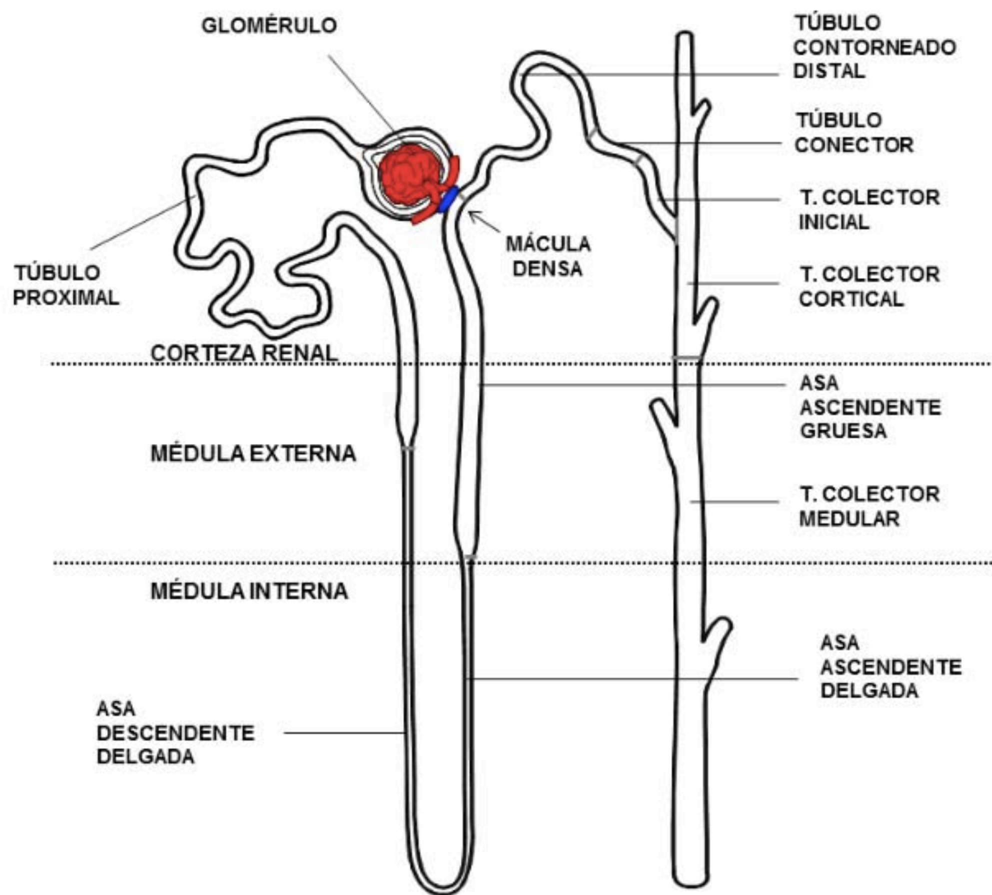

Figura 2. Esquema de los segmentos funcionales de la nefrona.

Los cotransportadores electroneutros acoplados a Cl^-

Los cotransportadores catiónicos acoplados a cloruro están ampliamente expresados en el organismo, son proteínas de membrana que mueven Na^+, K^+ y Cl^- a través de las membranas de manera electroneutra, es decir, ingresan el mismo número de cargas positivas y negativas a la célula (3). Este transporte es de tipo secundario, ya que utilizan el gradiente generado de sodio de la Na^+/K^+-ATPasa. La familia de los cotransportadores de cationes acoplados a Cl^- tiene al menos nueve miembros (Tabla 1) (158). Las funciones más relevantes de los cotransportadores son: 1) El transporte epitelial, como la reabsorción de sodio por el AAH y el TCD (61), la reabsorción de K^+ por el plexo coroideo (152), la secreción de K^+ por el oído interno y la secreción de cloro en el sudor y la saliva; 2) la regulación del gradiente de Cl^- y por lo tanto de la excitabilidad de las neuronas en el sistema nervioso (12) y 3) La regulación del volumen celular (175).

Los genes de la familia SLC12 codifican para un grupo de cotransportadores electroneutros acoplados a Cl^-. Esta familia está compuesta por 9 genes de los cuales 7 se han caracterizado extensivamente desde el punto de vista funcional. La familia está dividida en dos ramas (Tabla 1):

1. Cotransportadores electroneutros acoplados a Na^+:
    a. NKCC2 (SLC12A1): El transportador de Na^+:K^+:2Cl^- tipo 2, se expresa exclusivamente en el AAH y juega un papel determinante en los mecanismos de reabsorción renal de sal y cationes divalentes, así como en la concentración urinaria (57).
    b. NKCC1 (SLC12A2):El transportador de Na^+:K^+:2Cl^- tipo 1, es de expresión ubicua, involucrado en funciones como la percepción del dolor, la regulación del Cl^- y del volumen intracelular en el sistema nervioso (41).

c. NCC (SLC12A3): El transportador de Na^+:Cl^- Se expresa en el TCD en el riñón, cuya función es clave para la regulación del volumen extracelular y la presión arterial (PA).

2. Cotransportadores electroneutros acoplados a K^+:
   a. KCC1-KCC4 (SLC12A4-SLC12A7): Los transportadores de K^+:Cl^- 1 a 4 se expresan en diversos tejidos incluyendo eritrocitos (KCC1), sistema nervioso central (KCC2) y riñón (KCC3 y KCC4) (61).

Gen	Proteína	Locus en humano	Tipos de transporte	Expresión en tejidos
SLC12A1	NKCC2	15q21.1	$1Na^+$:$1K^+$:$2Cl^-$	Específico de riñón
SLC12A2	NKCC1	5q23.3	$1Na^+$:$1K^+$:$2Cl^-$	Ubicua
SLC12A3	NCC	16q13	1 Na^+:1 Cl^-	Riñón y hueso
SLC12A4	KCC1	16q22	$1K^+$:$1Cl^-$	Ubicua
SLC12A5	KCC2	20q13	$1K^+$:$1Cl^-$	Específico de cerebro
SLC12A6	KCC3	15q14	$1K^+$:$1Cl^-$	Extensivo
SLC12A7	KCC4	5p14	$1K^+$:$1Cl^-$	Extensivo; poca expresión en cerebro
SLC12A8	CCC9	3q21	Desconocido	Extensivo
SLC129	CIP	7q22	Desconocido	Extensivo

Tabla 1. Resumen de las características principales de la familia de contransportadores acoplados a cloro. Modificado de: (3).

El Sodio y el riñón: Cotransportador renal Na^+:Cl^- (NCC)

Manejo de Na^+

Los riñones ayudan a mantener el volumen del líquido extracelular del organismo regulando la cantidad excretada de Na^+ en la orina. El Na^+ es el catión más importante que contribuye al mantenimiento de la osmolaridad del LIC, debido a que a donde el Na^+ vaya siempre será seguido por el agua. La excreción diaria normal de Na^+ es sólo una pequeña del Na^+ total filtrado por los riñones. La carga filtrada de sodio es el producto del filtrado glomerular (180L/día) y la concentración plasmática de Na^+ (142mM) es decir, 25,500 mmol/día aproximadamente (Figura 3).

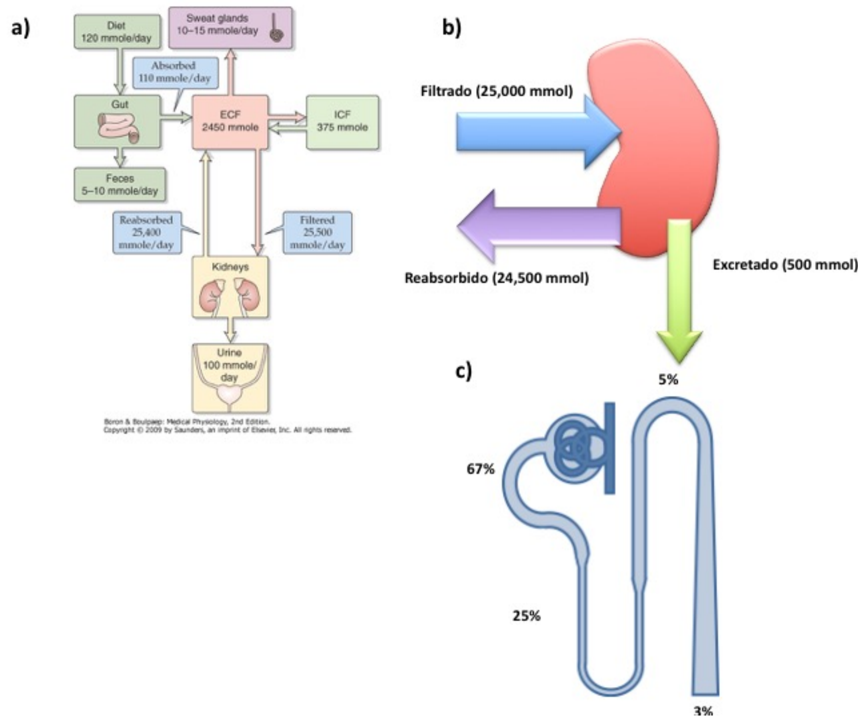

Figura 3. Manejo renal del Na^+. a) Distribución corporal de Na^+, *traducciones*: Diet, dieta; Sweat glands, glándulas sudoríparas; Urine, orina; Kidneys, riñones; Reabsorbed, reabsorbida; Filtered, filtrado; Feces, heces; ECF, LEC; ICF, LIC; Gut, intestino; Absorbed, absorbido.Tomado de: (15) b) Milimoles de Na^+ filtrado, reabsorbidos y excretados por el riñón. c) Porcentajes de Na^+ absorbidos en los distintos segmentos de la nefrona.

Con una dieta occidental típica con 120 mmol de sodio, los riñones reabsorben el 99.6% del Na^+ filtrado, por lo tanto cualquier mínima variación en la tasa de reabsorción puede llevar a cambios en la cantidad total de Na^+ que alteraría pronunciadamente al volumen del LIC. La reabsorción sistemática de Na^+ es favorecida por una nefrona compartamentalizada y altamente específica en cada uno de sus segmentos. Mientras que el TP reabsorbe la mayor parte (67%) junto con el (AAH) (25%) el TCD reabsorbe sólo un 5% dejando un 3% a los túbulos y conductos colectores (15).

Aunque el TCD sólo reabsorbe sólo una pequeña cantidad de Na^+, es un importante sitio de regulación fina de la reabsorción de Na^+ ya que al estar fuera del alcance de la regulación glomerulotubular puede establecer un gradiente de concentración transepitelial, responde a varias hormonas incluyendo mineralocorticoides y a la HAD, y es sitio de acción de antidiuréticos ampliamente utilizados en la clínica llamados tiazidas. Esta idea se ve reforzada por el hecho de que todos los desordenes monogénicos caracterizados a la fecha con alteraciones en la PA son causados por mutaciones en los genes que codifican para proteínas que afectan el manejo renal distal de sodio (26). Por lo anterior la reabsorción de sal en el TCD puede afectar de manera significativa el balance del líquido extracelular y por lo tanto de la PA.

La reabsorción en el TCD de Na^+ es exclusivamente un proceso transcelular impulsado por la actividad de la Na^+/K^+-ATPasa, la cual saca Na^+ hacia el espacio extracelular generando un gradiente de concentración que favorece la entrada de Na^+ por la membrana apical a través de dos diferentes vías: el cotransportador electroneutro Na^+: Cl^- sensible a tiazidas (NCC) y del canal de Na^+ epitelial sensible a amilorida (ENaC) que reabsorbe Na^+ de manera electrogénica (45, 55, 161, 167).

El contransportador Na^+:Cl^-, NCC

NCC es sensible a los medicamentos tipo tiazida como hidroclorotiazida, clortalidona, bendroflumetiazida, metolazona, etc (29). Está localizado en la membrana apical de las células del TCD y depende del gradiente de reabsorción de Na^+ del lado apical al basolateral generado por la Na^+/K^+-ATPasa basolateral.

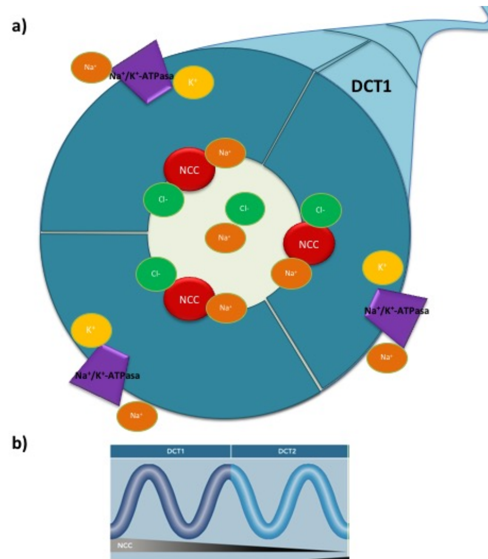

Figura 4. a) El cotransportador de Na^+:Cl^- (NCC) es un transportador secundario, dependiente de la función de Na^+/K^+-ATPasa. Se expresa en el lado apical del túbulo contorneado distal. b) Expresión de NCC a lo largo de DCT1 y DCT2. Modificado de: (5).

Debido a que la vejiga urinaria de *P. americanus* se puede considerar desde un punto de vista embriológico como una especie de TCD primitivo, se pudo utilizar como un modelo para aislar el ADNc[1] que codifica para la proteína responsable del transporte de Na^+:Cl^- de la vejiga urinaria de *P. americanus* siguiendo una estrategia de expresión funcional en ovocitos de *Xenopus laevis* como sistema de expresión heterólogo donde encontraron inducía la aparición de un cotransportador de Na^+:Cl^- sensible a tiazidas. Se identificó una clona de 3686 p.b. que codificaba para 1023 a.a. y un peso molecular de 110 kDa, la

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[1] El ADN complementario o ADN copia (ADNc) es una hebra de ADN de doble cadena una de las cuales constituye una secuencia totalmente complementaria del ARN mensajero a partir del cual se ha sintetizado.1 Se suele utilizar para la clonación de genes propios de células eucariotas en células procariotas, debido a que, dada la naturaleza de su síntesis, carece de intrones.

cual al ser expresada en ovocitos incrementó la captación de $^{22}Na^{+}$ 200 veces en comparación con los ovocitos inyectados con agua. La captación era dependiente de Cl^{-} e inhibida de manera selectiva por tiazidas y no así por furosemida o acetazolamida (59). El análisis de hidropatía sugirió la presencia de un extremo amino terminal intracelular corto hidrofílíco, sin péptido señal, lo que sugería que su ubicación era intracelular (95).

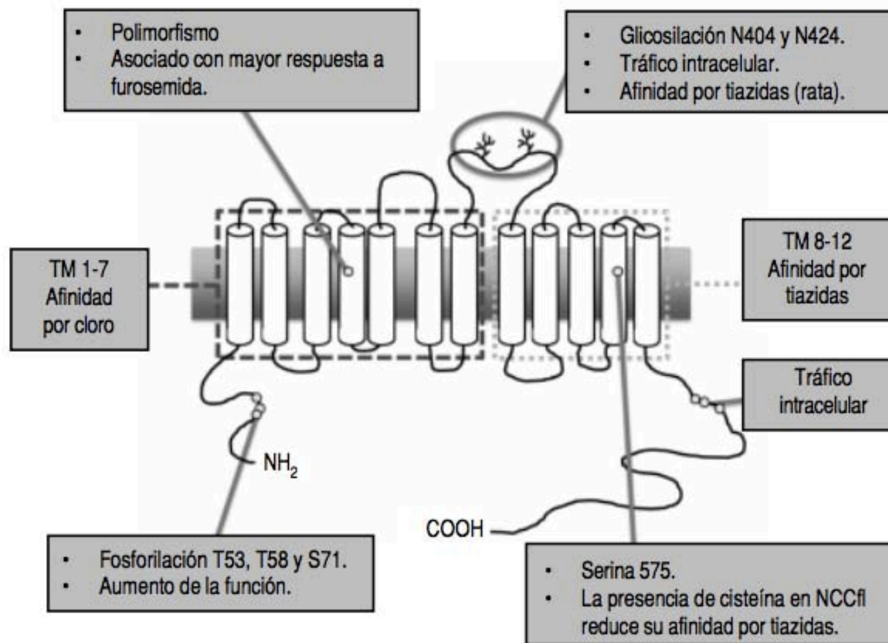

Figura 5. Modelo de la estructura de NCC. Tomado de: (129)

Este fragmento es seguido de 12 hélices, que se predijeron como transmembranales. Existe un asa hidrofilíca con tres posibles sitios de N-glucosilación (N403, N414 y N432) localizadas entre las α-hélices extracelulares. Finalmente, las α–hélices son seguidas del extremo carboxilo terminal intracelular largo de aproximadamente 450 a.a. (Figura 5).

Posterior a la identificación de la secuencia para NCC de lenguado, se reportó la identificación de la secuencia de NCC de rata con 61% de homología con el NCC de lenguado, del cual se identificaron dos variantes: una variante A de 4,394 p.b. con un marco de lectura abierta de 3,006 bases y una proteína de 1,002 a.a. con un peso de 110 kDa y una variante B de 3, 315 p.b con un marco de lectura idéntico (58).

El NCC de ratón fue identificado vía PCR donde reportaron una proteína con 95% de homología con el NCC de rata (94). El NCC humano identificado por dos grupos, hallaron dos transcritos codificados en el cromosoma 16q13, uno con un marco de lectura abierta de 3, 090 a.a. que codifica para una proteína de 1,030 a.a. (183) (112). Las diferencias en longitud de NCC de humano vs el de rata o ratón se debe a que es producto de la expresión del exón 20 (presente en humano, no así en rata o ratón) que agrega 17-26 a.a.

Regulación postraduccional de NCC

La actividad de NCC básicamente define la función del TCD, por lo que cualquier alteración modifica sustancialmente la reabsorción aguda de Na^+, mientras que cambios crónicos pueden causar la aparición de hipertensión o hipotensión arterial dependiendo de las modificaciones sufridas sobre NCC (ver Sx. Gordon y Sx. De Gitelman, abajoÇ). NCC es una proteína pleiotrópica en el sentido que muchos estímulos diferentes pueden activar a NCC para incrementar la reabsorción de Na^+, los estímulos conocidos hasta ahora son: dieta baja en Na^+(37), dieta baja en K^+ y dieta alta de citrato de potasio (27), inhibidores de calcineurina (78), acidosis metabólica (49) y hormonas, incluyendo aldosterona (199), angiotensina II (Ang II) (198), glucocorticoides (200), estrógenos (201), insulina, norepinefrina (133) y hormona antidiurética (HAD) (136). El complejo de regulación de NCC comprende cinasas y ubiquitilasas. Además de las cinasas WNK's, SPAK/OSR1 y las del complejo de ubiquitilación KELCH-CUL3 (descritos previamente), NCC puede ser regulado por la cinasa de glucocorticoides en Suero tipo 1 (*Serum- and glucocorticoid-inducible kinase 1) o* SGK1 y por la ubiquitilasaLos complejos de regulación ejercen sus efectos sobre NCC a través de tres mecanismos intracelulares de modificación postraduccional estudiados hasta la fecha: glicosilación, fosforilación y ubiquitilación (125):

Glicosilación: NCC es una glicoproteína de membrana con capacidad de formar dímeros lo que sugiere que la forma activa de NCC es en dímeros (40). Entre el segmento transmembrana 7 y el 8 hay un asa hidrofilíca que contiene dos sitos de glicosilación (N404 y N424) (Figura 5) (79), estos sitios de glicosilación son esenciales para que NCC

pueda anclarse a la membrana y funcionar (75). Cuando los sitios de glicosilación son eliminados, las tiazidas tienen mejor acceso al sitio de unión en NCC, lo que sugiere que la glicosilación bloquea la afinidad por tiazidas (79).

Fosforilación: La fosforilación en NCC ocurre en residuos de treoninas, tirosinas y serinas altamente conservadas en el dominio amino terminal, sin embargo tres han sido relacionados de manera universal con la activación de NCC; la treonina 45, 55 y 60 (171) (149). La fosforilación actualmente es considerada como un signo claro de su actividad, en especial la fosforilación de la treonina 60 en humanos o 58 en ratas. En particular la mutación en la treonina 60 causa un fenotipo de Síndrome de Gitelman (99) y el modelo knockin murino de NCC, desarrolla también un fenotipo de Síndrome de Gitelman; con un NCC atrapado en vesículas en citosol (211). En relación a este último hallazgo, existe evidencia de que sólo el NCC en membrana está listo para ser activado/fosforilado (156). Esto sugiere que aparte de la fosforilación, el tráfico de NCC desde las vesículas subapicales a la membrana plasmática es un requisito importante para su función (62).

Cuando no se contaban con fosfoanticuerpos específicos, la manera de poder analizar la fosforilación de NCC fue a través del fosfo-anticuerpo R5 de NKCC1(53), para que pudiese reconocer a NCC se mutó un aminoácido en el NCC de rata, la tirosina 56 por una histidina[2], la cual no alteró la función de NCC, además en otro trabajo subsecuente se observó que la actividad de NCC se redujo significativamente con la co-expresión de la fosfatasa de proteínas tipo 4 (64), confirmando la importancia de ese sitio de fosforilación para la actividad de NCC.

El estudio de NCC avanzó gracias al desarrollo de fosfoanticuerpos específicos para los residuos fosforilados de NCC (171). Mediante análisis de espectrometría de masas de NCC desde una proteína extraída de células HEK-293 transfectadas con NCC y estimuladas con un estrés hipotónico bajo en cloro, se encontraron los sitios treonina 46, 50, 60 (T46, T50 y T60) y la serina 91 (S91) fosforilados. Dicho resultado confirmó la importancia de la T53 y a T58 de rata (equivalentes a la T55 y T60 en humano) además de añadir dos sitios más. Esto condujo a la creación de fosfoanticuerpos para cada uno de

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[2] Sitio conservado en NKCC1 y NKCC2.

los sitios, los fosfo-anticuerpos fueron validados en células HEK-293 transfectadas con NCC, dichos experimentos confirmaron la importancia de la T60 como la treonina maestra de la fosforilación de NCC ya que su ausencia (por una mutación puntual) evita que los otros sitios puedan fosforilarse. La generación de fosfoanticuerpos creó herramientas poderosas para detectar la fosforilación de NCC y por consiguiente su activación. La tabla 2 muestra una compilación de los estudios que han utilizado a los fosfoanticuerpos para analizar la actividad de NCC.

Ubiquitilación: La evidencia de la reducción en la expresión de NCC por ubiquitilación a través de un fenómeno no genómico fue sugerido en 2007, pero el mecanismo fue descubierto hasta el 2011, NCC es regulado por el complejo ubiquitin ligasa Nedd4-2/SGK1 a través de la disminución de la expresión de NCC en la membrana (4). Ahora existe evidencia de un posible mecanismo de regulación fina entre los niveles de fosforilación y de ubiquitilación (211). Además se ha demostrado que el sistema SGK1/Nedd4-2/NCC puede ser regulado por WNK1, WNK3 y WNK4 (208) (96). Un año después de la publicación del sistema Nedd4-2/NCC un nuevo complejo de ubiquitin ligasas, KELCH/CUL3, se añadió a los mecanismos de regulación de NCC a través de modular la expresión de las WNK's (17, 104, 181).

Modelo experimental	Estímulo	Sitio fosforilado	Referencia
***Xenopus* ovocitos**	Disminución del Cl^- intracelular	T55, T60, S73	(149)
Ratón	Knockin de WNK4$^{(D561A/+)}$	S73	((212)
HEK-293	Disminución del Cl^- intracelular	T46, T55, T60, S91	(173)
Rata	Dieta baja en sodio	T55, T60, S73	(37)
Orina humana	No hubo estímulo	S8111	(67)
***Xenopus* ovocitos/ células mpkDCT**	Angiotensina II	T55, T60	(179)
Ratón	Dieta baja en potasio	T55, T60, S73	(196)
Ratón	Hipo mórfico de WNK4	T55, T60, S73	(145)
Ratas Brattleboro	HAD	T55, S73	(136)
Ratas Brattleboro/ ratas Wistar	HAD	T55, T60	(156)
Ratón	SPAK knockin	T55, T60, S91	(164)
Células mpkDCT /ratón	Angiotensina II/Aldosterona	T55, T60, S73	(192)
Ratas adrenolectomizadas	Angiotensina II/Aldosterona	T55, T60	(198)
Ratón	KS-WNK1 knockout	T45, T55, T60, S73	(73)
Ratón	KS-WNK1 knockout	T60	(102)
En tubo de ensaye	Incubación con MO25	T45, T55, T60, S91	(52)
Ratón	SPAK knockout	T55, T60, S73	(213)
Ratón	SPAK knockout	T55, S73	(112)
Células mDCT/ratas	Ciclosporina	T55, T60, S73	(121)
Ratón	Tacrolimus	T55	(78)
Ratón	Transgénico de NCC	T55	(118)
Ratón	WNK4 knockout	T60	(28)
Células mpkDCT/ratón	Insulina	T55, T60, S73	(184)
***Xenopus* ovocitos/ Riñón de rata ex vivo**	Insulina	T60	(31)
Ratas ZO (obese Zucker)	Hiperinsulinemia	T55	(92)
Ratón	KS-OSR1 knockout	T55	(100)
Ratón	Hipertensión sensible a sal asociada a la activación de los receptores adrenérgicos	T55, S73	(133)
En tubo de ensaye	WNK4/Ca^{2+}	T60	(138)
***Xenopus* ovocitos**	WNK3	T60	(150)
Ratas	Proteomica en masa	S124	(50)
Ratas Brattleboro	HAD	S124	(174)
***Xenopus* ovocitos/ratones**	WNK1 y WNK4	T53, T60	(32)
Ratones	Dieta alta y baja en potasio	T45, T55, T50	(27)
Orina humana	Aldosteronismo	T60	(197)

Tabla 2. Estudios de la actividad de NCC a través del uso de fosfo-anticuerpos. Modificado de: (62).

Enfermedades asociadas a NCC

Alteraciones en la función de NCC dan origen a enfermedades que se asocian con trastornos hidroelectrolíticos. Las alteraciones que generan menor actividad del cotransportador producen el síndrome de Gitelman mientras que aquellas que aumentan su actividad se asocian con el síndrome de Gordon. El síndrome de Gitelman imita los efectos de las tiazidas pérdida de sal, hipocalcemia, alcalosis metabólica e hipocalciuria (63) mientras que el síndrome de Gordon genera las alteraciones opuestas, es decir, retención urinaria de sal con hipertensión arterial, hipercalcemia, acidosis metabólica e hipercalciuria (69) .

Síndrome de Gordon (PHAII)	Síndrome de Gitelman
Autosómico dominante	Autosómico recesivo
Hipertensión arterial	Hipotensión arterial
Acidosis metabólica	Alcalosis metabólica
Hiperkalemia	Hipokalemia
Hipercalciuria	Hipocalciuria

Tabla 3. Características del Síndrome de Gordon y del Síndrome Gitelman. Los fenotipos son opuestos.

Cinasas "With no Lysine", WNK's

Las WNK's son una familia de serina/treonina cinasas con 4 miembros identificados: WNK1, con sus variantes L-WNK1 y KS-WNK1, WNK2 (Cromosoma 9), WNK3 (Cromosoma X) y WNK4 (116, 119, 178). Todas las cinasas comparten una estructura en común la cual está compuesta por un extremo amino terminal (146-200 a.a.), un dominio cinasa altamente conservada entre las WNK's (274 a.a.) un dominio autoinhibitorio, dominios *coiled-coil* que se localizan en el dominio carboxilo terminal (786-1888 a.a.) (170).

Las WNKs modulan el tráfico y la activación/fosforilación de NCC (77). La mayor parte de la evidencia proviene de estudios en ovocitos de *Xenopus laevis*. La regulación del tráfico de NCC por las WNK`s involucra una cascada en la cual WNK1 y WNK4 (a través de la estimulación por angiotensina II (179)) fosforilan y de esta manera activan a NCC (32) a través de otra cinasa llamada SPAK (171) (de la cual se hablará en más detalle en la siguiente sección).

Sin embargo existe también evidencia de un efecto inhibidor de WNK4 sobre NCC (210), dicha inhibición por el WNK4 es promovida por degradación lisosomal (190). Por otro lado WNK3 estimula a NCC (173). Por último no se han encontrado efectos de WNK2 en la nefrona.

Cinasas Ste20-related proline-alanine rich kinase y oxidative stress response 1, SPAK y OSR1

Las cinasas *Ste20-related proline-alanine rich kinase* (SPAK) y *oxidative stress response 1* (OSR1) son dos cinasas que éstas pertenecen a la familia de cinasas relacionadas con Ste20(193, 195) (Sterile-20) (56). SPAK está conformada por 553 a.a. y tiene un peso molecular aproximado de 61kDa, mientras que OSR1 contiene 527 a.a. y tiene un peso de aproximadamente 58 kDa.

SPAK se expresa en el TCD y el TC (204). Sin embargo, en un trabajo más reciente, se generó un anticuerpo que demostró ser más específico para SPAK y no reconoce a OSR1; con dicho anticuerpo se realizó un análisis de western blot de proteínas extraídas a partir de segmentos específicos de la nefrona fueron cuidadosamente disecados, de esta manera se observó expresión de SPAK exclusivamente en TCD y AAH y no en los conductos colectores[3] .

Estudios fisiológicos en animales genéticamente modificados sugieren que OSR1 juega un papel esencial en la regulación de la función de TAL, mientras que en TCD su función parece ser redundante (100).

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[3] Dicho anticuerpo fue utilizado en este trabajo.

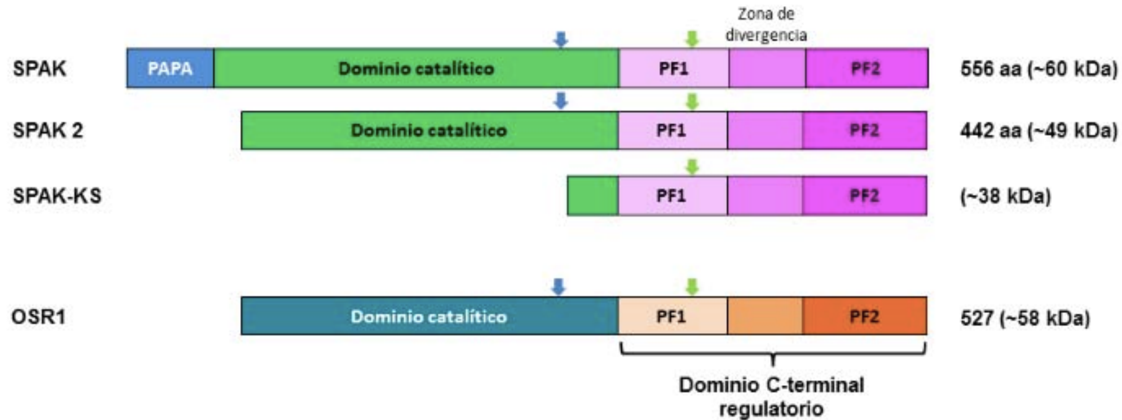

Figura 7. Esquema general de los dominios estructurales presentes en las cinasas SPAK y OSR1. También se muestran las distintas isoformas de SPAK que se han identificado (117). Las flechas azules indican la localización aproximada de la treonina del asa de activación o "asa T" (T-233 en SPAK de humano y T-185 en OSR1). Las flechas verdes indican la localización aproximada de la serina del "motivo S" (S-373 en SPAK de humano y S-325 en OSR1). Ambos sitios son blanco de fosforilación por las cinasa WNK's.

Los trabajos se han centrado en estudiar específicamente a la regulación de NCC por la vía de WNK's/SPAK-OSR1, se demostró que la fosforilación de NCC es dependiente de SPAK y OSR1 en los sitios previamente descritos en NKCC1 (en NCC de humano corresponden a la T55, T60 y S73) y que WNK1 fosforila a SPAK y OSR1 en un residuo evolutivamente conservado fuera de la región cinasa (S325 en SPAK de rata y S380 en OSR1 de ratón), sin embargo, la mutación de este sitio no pareció afectar de manera importante la actividad de OSR1. (131). Posteriormente, se demostró la importancia de la fosforilación en estos sitios para mantener la actividad basal de NCC observada en ovocitos de *X. laevis* (que disminuye al mutarse esos sitios), así como su relevancia para la activación del cotransportador por la depleción de Cl^- intracelular en este mismo sistema. Los cambios en la actividad observados y, por lo tanto, los niveles de fosforilación no se relacionaron con cambios en la expresión a nivel de membrana celular (149). Más adelante se identificó que las cinasas WNK1 y WNK4 fosforilan a SPAK y OSR1 en un residuo de treonina en el asa de activación o "asa T" dentro de la región cinasa (T-233 en SPAK y T-185 en OSR1 de humano) y en un residuo localizado fuera de la región cinasa dentro de lo que llamaron el "motivo S" (S-373 en SPAK y S-325 en OSR1 de humano); además demostraron que el nivel de fosforilación del asa de activación está íntimamente relacionado con la actividad de la cinasa mientras que la fosforilación del "motivo S" no parecía afectar la actividad de manera importante (202).

Se demostró que la fosforilación de los residuos identificados en NCC es importante, tanto para la actividad basal observada, como para la activación de NCC ante la exposición de las células al estrés hipotónico bajo en Cl^- (171).

Existen al menos dos isoformas de SPAK adicionales a la isoforma inicialmente descrita llamadas SPAK2 y SPAK-KS ("kidney specific") (117). La isoforma SPAK2 se propuso a partir de un estudio previo (157) en el que se demostró que SPAK puede ser generada a partir de dos codones de inicio distintos y que la generación de SPAK a partir del segundo codón produce una isoforma carente de la caja PAPA y del primer segmento del dominio cinasa, por lo que se predice que es catalíticamente inactiva. Al aislar el mRNA para la segunda isoforma, SPAK-KS, (117). Este transcrito contiene un primer exón alternativo, el exón 5a, localizado dentro del intrón 5, en el gen de SPAK. La presencia del exón 5a en el transcrito no provoca que SPAK-KS contenga una segmento peptídico novedoso ya que el primer codón de inicio se encuentra en el exón 6. Por lo tanto este transcrito sólo produce una isoforma truncada carente de región cinasa. Es importante recalcar que la isoforma larga de SPAK (SPAK-L) parece ser muy poco abundante en riñón en donde SPAK2 y SPAK-KS predominan. La isoforma SPAK-KS no se expresa en otros tejidos (117). Además la actividad cinasa de SPAK inmunoprecipitada de riñón parece ser muy baja, apoyando la idea de que SPAK2 es catalíticamente inactiva (165). En riñón, la isoforma SPAK-L parece ser más abundante en la corteza en donde se expresa NCC y en menor cantidad NKCC2, mientras que SPAK-KS predomina en la médula en donde se expresa de forma importante NKCC2 y no se expresa NCC.

Con toda esta información propusieron que en el AAH las isoformas SPAK2 y SPAK-KS ejercen un efecto dominante negativo sobre la activación de NKCC2 mediada por SPAK-L/OSR1 y que al eliminar todas las isoformas de SPAK en los ratones $SPAK^{-/-}$ la inhibición basal de SPAK2 y SPAK-KS sobre OSR1 se perdió, razón por la cual aumentó la actividad de NKCC2. La propuesta del efecto dominante negativo de SPAK2 y SPAK-KS sobre SPAK-L/OSR1 fue sustentada con ensayos *in vitro* de actividad cinasa en los que se observó que la fosforilación de NKCC2 por SPAK-L y OSR1 disminuía en presencia de cantidades crecientes de SPAK-KS. Esta propuesta conciliaba las

discrepancias con respecto al efecto en NKCC2 observado en los ratones $SPAK^{-/-}$ y SPAKKI, ya que en los ratones SPAKKI la mutación introducida en SPAK afectaba exclusivamente la actividad catalítica de SPAK-L (en TCD y AAH) sin alterar el papel negativo que SPAK2 y SPAK-KS juegan en AAH, explicándose así por qué en estos ratones se observaron menores niveles de NKCC2 y pNKCC2 .

Dimorfismo sexual y hormonas sexuales

Dimorfismo sexual

El dimorfismo sexual ocurre cuando los sexos de una especie son diferentes uno del otro. Es un fenómeno que se cree evolucionó para asegurar el éxito reproductivo de los individuos. El signo más visible del dimorfismo sexual son las características sexuales secundarias se desarrollaron para poder asegurar el cortejo y la reproducción y que están reflejadas principalmente en los órganos reproductores. Los mamíferos pueden diferir en muchas más maneras entre ambos sexos que van más allá de los órganos reproductivos, incluyendo características anatómicas, psicológicas y fisiológicas (120).

Los cambios dimórficos sexuales se deben al efecto sistémico de las hormonas predominantes en cada uno de los sexos, en el caso de mamíferos las hembras producen en los ovarios dos tipos de hormonas: estrógenos y progestágenos. En el caso de los machos son los andrógenos (principalmente testosterona) secretados y producidos por los testículos(74).

Hormonas sexuales esteroideas

Los estrógenos en las mujeres son sintetizados en el ovario y en la glándula suprarrenal de la conversión periférica en el tejido adiposo. En las mujeres no embarazadas solo tres estrógenos están presentes en cantidades significativas en el plasma: 17ß-estradiol (E2), estrona y estriol. El estrógeno principal secretado por los ovarios es el E2. Pequeñas cantidades de estrona son también secretados, pero la mayor parte están formados en los tejidos periféricos desde andrógenos. La potencia estrogénica del E2 es 12 veces mayor que la estrona y 80 veces que la del estriol. Considerando las potencias relativas, el E2 es

el principal efector biológico estrogenico. La progesterona (P4) es secretada en cantidades significativas durante la última mitad del ciclo ovárico cuando es secretado por el cuerpo lúteo así como en el cuarto mes de gestación (29).

El período menstrual

Ovulación

La ovulación ocurre a la mitad de cada ciclo menstrual y es estimulado por la liberación de hormona leutinizante (LH) la cual a su vez estimula la liberación de LH. La secreción de E2 por el folículo dominante[4] se incrementa a final de ciclo. La liberación de E2 inicia un proceso de retroalimentación positiva en la hipófisis anterior que la sensibiliza a la horomona liberdora de gonadotrofina (GnRH). El efecto neto de la elevación de las concentraciones de E2 es la inducción de LH. La liberación de LH se inicia generalmente después del pico de 24-36 horas después del pico de secreción de E2, y la ovulación ocurre a su vez 36 horas después del comienzo de la liberación de LH y 12 horas después del pico de LH. De esta manera el desarrollo del folículo por el incremento en la secreción de E2, le indican al eje hipotálamo-hipófisis (EHE) que el folículo está maduro y poder líberar otro bolo de GnRH para inducir la ovulación. La liberación de LH termina en parte por la elevación en las concentraciones de P4, por retroalimentación negativa y por la pérdida de la retroalimentación positiva proveniente del E2. La disminución de los almacenes de GnRH en la hipófisis anterior contribuye también a la terminación de la liberación de LH.

Después de la expulsión del ovocito las células de la granulosa y la teca son arrojadas en el espacio que ocupa la cavidad folicular y forman el CL, un órgano temporal endocrino cuyo principal producto es la P4. Aunque el CL produce tanto E2 como P4, la fase lútea es dominada primariamente por la secreción de P4. Después de la ovulación, la elevación de la P4 se eleva durante 7 días. La P4 actúa localmente para inhibir el crecimiento folicular durante la fase lútea. Además, la P4 puede actuar

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[4]Es el folículo destinado a ser ovulado, ya que durante las fases tempranas del ciclo, alcanza dominancia sobre los demas folículos, que eventualmente se atrofian.

centralmente al inhibir la secreción de gonadotrofina. La P4 es además anti-estrógenos. Como resultado la P4 actúa localmente para disminuir la cantidad de receptores de estrógenos, disminuyendo el efecto del E2, y por tanto la foliculogénesis.

Las concentraciones de E2 también se elevan durante la fase lútea, lo cual es necesario para inducir los cambios dependientes de P4 del endometrio. Si no existe la fecundación y la implantación, la producción de hormonas por el CL es degenerado, dejando un fibrótico *corpus albicans*.

El ciclo menstrual

El ciclo menstrual involucra cambios cíclicos en dos órganos: el ovario y el útero. El ciclo endometrial incluye las fases menstrual, proliferativa y secretora. A pesar que los ciclos menstruales son generalmente regulares durante los años reproductivos la duración del ciclo menstrual puede ser altamente variable debido a disturbios neuroendócrinos. La primera fase del ciclo ovárico es la fase folicular durante la cual la FSH (hormona folículo estimulante) estimula el completo desarrollo del folículo. La fase folicular empieza con la iniciación de la menstruación, la duración de la fase folicular es la más variable en longitud del ciclo. Durante la foliculogénesis, las células de la granulosa de los folículos incrementan la producción de E2, la cual estimula al endometrio para su maduración. Este período es llamado fase proliferativa del ciclo endometrial. Un rápido incremento en la secreción ovárica de E2 eventualmente dispara un pico de LH (hormona leutinizante), lo cual provoca finalmente la ovulación.

Las células lúteas del CL producen P4 y estrógeno, lo que estimula aún más el crecimiento endometrial. Este período es la fase secretora del ciclo endometrial. Por algunas razones desconocidas, el CL rápidamente disminuye la producción de E2 y P4, lo que resulta en la degeneración del endometrio que conlleva al sangrado menstrual.

Receptores de estrógenos

El mecanismo clásico de acción estrogénica es la difusión de estrógenos dentro de la célula donde se une al receptor de estrógenos que se localiza en el núcleo. La unión del receptor de estrógenos (ER) a los estrógenos se le llama complejo estrógeno-ER. El

complejo estrógeno-ER se une a la secuencias de elementos de respuesta a E2, directa o indirectamente a través de interacciones proteína-proteína vía la proteína activador 1 (AP1) en la región promotora de los genes responsivos a estrógenos, resultando en el reclutamiento de coactivadores o correpresores al promotor, incrementando o disminuyendo respectivamente los niveles de ARN y la producción de la proteína asociada y su respuesta fisiológica (42). El mecanismo clásico o genómico ocurre típicamente en un transcurso de horas.

En contraste, los estrógenos pueden iniciar respuestas fisiológica específicas en muchos tejidos en los primeros segundos a minutos después de la unión con su ligando, es decir un mecanismo no-genómico, por los ER localizados en o junto a la membrana o a través de receptores de membrana acoplados a proteínas que no son ER. Las cascadas de señalización intracelular ligadas a la activación del receptor de estrógenos han sido muchas incluyendo MAPK, PI3K/AKT, activación de canales iónicos y receptores acoplados proteínas G, receptores de crecimiento y cambios en las concentraciones de Ca^{2+}. Las proteínas candidato involucradas en dichas cascadas son: proteínas G, proteína de choque térmico 90 (Hsp90), caveolina-1, metaloproteinasas de matriz, etc. (130). Por otra parte los ER pueden estar unidos a la membrana mediante adaptadores de proteínas, como la caveolina-1 (Figura 12) (134).

Los genes regulados por estrógenos están ampliamente distribuidos en el organismo. Los órganos principales en orden son: el útero, la hipófisis anterior y el riñón (88).

Dado lo anterior se han clasificado las acciones del receptor de estrógenos con base en la localización de su acción: membranal, citosólico o nuclear (139):

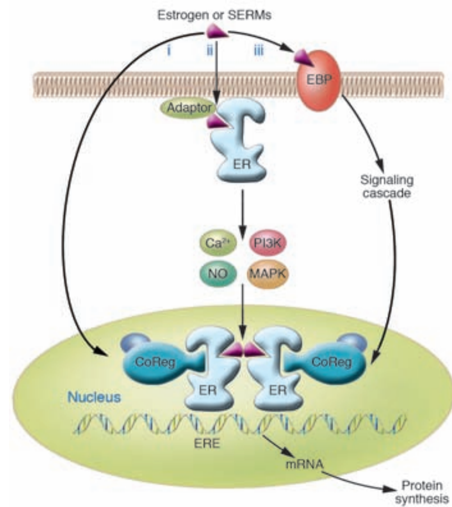

Figura 8. Modelo de acción de los estrógenos. En la vía clásica la vía de acción del receptor de estrógenos (i) u otros moduladores selectivos de receptores de estrógenos (SERMs) que se unen al ER, un factor de trascripción unido a un ligando regula la transcripción de genes blanco en el núcleo por unión a los elementos de respuesta a estrógenos (ERE), que son secuencias regulatorias de los genes blanco y que pueden reclutar proteínas regulatorias (CoRegs) como coactivadores o corepresores. Los efectos rápidos "no genómicos" de los estrógenos pueden ocurrir a través de ER localizados en o junto a la membrana plasmática (ii) que requieren de moléculas de señalización y estimulantes de la actividad de cinasas, que a su vez pueden afectar la transcripción. Otros receptores de estrógenos, no ER, asociados a membrana pueden también disparar respuestas intracelulares (iii). Tomado de: (134).

Efectos nucleares: los receptores suelen funcionar como "lanzadera" (en ingles shuttles) entre el citroplasma y el núcleo, en ausencia de ligandos. Una vez que se unen al ligando se traslocan al núcleo para servir como factor de transcripción e iniciar la transcripción (8). Existen dos tipos de ER: ERα y ERß, que tiene distintos patrones de expresión; existen variantes por corte y empalme que suelen co-expresarse con su contraparte silvestre, sin embargo, su función ha sido poco estudiada. A pesar que ERα y ERß comparten una alta homología en sus secuencias difieren de manera importante en los dominio de unión a ligando, dichas diferencias han llevado a la creencia que cada uno de los ER pueden regular genes diferentes incluso opuestos en su función al mismo tiempo. En el caso de ERß su afinidad por E2 es más baja en comparación al ERα. Los ER´s pueden forman homodímeros o heterodímeros (93).

Efectos membranales: Se ha reportado que el E2 es un antagonista alostérico de los receptores N-methyil-D-aspartato y los receptores de serotonina así como de canales iónicos (139).

Además existen proteínas de membrana que cruzan con anticuerpos para el ERα que se sabe están unidas a proteínas G. Utilizando una técnica de clonación GPCR-30 fue aislado de células de mama, el cual al ser más estudiado fue renombrado a receptor de estrógenos acoplado a proteínas G tipo 1 (GPER-1); sus funciones han sido reportadas en el sistema nervioso central, inmune, cardiovascular, reproductivo y renal. Además la afinidad de GPER-1 por estrógenos naturales y artificiales es diferente del de los ER's[5] (51). Otra característica del GPER-1 es que existe evidencia de ser activado por otros esteroides, como por la aldosterona, en un mecanismo de acción rápida independiente del receptor de mineralocorticoides vía la cascada de señalización ERK-1/2 (72).

Efectos citoplasmáticos: Los estrógenos también tienen blancos en membrana con localización y función citosólico, como las vías de la cinasa, MAPK (124). Bajo condiciones no estimuladoras los dominios catalíticos de la cinasa Src,[6] cambian su conformación al estado activo, por la estimulación con E2. Otra vía citosólica regulada es la de la modulación de oxido nítrico[7] en las células vasculares, independiente del efecto de los ERα sobre las concentraciones oxido nítrico sintasa (122).

Receptores de Progesterona

Como con los ER`s, los receptores de progesterona (PR) son factores de transcripción que consisten en un dominio de unión a ADN rodeado de una región amino terminal que contiene las funciones *activantes* (AF) e *inhibientes* (IF) y una región de bisagra río abajo y carboxilo terminal que es el dominio de unión a ligando. Existen dos isoformas del PR: PRA y PRB, las cuales sólo difieren en que el PRB humano contiene 164 a.a. adicionales arriba de la región amino-terminal llamada “segmento B rio arriba”, dicha secuencia le

[5] La afinidad de ER por 17ß-Estradiol está en el orden de nanomolar (constante de disociación (K_d) = 0.1-1.0 nM, mientras que por GPER-1 es de 3.7-4 nM.
[6] Es miembro de la familia de cinasas, tirosin cinasas no receptores.
[7] Relajante del musculo liso vascular.

confiere una actividad funcional 3. En tejidos normales y sanos, incluyendo la glándula mamaria, PRA y PRB se expresan de manera similar. La transcripción mediada por PR es: 1) El ligando se une al receptor que está en el citoplasma creando un complejo, cuando el PR no está unido a ligando, está unido a proteínas de shock térmico. 2) El complejo liberado de las proteínas de shock térmico forma dímeros y se transloca al núcelo. 3) En el ADN existen secuencias palíndromas llamadas elementos de respuesta a progesterona en promotores de genes regulados por PR. 4) La transcripción de genes regulados por P4 es iniciada (87).

Aparte de los efectos clásicos o genómicos existen efectos no genómicos mediados por efectores membranales sensibles a P4 (14). Se nombró a este, receptor membranal de progesterona (mPR). Dicho receptor tiene tres isoformas mPRα, mPRß y mPRγ. Los mPR son miembros, de la familia de receptores de progestina y adiponectina Q, y sus cascadas de señalización están conectadas a la activación de proteínas G (43).

Los PR's, están expresados en los tejidos reproductivos femeninos, pero también está ampliamente distribuido en tejidos no reproductivos como el timo, el hueso, los vasos sanguíneos, el sistema nervioso central (166), y el riñón (22).

Prolactina

La prolactina (PRL) fue descubierta hace más de 70 años (172). Clásicamente la PRL fue definida como una hormona polipeptídica secretada por la hipófisis anterior, y liberada al organismo a través del torrente sanguíneo. Su nombre fue tomado debido a que la primera función descrita fue la estimulación de la lactancia (10). El gen de la PRL tiene seis exones y cuatro intrones de una longitud de 10 kb. Cuando se procesa se remueve el péptido señal (28 a.a.), y su forma madura tiene 199 a.a. (23 kDa). Hay muchas variantes de la PRL debido a modificaciones postraduccionales, varias de ellas con gran importancia para la función en los tejidos diana (65). La secuencia de a.a. es similar a otras dos hormonas polipeptídicas; la hormona del crecimiento y el lactógeno placentario. Tanto la PRL/GH/PL pertenecen a la misma familia referida como citoquinas hematopoyéticas. Se considera a la PRL como una citoquina basado en sus características moleculares y funcionales.

Está ampliamente aceptado que la secreción de PRL de la hipófisis anterior es regulada positiva y negativamente, pero es principalmente controlada por factores inhibitorios originados en el hipotálamo, el más importante es la dopamina, a través de los receptores de dopamina tipo 2, expresados en los autótrofos[8]. El ratón knockout para el gen del receptor de prolactina (PRLR) es hiperprolactinémico sugiriendo que la PRL regula su propia secreción (11). La secreción de PRL es pulsátil y regulada circadianamente, las concentraciones más bajas se observan en las primeras 2-3 horas tras despertar y el pico más alto es durante el sueño profundo; las concentraciones de PRL son más altos en mujeres[9] que en hombres[10],y en el caso de las mujeres se incrementa durante la ovulación (84) . Se sabe que la liberación de PRL puede ser modulada por la estimulación de los lactótropos por neurotransmisores y por otras hormonas como el E2 (182). El efecto de la PRL sobre las gónadas es básicamente disminuir la sensibilidad del ovario por FSH y LH (13).

El receptor de prolactina (PRLR) es miembro de la superfamilia de receptores de citoquinas (65). Existen isoformas cortas, medianas y largas del PRLR. Entre las distintas especies estudiadas el dominio de unión a ligando es idéntico entre todos ellos, mientras que el dominio carboxilo es diferente entre ellos. Se ha encontrado que la expresión de las distintas isoformas del PRLR varia a lo largo de los estados hormonales como el ciclo estral, el embarazo y la lactancia (13). El PRLR se expresa ampliamente en órganos periféricos como la hipófisis, el corazón, los pulmones, el timo, el bazo, el páncreas, las glándulas renales, el útero, el músculo esquelético, la piel y el riñón (140). Existen diversos estudios que sugieren que el PRLR forma dímeros a través de la unión de un solo ligando (Figura 9). Lo que involucra dos regiones, cada una interactuando con otra molécula a través de la PRL (66).

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[8] Es decir la prolactina se secreta constitutivamente y su regulación consiste en bloquear dicha secreción a diferencia de otras hormonas, donde lo que se estimula es la secreción de dicha hormona.
[9] En la fase folicular <20 ng/ml y en la fase lútea <40ng/ml.
[10] 15ng/ml.

La cascada de activación del PRLR por la PRL se inicia una vez que la PRL se une al PRLR. Como todos los receptores de citosinas, en el PRLR la ausencia de estímulo se asocia a la falta de actividad intrínseca enzimática del PRLR y, una vez que se activa por la unión del ligando, se traduce en un mensaje que se transmite al interior de la célula y se asocia a cinasas, las cuales activan efectores río abajo (Figura 9). La parte intracelular del PRLR tiene dominios ricos en prolina llamados "Box-1" en los que la cinasa Jak-2 esta anclada mientras el receptor está inactivo (84). La vía principal es la de Jak2/Stat5 (13). Se cree que el PRLR puede cruzar con otros miembros de los receptores de tirosina cinasas y también de receptores nucleares, además las interacciones de las Stat`s activadas con otros receptores representan una posible manera de regular funciones multi-hormonales en donde esta involucrada la PRL (65).

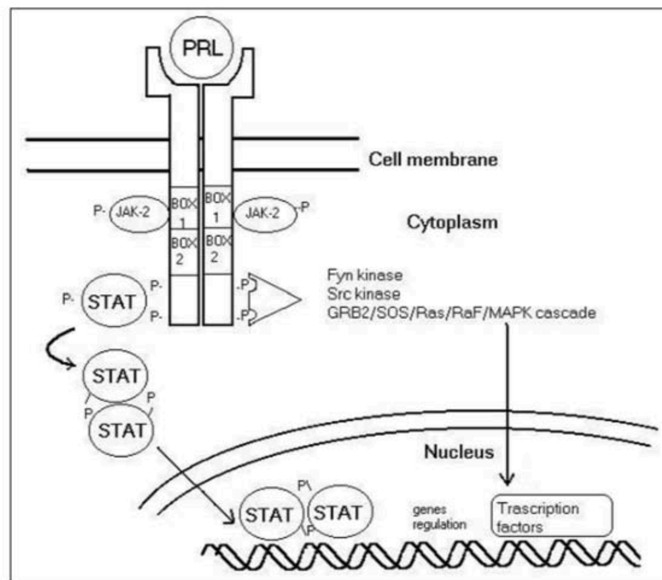

Figura 9. Esquema de la cascada de señalización principal para la activación del PRLR. Tomado de: (84).

Gran parte de las funciones ejercidas por la prolactina se han descubierto gracias a modelos transgénicos animales como el knockout para PRL (81) y para el PRLR (148). Estas funciones son la modulación del comportamiento del sistema nervioso central, metabolismo, la respuesta inmune y el balance electrolítico (13). Hasta el momento entre el número de funciones y blancos en el organismo se han calculado 300 acciones separadas en diferentes especies por parte de la prolactina. Muchas de estas funciones sólo han sido caracterizadas en especies animales y, debido a la falta de evidencia en

humanos, siguen existiendo dudas de su verdadera relevancia en humanos. Una dificultad para su estudio, es que no existen enfermedades genéticas asociadas a mutaciones en el gen de PRL o su receptor (16).

La única enfermedad asociada a la disfunción de la PRL es la hiperprolactinemia definida como concentraciones de prolactina elevados sobre el rango normal, que generalmente ocurre bajo condiciones fisiológicas como el embarazo y la lactancia, sin embargo, las concentraciones de PRL pueden elevarse patológicamente debido a tumores en la hipófisis en las células lactótrofas (prolactinomas), como resultado los pacientes tienen galactorrea, oligo/amenorrea en mujeres, impotencia en hombres y pérdida de libido y fertilidad en ambos sexos (126). El tratamiento usual es el uso de análogos de dopamina que ejerce un efecto fisiológico negativo y regulador sobre la producción de PRL por parte de los lactótrofos (127).

Efecto osmoregulador de la PRL

La PRL tiene diversos efectos, uno es la osmoregulación en mamíferos, el cual lo hace a través de la reducción renal de la excreción de Na^+ y K^+ y el estímulo de la Na^+/K^+-ATPasa en la médula externa del riñón de rata (159). Además la PRL incrementa la excreción de iones Na^+ y Cl^- con el sudor, incrementa la absorción de agua en todos los segmentos de intestino y disminuye el transporte de agua en la membrana amniótica humana (54), en un transporte que podría estar contribuyendo a la madre embarazada para la lactancia. Un argumento similar puede ser hecho desde la evidencia del efecto de la PRL en el TP para promover la retención de Na^+, K^+ y agua (187).

La PRL en el embarazo y la lactancia

Las concentraciones maternas de PRL empiezan a elevarse en la semana 6-8 de gestación y se incrementan progresivamente hasta alcanzar 200-300 ng/ml al término de la misma (Figura 10). Durante el embarazo el incremento en la secreción de estrógenos estimula el crecimiento de lactótrofos incrementando la secreción de PRL, de hecho en la mujer embarazada la hipófisis anterior duplica su tamaño para preparar la intensa secreción de PRL para la lactancia durante el postparto (91). La PRL también se eleva en la circulación fetal en la semana 20-24 y se incrementa continuamente desde la semana 30

cuando alcanza concentraciones similares a los de la madre. En los humanos, la decidua[11] produce grande cantidades de PRL que se acumulan en el líquido amniótico llegando a alcanzar 4000-5000 ng/ml entre la semana 16-22 de gestación y reduciéndose a 400-500 al termino del embarazo (9).

La síntesis de P4 por el CL produce andrógenos y estrógenos, pero no existe evidencia de que la PRL estimule al CL, como sí lo hace la LH después de la implantación (203).

Es bien conocida la producción de PRL por la decidua humana, pero se conoce acerca de las funciones exactas de la PRL en el líquido amniótico. La PRL es la principal proteína sintetizada y secretada durante la decidualización y su expresión es detectable en el endometrio durante la mitad del fase secretoria del ciclo menstrual. Se asume que la PRL decidual actúa localmente como un agente parácrino/autócrino y además sirve como fuente de PRL del líquido amniótico, aunque no se sabe si la PRL decidual escapa a la circulación materna. La PRL del fluido amniótico también ha sido relacionado a la regulación osmótica y electrolítica del amnios (86).

La glándula mamaria sufre un cambio estructural importante durante el embarazo para la preparación a la lactancia que incluyen a grandes rasgos, un incremento en el ramificado y la emergencia de los alveolos mamarios. El control hormonal es complejo y depende de la comunicación entre PRL, el PL, P4, insulina, GH, las hormonas tiroideas y las corticosteroideas. En la glándula mamaria la expresión del PRLR es baja durante la mayor parte del embarazo. Por otro lado no se sabe nada del PRLR en la mama humana durante el embarazo y la lactancia (83).

Para la generación de la leche se requiere de una combinación de PRL con glucocorticoides e insulina así como de factores de crecimiento. La P4 inhibe la lactogénesis en los humanos, para que se inicie la lactancia debe disminuir la P4, ya que inhibe la expresión de PRLR, antagoniza la expresión de caseína producida por la PRL y

[11] Es la recubierta uterina (endometrio) específicamente durante la gestación, el cual da origen a la porción materna de la placenta.

compite por los receptores de glucocorticoides (143). Para la lactancia, la PRL es primordial, ya que actúa como un sensor fisiológico ante la demanda de leche favoreciendo su producción a través del metabolismo de lípidos de las glándulas mamarias. La producción de leche en las mujeres inicia después del día 2 de haber dado a luz, cuando las concentraciones de P4 bajan. Además existen cantidades significativas de PRL en la leche materna (44).

PRL y ciclo menstrual

Las concentraciones de PRL durante el ciclo menstrual tienen grandes variaciones entre las especies a diferencia de los perfiles de E2 y P4, los cuales son muy similares entre humanos y roedores (ver arriba). Prácticamente las concentraciones de PRL no varían en el ciclo menstrual del humano, a diferencia de los roedores en donde existe un pico de PRL en el ciclo estral (Figura 10). La ausencia de la elevación de PRL en el ciclo no excluye la idea de una posible función sobre los ovarios (9).

Ciclo estral en la Rata

El ciclo reproductivo de la rata tiene 4 fases: proestro, estro, diestro 1 y diestro 2 (Figura 10 panel inferior). Estas fases se caracterizan por cambios temporales en la liberación de LH, FSH, PRL, estrógenos y progesterona. La ovulación ocurre del inicio del proestro al fin del estro. Desde el inció de la madurez sexual hasta los 12 meses, el ciclo promedio dura 4 días.

Las concentraciones de PRL son bajos durante la mayor parte del ciclo, excepto durante la tarde del proestro, cuando el pico de PRL coincide con el pico pre-ovulatorio de LH. A pesar del brusco y simétrico pico de LH, el incremento trifásico de PRL tiene un pico, una meseta y una fase final. El aumento en las concentraciones de PRL es consecuencia a su vez de un aumento de las concentraciones de estrógenos, en la mañana del proestro. La acción de los estrógenos esta acoplada al ciclo circadiano. Antes de la ovulación, los estrógenos son producidos por las células de la granulosa de los folículos estimulados por la FSH. Después de la ovulación, las células de la granulosa se transforman en luteinizadas, y cada folículo es transformado en CL. Los roedores tienen

cuatro tipos de CL, durante el ciclo estral, el pseudo-embarazo, el embarazo y la lactancia, los cuales difieren en su duración y en la producción de esteroides. Si no hay apareamiento, el CL se deshecha en el siguiente ciclo de ovulación. Los apareamientos estériles activan el estimulo cervical que produce pseudo-embarazo que dura de 11-12 días- En el caso de un apareamiento fértil, la duración del CL se extiende al final de la gestación, asegurando un aporte continuo de progesterona (105) (109).

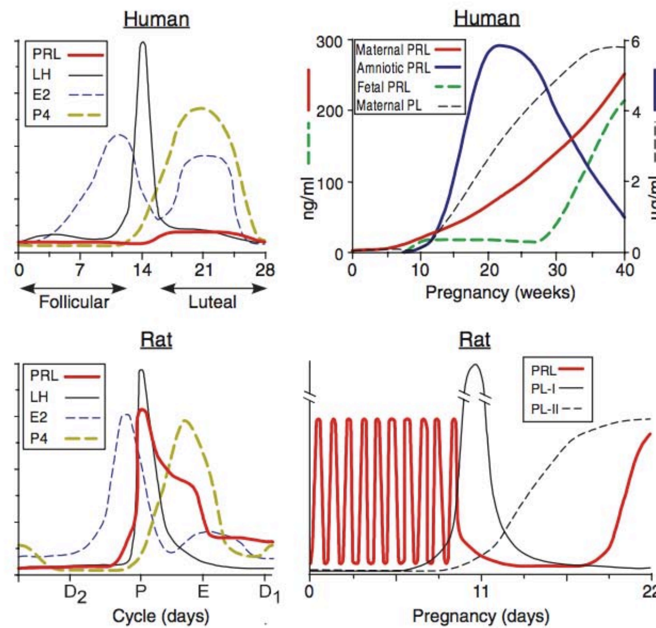

Figura 10. Comparación de los perfiles hormonales durante el ciclo menstrual (paneles de la izquierda) y durante el embarazo (paneles el derecha) en humanos y ratas. El ciclo menstrual humano es de 28 días, consta de las fases folicular, ovulatoria y lútea. En las ratas el estro es de 4 días y se compone de diestro 2 (D2), proestro (P), estro (E) y diestro 1 (D1). En humanos sólo existe un ligero incremento de PRL durante la fase lútea, mientras que el pico de estrógeno (E2) induce un pico de PRL en el proestro de la rata, seguido de una meseta y una fase de terminación extendida. En el embarazo humano al inicio existen concentraciones bajas de PRL que se elevan gradualmente en la semana 6-8, mientras que se eleva en el suero fetal gradualmente a partir de la semana 30. La PRL producida por la decidua se acumula en el liquido amniótico en la semana 10 y alcanza concentraciones altas durante la mitad del embarazo. La hormona lactógeno placentario eleva a la PRL decidual. Durante el embarazo de la rata existen dos elevaciones diarias de PRL por 10 a 11 días, seguidas de la supresión de la PRL por otras hormonas (PL-I y PL-II) (9).

Regulación sexual de NCC

Chen et al. en 1994 fueron los primeros en mostrar evidencia que la respuesta urinaria a tiazidas, así como la saturación de la unión al diurético tipo tiazida, [^{3}H]Metolazona en homogenados de corteza renal era mayor en ratas hembras en comparación con los machos (34). Al inicio el estudio de NCC por parte de los investigadores se hacía de manera funcional[12] o través de la densidad de NCC en membrana, por marcaje radiactivo, debido a la falta de anticuerpos específicos.

En el estudio se encontró que la densidad de la unión a los sitios de [^{3}H] Metolazona por miligramo de membrana fue un 78% mayor en hembras en comparación de los machos (*P*<0.001) en animales de la misma edad. La gonadectomía (Gdx) produjo un intercambio en las proporciones de la densidad de los sitios de unión a [^{3}H] Metolazona en ambos géneros. En machos el máximo número de sitios se incrementó significativamente (*P*<0.001) sobre los machos intactos (Figura 11). En contraste en las hembras disminuyó el número de sitios con la Gdx (*P*<0.01).

Parameter	Intact Male	Orchiectomy	Intact Female	Ovariectomy
Body Wt (g)	399 ± 7.1	386 ± 5.9	257 ± 7.8^b	295 ± 4.3^c
Kidney Wt (g) (single)	1.65 ± 0.030	1.35 ± 0.031^b	1.14 ± 0.034^b	1.14 ± 0.033
Kidney Wt per 100 g Body Wt	0.828 ± 0.015	0.699 ± 0.019^b	0.890 ± 0.015^b	0.787 ± 0.013^c
B_{max} for (^{3}H)Metolazone (pmol/mg)	0.433 ± 0.040	0.793 ± 0.076^b	0.770 ± 0.48^b	0.529 ± 0.051^c

Figura 11. Resultados observados en el estudio de *Chen et al.,* sobre el efecto del genero y la Gdx en la unión a los sitios de [^{3}H]Metolazona. Tomado de (34).

Estos animales también fueron sometidos a estudios metabólicos de sensibilidad a tiazidas utilizando bendroflumetiazida (BFTZ), en donde se midió la excreción urinaria y los electrolitos excretados en la orina y se analizó el cambio en ellos al restar los valores 2 h antes y 2 h después de la administración de la BFTZ (Figura 12). En las hembras de manera basal se observa una mayor excreción de Ca^{2+} y K^+ en comparación con los

[12] Estudio de sensibilidad a tiazidas, véase más detalle en Material y métodos.

machos. La BFTZ incrementó la excreción de orina, así como de Na^+, Cl^- y amonio en las hembras en comparación con los machos. Además disminuyó la calciuria sólo en hembras. En los animales con Gdx, basalmente, el efecto de la BFTZ fue mayor en hembras en comparación con los machos, de esta manera este trabajo concluyó que la función de NCC, tiene una mayor función en hembras en comparación de los machos y dicha diferencia se debe al efecto de las hormonas sexuales.

Parameter	Male	Orchiectomized	Female	Ovariectomized
Urine Volume (μL/100 g)				
Before BFTZ	1,240 ± 350	1,100 ± 252	666 ± 95	1,170 ± 118
After BFTZ	2,890 ± 368	3,110 ± 549	2,820 ± 341	2,740 ± 308
BFTZΔ	1,650 ± 349[b]	2,010 ± 636[b]	2,160 ± 303[b]	1,570 ± 253[b]
$U_{Na}V$ (μmol/100 g)				
Before BFTZ	17.5 ± 4.68	14.4 ± 3.40	17.2 ± 2.74	30.4 ± 8.81
After BFTZ	105 ± 10.4	109 ± 18.8	162 ± 25.1[c]	125 ± 14.8
BFTZΔ	87.6 ± 12.7[b]	94.7 ± 20.6[b]	144 ± 24.8[b,c]	94.1 ± 17.6[b]
U_KV (μmol/100 g)				
Before BFTZ	42.8 ± 4.62	60.3 ± 14.5	47.9 ± 5.56	63.2 ± 11.9
After BFTZ	86.1 ± 7.56	88.6 ± 12.2	91.1 ± 9.98	89.0 ± 8.30
BFTZΔ	43.3 ± 6.08[b]	28.3 ± 22.6	43.2 ± 6.03[b]	25.8 ± 10.2[b]
$U_{Cl}V$ (μmol/100 g)				
Before BFTZ	21.2 ± 2.60	27.9 ± 5.84	26.0 ± 2.57	37.6 ± 7.29
After BFTZ	137 ± 23.8	150 ± 22.4[d]	224 ± 26.4[c]	184 ± 18.2
BFTZΔ	116 ± 22.8[b]	122 ± 24.8[b,d]	198 ± 24.5[b,c]	146 ± 14.8[b]
$U_{Ca}V$ (μmol/100 g)				
Before BFTZ	0.688 ± 0.205	2.01 ± 0.405	3.14 ± 0.495[c]	2.90 ± 0.630[c]
After BFTZ	0.710 ± 0.110	1.42 ± 0.208[c]	1.45 ± 0.195[c]	1.40 ± 0.128[c]
BFTZΔ	0.022 ± 0.160	−0.585 ± 0.338	−1.68 ± 0.335[b,c]	−1.50 ± 0.590[b,c]

Figura 12. Resultados observados en el estudio de *Chen et al.,* sobre el efecto del género y la Gdx en la respuesta a BFTZ. Se muestran los valores antes, después y el delta de la administración de BFTZ. Tomado de: (34).

Apoyando estos hallazgos *Verlander et al.* en 1998, observaron por w.b. y eletro-inmunoscopia con anticuerpos acoplados a oro contra el amino terminal de NCC que la ovariectomía reduce la expresión de NCC en las células del TCD y que la administración de E2 en ratas hembras ovariectomizadas restauran las concentraciones normales de expresión de NCC (201).

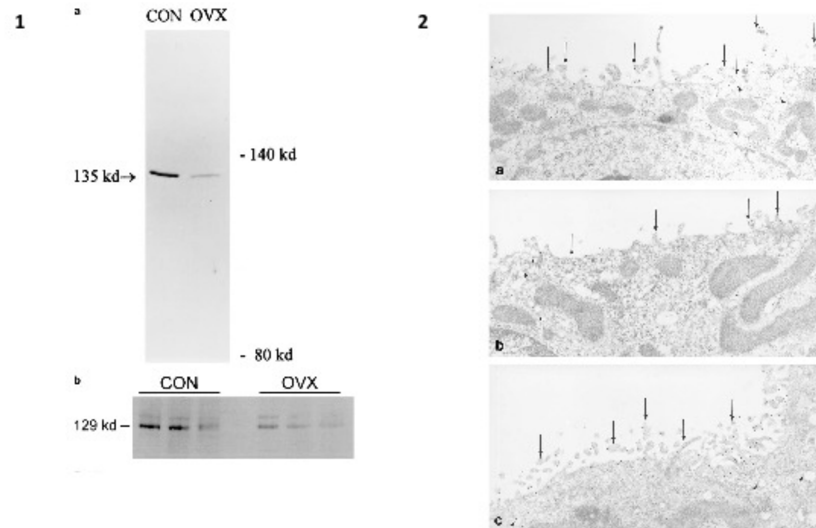

Figura 13. Resultados del trabajo de Verlander et al., en1998. 1) W.b. de NCC en hembras intactas y Gdx. 2) Microscopia con anticuerpos acoplados a oro en: a, ratas hembra intactas; b, ratas hembras Gdx y c, ratas hembras Gdx+E2.

Aunque los machos no están incluidos en este estudio las observaciones sugieren que el género femenino está asociado con la mayor expresión de NCC. Si bien no existen explicaciones de esta relación, por un lado la mayor respuesta a tiazidas en las ratas hembras puede deberse a la regulación dimórfica sexual de la secreción en el TP de los diuréticos a través de los transportadores orgánicos de aniones que se sabe se encuentran regulados por las hormonas esteroideas (103). Por otro lado la mayor respuesta a tiazidas en las ratas hembras puede ser explicado por un incremento en la reabsorción de sodio por NCC en el TCD y/o una menor compensación de la natriuresis inducida por tiazidas en regiones distales de la nefrona. Además la expresión del ARNm de NCC, así como la expresión y activación/fosforilación de la proteína, nunca han sido estudiados en ratas machos y hembras. Por lo anterior, se desconoce si la regulación sexual dimórfica de NCC ocurre a nivel transcripcional, traduccional o post-traduccional.

Hipertensión en el transplante renal

La enfermedad renal crónica (ERC) se define como la presencia de un daño renal (usualmente detectado por una excresión urinaria de albumina de ≥30 mg/día) o por una disminución de la función renal (una tasa de filtrado glomerular (TFG) menor de 60ml/min/1.73m^2) y/o daño renal determinado por proteinuria, hematuria, alteraciones estructurales en imagen o histológicas, por un periodo igual o mayor a 3 meses. La enfermedad renal crónica terminal (ERCT) representa el final del espectro de la ERC (2, 6).

Actualmente la ERCT es un problema importante de salud pública. Los tratamientos de la ERCT involucran la sustitución de la función renal en menor o mayor medida con hemodiálisis o diálisis peritoneal y trasplante renal. Esta última siendo la forma de sustituir la función renal con mejores desenlaces en términos de mortalidad y calidad de vida. Los pacientes entran a una larga lista de espera, en donde es usual colocar a los pacientes relativamente más sanos en la lista de espera mientras reciben dialisis y los pacientes menos estables como candidatos de donaciones cadavéricas (206). Sin embargo, la vida media del injerto es limitada con 11.9 años para injertos de donador vivo y 8 años para injertos de donador cadavérico (97) .

Un punto primordial dentro del transplante, es el tratamiento inmunosupresivo, que básicamente sirve para prevenir un rechazo agudo y por lo tanto la pérdida del injerto. Los fármacos inmunosupresores son básicamente combinaciones de régimenes de coticoesteroides como prednisona, azathioprine, micofenolato de mofetil, micofenolato sódico, ciclosporina, tacrolimus, everolimus, sirolimus y belatacept. El uso de inmunosupresores disminuye las tasas de *rechazo agudo* y aumenta la sobrevida del injerto a un año del 80% a 15% y del 45% a 95%, respectivamente (108). Sin embargo la *sobrevida a largo plazo* del injerto no ha aumentado, por ejemplo en Estados Unidos la vida media del injerto de un donador fallecido fue de 6.6 años en 1989, 8.2 años en 2000 y 8.8 años en 2005, mientras que cuando proviene de un donador vivo no ha existido una mejora significativa (11.4 años en 1989 en comparación con 11.9 años en 2005) (97).

Las causas de pérdida del injerto son: la muerte del paciente con injerto funcional, nefropatía crónica del injerto y recurrencia de la enfermedad de base. De éstas, la nefropatía crónica del injerto es la causa mas frecuente de pérdida del injerto. A su vez las etiologías pueden ser inmunológicas (rechazo) o no inmunológicas (factores hemodinámicos) como la hipertensión. De hecho la aterosclerosis es la principal causa de muerte en pacientes trasplantados (151) .

La hipertensión arterial (HTA) contribuye tanto en la nefropatía crónica del injerto como a la morbilidad/mortalidad cardiovascular de pacientes trasplantados con injerto funcional. La HTA post-trasplante ocurre con una frecuencia de 75 a 90% (24). Se ha asociado el aumento en la presión arterial con el deterioro de función del injerto. De la misma forma el control de la HTA en pacientes postransplantados se asocia a una mejor sobrevida del injerto en comparación con pacientes con HTA no controlada (147).

Los mecanismos involucrados para la génesis de la HTA post-trasplante son variados. En el ambiente multifactorial, se desconocen los principales causales. Los factores mencionados ocurren pretrasplante (rigidez vascular, etiología de ERC, historia de hipertensión), post-trasplante (función retardada injerto, calidad del órgano, daño crónico), intrínsecos (rechazo, recurrencia enfermedad de base) y extrínsecos (estenosis arterial renal y medicamentos) (47, 106).

Uno de los factores importantes en el post-trasplante renal como causantes de HTA son los inmunosupresores, particularmente los inhibidores de calcineurina[13] (ICN), como la ciclosporina y el tacrolimus. La prevalencia de HTA post- trasplante previo a la introducción de ciclosporina era de 40-50%, elevándose hasta un 90% posterior a su inclusión. Esto se vio reflejado en HTA post-trasplante en otros órganos (106, 194).

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[13] La calcineurina (calcineurina serina-treonina-fosfatasa) es una enzima, dependiente del calcio y de la calmodulina, que efectúa una regulación positiva de las vías de transducción de señales en los linfocitos T, favoreciendo la transcripción de los genes de diversas citocinas dependientes del factor nuclear de las células T activadas (NF-AT), entre las que se encuentran las interleucinas (IL) IL-2, IL-3, IL-4 e IL-5, el factor estimulador de colonias de granulocitos y macrófagos, el factor de necrosis tumoral alfa y el interferón gamma

Los mecanismos propuestos por los cuales los ICN producen HTA son mecanismos vasculares por el desequilibrio de factores vasoactivos, mientras que otros favorecen la posibilidad de que exista alteración en la curva de natriuresis de presión, con la consecuente HTA sensible a sal. Al estudiar 30 sujetos hipertensos, con función renal normal después de 12 meses del trasplante. Ambos incluyeron esteroide como parte del esquema inmunosupresor, 15 sujetos con azatioprina y 15 sujetos con ciclosporina. El grupo con el ICN no respondió a captopril, mejoró con dieta baja en sal y se encontró un balance positivo de sodio con disminución de su excreción renal, contrario a lo encontrado en el grupo con azatioprina, sugiriendo un tipo de HTA diferente en el post-trasplante muy similar la génesis de una hipertensión sensible a sal, similar a la HTA esencial (39).

Ratas expuestas a ICN presentaron HTA con daño estructural renal (nefrotoxicidad) y revirtiendo la hipertensión al retirar el ICN (151). Los inhibidores de calcineurina inducen un cuadro de HTA que se acompaña con frecuencia de disfunción tubular renal manifestada por hiperkalemia, hipercalciuria y acidosis metabólica cuadro similar al síndrome de Gordon.

Efecto de tacrolimus sobre la activación de NCC

Dado los antecedentes observados sobre el desarrollo de HTA con desórdenes electrolíticos similares al síndrome de Gordon en pacientes renales post-trasplantados el grupo de Ellison desarrollo un trabajo sobre la hipótesis que los ICN inducen la HAT a través de estimular a NCC. Para ello utilizaron ratones silvestres y los trataron con tacrolimus, en ellos analizaron la PA, la excreción de Na^+ y K^+ así como las concentraciones de fosforilación mediante w.b. en homogenados de riñón. Lo que observaron fue que el tacrolimus indujo un fenotipo similar al síndrome de Gordon, con un aumento en la PA, disminución de la excreción de Na^+ y de K^+ en orina así como un aumento en la fosforilación de NCC (Figura 18) (78).

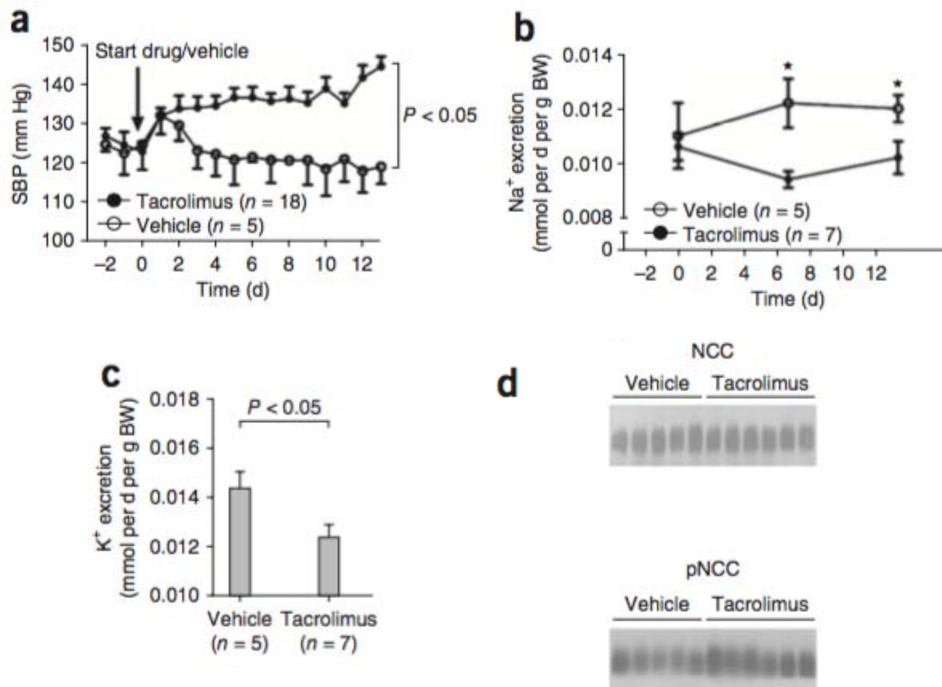

Figura 14. Resultados del trabajo de (78) .a) PA sistólica en ratones silvestres y tratados con tacrolimus. b) Excreción de sodio y c) potasio urinario en ratones silvestres y tratados con tacrolimus. W.b. contra NCC y NCC fosforilado en ratones administrados con tacrolimus. Tomado de (78).

Aún más, utilizaron un ratón knock-out para NCC que fue resistente a la hipertensión producto del tratamiento con tacrolimus, mientras que en ratones que sobreexpresan NCC, por manipulación genética, la respuesta hipertensiva al ICN fue exagerada. Por último el tratamiento con tiazida revirtió el efecto hipertensivo del tacrolimus (Figura 19) (78).

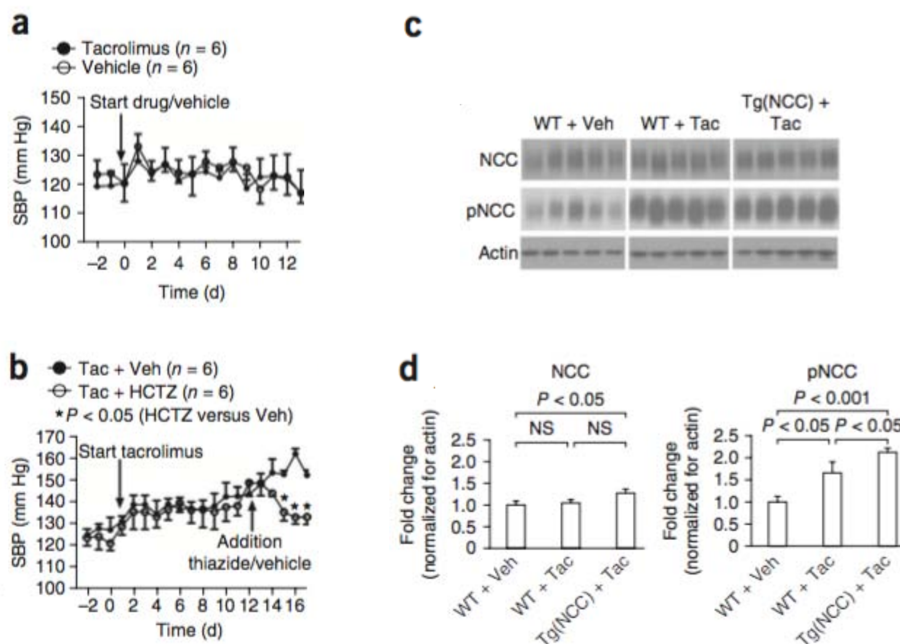

Figura 15. Resultados del trabajo de (Hoorn et al. 2011). a) Registro de la PA sistólica de ratones knock out para NCC tratados con tacrolimus y vehiculo. b) Registro de la PA sistólica en ratones silvestres tratados con tacrolimus+vehiculo y tacrolimus+ tiazida (HCTZ). c y d) W.b para NCC total y su forma fosforilada en ratones transgénicos que sobreexpresan a NCC tratados con tacrolimus y análisis densitométrico. Tomado de (78) .

Estas observaciones fueron estudiadas también en pacientes post-strasplantados tratados con tacrolimus que fueron voluntarios para un estudio de sensibilidad a tiazidas, BFTZ, utilizando como controles voluntarios sanos que no fueron tratados con tacrolimus y en pacientes tratados con otro inmunosupresor, sirolimus; en donde verificaron que NCC es activado específicamente por tacrolimus, es decir la excreción de cloro fue mayor en los pacientes tratados con tacrolimus (78).

Recientemente se realizó un estudio en pacientes, donde se buscó la relación entre la administración de ciclosporina y la expresión de NCC en exosomas urinarios de pacientes con trasplante renal, sin embargo no se halló una correlación de la expresión de

NCC con el desarrollo de hipertensión en los pacientes tratados con el ICN. Este análisis se baso en datos de una población de manera retrospectiva reunida con un promedio de 10 años de haber recibido el injerto, de esta manera pudieron intervenir múltiples factores en el desarrollo de la hipertensión (46).

Análisis de NCC en exosomas urinarios

Los exosomas son vesículas de membrana exocitadas y se originan de cuerpos multivesiculares a través del recambio de la membrana plasmática por endocitosis. Son secretados por células epiteliales incluyendo el epitelio renal (90). El hallazgo de exosomas en orina, con proteínas características de la membrana apical de la nefrona trajo consigo la posibilidad de estudiar cambios in vivo en organismos con vida de manera no invasiva incluyendo al ser humano (160). NCC fue identificado a través de w.b. dentro de las proteínas secretadas en la orina, lo cual trajo la posibilidad de unir dos técnicas, el aislamiento de exosomas(1) y el análisis de proteína (Western blot) utilizando anticuerpos y fosfoanticuerpos para NCC. Ahora ya se ha corroborado en exosomas urinarios de humanos los hallazgos en modelos experimentales: NCC se encuentra fosforilado en pacientes con aldosteronismo (85) (197) y con síndrome de Gordon (85, 114). Tiene una menor expresión en pacientes con Sx. Gitelman (38). Además se tienen datos de una regulación circadiana a través de la excreción de NCC en orina a lo largo del día (25).

Planteamiento del Proyecto

Regulación por hormonas femeninas

Dado los hallazgos encontrados por *Verlander et al.* en 1998 (201) la posibilidad de que los estrógenos modifican la transcripción de NCC suena coherente, sin embargo, una revisión profunda de las evidencias mostraba que no se había demostrado con precisión la presencia de receptores de estrógeno en las células epiteliales del TCD (209), pese a que es bien sabido que el riñón tiene receptores de estrógenos principalmente del tipo alfa (ER-α) (88).

Además, si bien existe una gran cantidad de información que muestra una relación entre el ciclo menstrual y la retención de Na^+ por parte del riñón en específico en la fase folicular, que es cuando existe el pico más grande de estrógenos (155) la evidencia fisiológica de que NCC sea regulado exclusivamente por estrógenos de manera positiva en la hembras es incongruente con la evidencia sobre el incremento del riesgo de hipertensión arterial y de enfermedades cardiovasculares durante la menopausia (123) así como de osteoporosis (101), datos que son totalmente opuestos a una pérdida de la función de NCC por el decremento de las concentraciones de estrógenos, en donde la pérdida de función de NCC como en el Sx. de Gitelman favorecen la densidad ósea y disminuyen la retención de Na^+ hechos que se reflejarían en una disminución de la presión arterial (177).

Otra hormona femenina que pudiese estar regulando a NCC de manera positiva es la P4 que junto con los estrógenos, se encuentra elevada en fenómenos en donde existen incrementos en la retención de líquidos y Na^+ como el ciclo menstrual y el embarazo (36) y, a diferencia de los estrógenos, existe evidencia clara de que el receptor de P4 se expresa en células del TCD (22). Sin embargo la información la sitúa más como una hormona con efectos antihipertensivos (176) debido a sus efectos diuréticos que resultan más evidentes durante la fase lútea (pico de P4 en el ciclo menstrual) y en el embarazo (163). Se cree que este efecto diurético se debe a que la P4 es un antagonista natural del receptor de mineralocorticoides evitando así que se una la aldosterona una hormona que favorece la retención de Na^+ en la nefrona distal (137).

Debido a las incongruencias fisiológicas entre una mayor expresión y función de NCC en ratas hembras vs machos y los efectos sistémicos de las hormonas esteroideas debe existir otra hormona con mayor concentración en las hembras que pueda ser responsable del dimorfismo sexual de NCC, es decir la hormona prolactina (54). La PRL es un osmoregulador bien conocido en vertebrados inferiores (207). En teleósteos, la PRL incrementa la reabsorción de sal y agua en la vejiga urinaria (76), órgano en donde la reabsorción de sodio es mediada a través de NCC (60, 168) (188).

Se ha encontrado que NCC es regulado de manera directa por PRL (187). Existe evidencia de que también en mamíferos la PRL afecta la función renal, en específico se encontró que la PRL incrementa la reabsorción de Na^+ en la nefrona distal de manera independiente de HAD (187). La PRL activa de manera significativa la actividad de la Na^+/K^+-ATPasa en el TCD de manera dosis dependiente (23). Por último se sabe que los receptores para PRL (PRLR) se expresan en el AAH, TCD y CC (48). En humanos la PRL puede producir retención de agua, sodio y potasio (80) y en pacientes hiperprolactinémicos por tumores secretores de PRL en la hipófisis se encontró que existe una disminución en la excreción urinaria de solutos en comparación con pacientes sanos (21).

Además la secreción de PRL por la adenohipófisis es constantemente inhibida por el factor inhibidor de prolactina o dopamina producida por las neuronas del núcleo arcuato en el hipotálamo (54), sin embargo, se han hallado estimulantes de la liberación de PRL, por ejemplo los estrógenos tienen una importante función en el control hipotalámico de la PRL. Se demostró que las ratas hembras exhiben mayores concentraciones de PRL en sangre en comparación con las ratas macho. La ovariectomía reduce tanto a las concentraciones de estrógenos como la PRL en sangre, mientras que la administración de E2 restaura no sólo las concentraciones de estrógeno sino también los de PRL (111) (82). Estudios en los que se utilizan hembras ovariectomizadas con y sin la administración de E2 no descartan la participación de la PRL sobre la regulación de la expresión/regulación de NCC.

Pregunta

Dado el hilo de evidencias planteado la pregunta que surgió para esta tesis fue:

¿Cuál es la hormona responsable de los efectos positivos sobre la expresión/actividad de NCC observados en las hembras?"

Hipótesis

Nosotros propusimos la siguiente hipótesis:

La expresión y actividad/fosforilación de NCC es regulada positivamente por las hormonas femeninas, estrógenos, progesterona y prolactina.

Objetivos

Objetivo general

Determinar la regulación dimórfica a nivel fisiológico y molecular sobre NCC.

Objetivos particulares

- Evaluar la función de NCC en hembras y machos con y sin gonadectomía en ratas.
- Determinar si existe una mayor expresión/activación de NCC en hembras vs machos, en ratas, ratones y humanos.
- Evaluar los efectos de las hormonas esteroideas en un modelo de gonadectomía sobre la fosforilación y expresión de NCC en ratas hembra.
- Evaluar los efectos de la prolactina sobre NCC utilizando un modelo de perfusión ex vivo de PRL en riñones de rata macho.
- Determinar los efectos de la ausencia del receptor de prolactina sobre la activación/expresión de NCC, en un modelo de ratones knock out para el receptor de prolactina.

Papel en la hipertensión arterial inducida por tacrolimus en pacientes con trasplante renal

Con la intensa actividad de trasplante renal que se lleva a cabo en México y en el mundo, la pérdida del injerto se ha convertido en una de las principales causas de ERCT. Por lo tanto, cualquier maniobra que alargue la vida media de los injertos renales tendrá un efecto positivo sobre la disminución de nuevos enfermos con ERCT que requieren sustitución de la función renal.

Aunque existen diversos fármacos antihipertensivos, a la fecha no hay forma de individualizar el tratamiento dado que son varios los potenciales mecanismos de la HTA y se conoce poco al respecto de estos. Mientras mejor aclaremos el o los mecanismos de HTA en cada paciente, podremos indicar terapias individualizadas que sean más efectivas en el manejo de la HTA y esto redundará en mejor supervivencia del injerto y del paciente

Estos trabajos sugieren que la activación del NCC puede ser un mecanismo importante en la HTA inducida por inhibidores de calcineurina, por lo que el tratamiento óptimo en estos casos sería con diuréticos de tipo tiazida.

Con estos antecedentes nosotros creemos que la HTA en al menos algunos pacientes con trasplante renal tratados con tacrolimus como inmunosupresor podría ser por activación del NCC. Si este es el caso, en dichos pacientes el tratamiento antihipertensivo de elección podrían ser los diuréticos de tipo tiazida.

Pregunta

Dado el hilo de evidencias planteado la pregunta que surgió para esta tesis fue:

¿La presencia de una mayor activación/fosforilación de NCC encontrada en ratones tratados con ICN, se puede extender en pacientes con HTA con trasplante renal?

Hipótesis

Nosotros propusimos la siguiente hipótesis:

La HTA postrasplante renal en pacientes tratados con tacrolimus como inmunosupresor se encuentra asociada a una mayor activación del NCC.

Objetivos

- Determinar la asociación de HTA y su relación con diversas variables demográficas, clínicas y bioquímicas.

- Determinar la asociación de la expresión/fosforilación del NCC con la presencia de hipertensión en pacientes post-transplantados renales tratados con el inhibidor de calcineurina, tacrolimus.

Material y métodos

Animales

El manejo de animales para los fines de este proyecto fue autorizado por la Comisión para el Cuidado y Uso de los Animales de Laboratorio del Instituto de Investigaciones Biomédicas de la UNAM y por el Comité de Investigación en Animales (CINVA) del Instituto Nacional de Nutrición Salvador Zubirán (INCMNSZ).

Las ratas y ratones nacieron y se mantuvieron en todo momento en el bioterio del INCMNSZ, expuestas a períodos de luz/oscuridad de 12:12 h, en condiciones constantes de temperatura y humedad relativa de 18º-19º y 65%, respectivamente. Todas las ratas y ratones tuvieron libre acceso a agua y comida estándar para rata.

Para el desarrollo del presente trabajo se utilizaron muestras de riñón de ratas y ratones hembras y machos. Todos los animales sometidos a algún protocolo fueron asignados a los grupos experimentales de manera aleatoria.

Estudios de sensibilidad a diuréticos:

Se estudiaron ratas hembras y machos de la cepa Wistar entre 250-300 g divididas en cuatro grupos de 10 ratas cada uno. Los grupos quedaron de la siguiente manera:

1) Machos control o intactos
2) Hembras control o intactas
3) Machos gonadectomizados
4) Hembras gonadectomizadas

Con el fin de analizar la función de NCC en la excreción urinaria de agua y electrolitos en ambos géneros, se analizó la respuesta a la administración aguda del diurético tipo tiazida, bendroflumetiazida (BFTZ), el cual inhibe específicamente la actividad de NCC.

Un día antes de la administración del diurético las ratas permanecieron en jaulas metabólicas para recolectar la orina de 24 h a partir de la cual se midió el volumen

urinario y la excreción urinaria de Na^+ y K^+. Al término de este tiempo los animales fueron inyectados con una dosis de 1mg/kg de BFTZ vía intraperitoneal. La BFTZ fue diluida en dimetilsulfóxido al 20%. Durante las siguientes horas, se recolectó la orina nuevamente, a partir de la cual se determinó nuevamente el volumen urinario y la excreción urinaria de electrolitos. La dosis y tiempo de respuesta máxima empleadas en los experimentos se deteminaron a partir de estudios previos de nuestro laboratorio llevados a cabo en ratas macho con BFTZ.

Dos o tres días después de la primera inyección de BFTZ, se llevó a cabo la gonadectomía (Gdx) de los grupos 3 y 4. Después de las Gdx´s, se dejaron transcurrir tres semanas para que las concentraciones de las hormonas sexuales de las ratas gonadecotimizadas disminuyeran y las ratas tuviera reposo postoperatorio. Una vez transcurrido ese tiempo, los 4 grupos experimentales fueron expuestos de nuevo a la administración aguda de BFTZ con el fin de determinar las diferencias en la respuesta al diurético entre hembras y machos antes y después de la Gdx.

Las ratas se sacrificaron al día siguiente de la segunda administración de BFTZ. Un día antes, se pesaron y colocaron en cajas metabólicas sin alimento para llevar a cabo una recolección de orina de 24 h. Se midió el volumen de orina y a partir de ésta se determinaron las concentraciones de creatinina. Las ratas se anestesiaron en cámara de éter y se tomaron muestras de sangre mediante punción de la vena cava para determinar las concentraciones de E2 y testosterona. Posteriormente ambos riñones fueron extraídos y separados en corteza y médula. Ambas regiones fueron congeladas en nitrógeno líquido y almacenadas a -80ºC para su posterior procesamiento.

Ya que en el presente estudio se analizó la función de NCC en la excreción urinaria de agua y electrolitos en ambos géneros, fue necesario determinar si la respuesta a BFTZ en las ratas hembra no variaba entre los diferentes días del ciclo estral. Para poder determinarlo, se llevó acabo un estudio piloto en el cual se analizó el efecto del estado hormonal sobre la respuesta a la administración de la BFTZ. Para realizarlo, se emplearon dos grupos de 10 ratas cada uno: Uno fue estudiado en estro el día del ciclo con menor nivel de E2 circulante y el otro fue estudiado en proestro el día del ciclo con mayor producción de E2. El día del ciclo fue determinado por frotis vaginal. Se tomó

una muestra de lavado vaginal mediante una pipeta con solución salina el cual fue sujeto a análisis citológico en un microscopio óptico. Las diferencias en el tamaño, forma y cantidad de células son indicativas del día del ciclo estral. Como puede observarse en la Figura 18 no se encontraron diferencias significativas en la respuesta de las ratas en fase de estro comparadas con las ratas en proestro, ni antes ni después del tratamiento con BFTZ. Lo anterior indica que la magnitud de la respuesta al diurético no es influenciada por cambios cíclicos de las hormonas sexuales ováricas. Con base en este antecedente en el presente proyecto las ratas no fueron cicladas.

Con el fin de comprobar si las diferencias encontradas en las ratas hembra y macho después de la administración de BFTZ son específicas para este tipo de diurético y no el reflejo de algún factor involucrado con su llegada y función en el túbulo distal se analizó también la respuesta a los diuréticos furosemida y amilorida en ratas hembra y macho. Ambos diuréticos se administraron por inyección intraperitoneal en una dosis única de 5 mg/kg y 1 mg/kg respectivamente. La furosemida inhibe la reabsorción apical de sodio en el AAH mientras que la amilorida lo lleva a cabo en el túbulo colector. De esta manera se analizó la reabsorción de sodio en la porción anterior y posterior al TCD. El volumen urinario y la excreción urinaria de los principales electrolitos renales se cuantificaron 2 h antes y 2 h después de la administración del diurético de la misma forma que se llevó a cabo con la BFTZ.

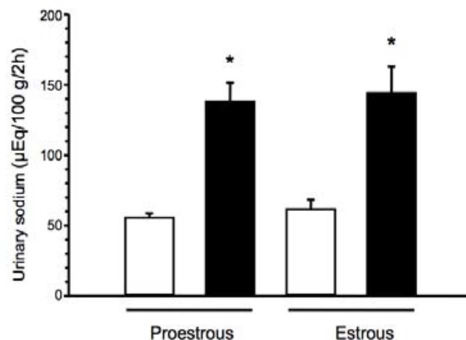

Figura 18. Excreción urinaria en ratas hembras antes (barras blancas) y después (barras negras) de la administración de BFT durante el proestro y estro.

Gonadectomía

Ratas hembras y machos de doce semanas fueron sometidas a gonadectomía bilateral u operación control (sham). Las ratas fueron anesteziadas con una inyección intraperitoneal de pentobarbital sódico en una dosis de 30mg/kg de peso corporal. Una vez que se confirmó la ausencia de reflejos, se rasuraron y se limpiaron con procedimientos para la asepsia del área. Se realizaron inciciones de 2 cm en la piel, para las hembras a lo largo de la linea media dorsal y para los machos en la linea media del escroto, los ovarios y testículos, respectivamente fueron expuestos, ligados y removidos. Para el caso de la cirugía sham, se siguieron todos los pasos anteriores excepto por la remoción. Los animales fueron suturados y aislados para su recuperación y observación por un mes.

Extracción de exosomas

Para evaluar el estado de NCC en humanos se aislaron exosomas a partir de muestras de orina (214). El protocolo consistió en la recolección de una muestra de orina de 30 ml, de preferencia fue la primera orina de la mañana. A esta orina se le agregaron inhibidores de proteasas de manera inmediata y se almacenó a -80°C.

Para la extracción de exosomas se descongeló la orina en hielo y se vortexeó intensamente para rehomogenizar la orina. Se tomó un total de 10 ml y se centrifugó a una velocidad de 17,500 g por 15 minutos a 4°C, para eliminar los sedimentos. De este procedimiento se aisló el supernadante (1) en un tubo limpio y el pellet (1) se procesó con 200 mg/ml de dedithiothreitol (DTT) disuelto en un volumen de 200µl de solución de aislamiento (10mM de trietanolamina y 250mM de sacarosa a un pH 7.6) e incubado durante 5 minutos a 37°C y posteriormente centrifugado a 17,500 g por 10 minutos a 37°C. De este procedimiento se obtuvó el supernadante (2) que se mezcló con el supernadante 1, mientras que pellet (2) se desechó.

La mezcla del supernadante se centrifugó a 200,000 g por 2 horas a 4°C, el supernadante (3) se desechó por decantación y el pellet (3) se disuelvió en 50µl de buffer de lisis con inhibidores de fosfatasas, el pellet resuspendido se analizó a través de western blot.

Microscopia de Inmunoflorescencia

Ratones machos fueron anestesiados con isofluorano y perfundidos a través de canulación al ventrículo izquierdo del corazón con formaldehído. Mediante un corte en cárotida inferior. Los riñones fueron lavados, extraídos y conservados en formaldehído, hasta ser crioseccionados con un criotomo, para poder ser incubados con anticuerpos para NCC (S965B) y para el carboxilo terminal del receptor de estrogenos acoplado a proteína G 1 (GPER-1) (35) y para parvalbumina (SWANT, PV 235), y revelados con los anticuerpos secundarios donkey anti-rabbit Alexa-Fluor 488 y donkey anti-mouse Alexa-Fluor 594 y donkey anti-sheep Aexa-Fluor 488 (Life technologies).

Tratamiento hormonal

Ratas hembras ovariectomizadas y ratas intactas fueron tratadas con 60µg/kg de 17-ß Estradiol (E2), 20 mg/kg progesterona (P4) o vehículo, tras el mes de recuperación. Los grupos quedaron de la siguiente manera:

1) Ovx+Vhl
2) Ovx+E2
3) Ovx+P4

Las drogas fueron disueltas en etanol al 10% y el resto del volumen con aceite de oliva y administradas intraperitonealmente todos los días por 3 semanas hasta su eutanasia.

En el caso del protocolo de administración de 60µg/kg de 17-ß Estradiol en ratas machos, el procedimiento fue similar, quedando los grupos como:

1) Macho + E2
2) Macho + Vhl

Para el caso de las ratas machos tratadas con 25µg/ kg de prolactina o con vehículo (agua biología molecular) por 2 semanas. La prolactina fue recombinante de murinos (Sigma-Aldrich), los grupos de tal manera fueron:

1) Macho + PRL
2) Macho + Vhl

Perfusión de riñón de rata *ex vivo*

Los riñones de ratas machos fueron montadas en un sistema de Langerdoff y perfundios con vehículo o prolactina (murine recombinat, Sigma-Aldrich) a una concentración de 0, 10 y 40 ng/ml concentraciones que no tuvieron efectos en la presión de perfusión. Tras 30 min de perfusión el riñón se separó manualmente en corteza y médula y congelados en nitrógeno líquido.

Determinaciones bioquímicas

Electrolitos: El Na^+, K^+ y Cl^- urinarios se determinaron mediante el potencial de ión selectivo en un analizador de electrolitos Synchron CX5, Beckman Coulter. Los electrolitos fueron determinados tanto en la orina de 24 h como en la orina de 2 h después de la administración de BFTZ.

Creatinina: Se determinó tanto en suero como en orina en un analizador de creatinina II de Beckman. Los resultados obtenidos fueron utilizados para calcular la depuración de creatinina según la fórmula U*V/P en donde U es la concentración urinaria, V el volumen urinario y P la concentración plasmática. La depuración de creatinina constituye un índice confiable de la filtración glomerular y se midió con el objetivo de analizar que la función renal en todas las ratas estudiadas fuera normal.

Hormonas sexuales: Se evaluaron por medio del radio-inmunoensayo con ^{125}I en la fase sólida Coat-a-Count diseñado para la determinación de testosterona P4 y E2 en muestras de suero hepanirizado sin necesidad de extracción. El procedimiento se basa en la utilización de tubos de propileno recubiertos con anticuerpos específicos para las hormonas antes mencionadas. La testosterona y el E2 marcados con ^{125}I compiten con los de las muestras durante un tiempo determinado por los sitios de unión en los anticuerpos.

A continuación el tubo se decanta para separar las hormonas sexuales unidas de las libres y se cuenta en un contador gamma. La cantidad de cuentas está inversamente relacionada con la cantidad de hormona presente en la muestra. La cantidad presente en las muestras se determina a partir de una curva de calibración.

Prolactina: Las concentraciones se evaluaron mediante ensayo por inmuno-absorción ligado a enzimas (ELISA) con un kit de para la detección de prolactina de rata de ALPCO®, el cual está basado en un formato de microplato cubierto con un anticuerpo monoclonal específico para prolactina de rata. Los controles y muestras son pipeteadas dentro del microplato para ser incubadas con el anticuerpo contra prolactina de rata durante 2 h. Después de lavar se incuba por 1 h con un segundo anticuerpo acoplado a la peroxidasa de rábano. Tras un segundo lavado se agrega un sustrato cromogénico TMB (3,3',5,5'-Tetra-Metil-Benzidina) por 30 min, durante los cuales el sustrato es convertido a un producto con color (azul). La intensidad de color es directamente proporcional a la concentración de prolactina de rata en la muestra. La densidad óptica del color de la solución es medida a 450 nm.

Western blot

Uno de los propósitos del presente trabajo fue el evaluar los niveles de expresión y de fosforilación de distintas proteínas de interés en los riñones de las ratas y ratones en condiciones basales y ante las diferentes manipulaciones experimentales propuestas. Para este fin utilizamos anticuerpos contra NCC y SPAK, así como fosfoanticuerpos que reconocen epítopes fosforiladas dentro de estas proteínas. Todos los anticuerpos fueron donados por el Dr. Dario Alessi de la Universidad de Dundee en Escocia, con quien mantenemos activa colaboración y, por lo tanto, ya se han caracterizado previamente y se ha demostrado su especificidad (165, 171). En el laboratorio se estandarizaron las condiciones en que se realizarían después los western blot y además se verificó nuevamente la especificidad mediante Western blot de proteínas sobre-expresadas en ovocitos de *X. laevis*.

Para los riñones de rata o ratón, se pesaron 50 mg de tejido. Se homogenizaron los tejidos con 500 µl de buffer de lisis con inhibidores de proteasas y fosfatasas [Tris-Cl. 50 mM (pH 7.5), EGTA 1 mM, EDTA 1 mM, fluoruro de sodio 50 mM, pirofosfato de sodio 5 mM, ortovanadato de sodio 1 mM, Nonidet P-40 al 1% (peso/vol), sacarosa 0.27 M, 2-mercaeptanol al 0.1% (vol/vol) e inhibidores de proteasas (Complete; Roche)]. Se centrifugaron las muestras a 10, 000 r.p.m. x 10 min, se recuperó el sobrenadante y se almacenó a -80ºC hasta la utilización de las muestras. La cantidad total de proteínas se cuantificó mediante el método de Larry. Se corrieron 60-120 µg de proteínas de cada muestras en geles de poliacrilamida-SDS al 7.5%. Posteriormente se transfirieron las proteínas de cada muestra en membranas de PVDF y éstas se bloquearon con un agente bloqueador (Vigorad) al 5%, disuelto en TBS-Tweed al 0.2%. Las incubaciones con los anticuerpos se realizaron de la manera que se muestra en la Tabla 5. Las bandas inmunoreactivas se detectaron mediante quimioluminiscencia utilizando sustrato (Luminata Forte Western HRP; Merck Millipore). Los western blots de las figuras en resultados, Fig. 1 A y 1 B, 2 B, 3 A y 4 A fueron expuestos en placas de rayos X y las densitometrías se hicieron en una sola exposición. El resto de las membranas fueron detectadas con un escáner de quimioluminiscencia (C-DiGit Blot Scanner; Li-COR) y analizadas con el software acompañante (ImageStudio; Li-COR). Los valores fueron normalizados al promedio de la intensidad medida en machos, ratas no Gdx, o tratadas con vehículo, definido como 1. Todas la comparaciones fueron realizadas entre grupos dentro de una misma membrana.

Diseño de la cohorte para el estudio de la actividad de NCC en pacientes post-trasplantados tratados con tacrolimus

Diseño del estudio: Estudio prospectivo, longitudinal, observacional.

Selección de pacientes. Pacientes trasplantados renal en el Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ) desde Enero 2013. Los cuales hayan aceptado ingresar al estudio con su consentimiento y contaran con un ICN dentro de su esquema inmunosupresor.

Definición de variables obtenidas

Variables clínicas. Del expediente clínico se recolectaron los siguientes datos: causa de enfermedad renal, tipo y tiempo de terapia sustitutiva renal, volumen urinario residual, así como medicamentos pretrasplante. Se determinó durante el reclutamiento y seguimiento, el tipo de trasplante, las características del donador, el tipo de inmunosupresión, el número de haplotipos compartidos y los eventos de rechazo por medio de biopsias protocolizadas.

Variables bioquímicas. Se registraron de forma basal y durante los meses 1,3,6 y 12. Biometría hemática completa, glucosa, nitrógeno ureico, creatinina, electrolitos séricos (sodio, potasio, cloro y CO_2 total) y albúmina. Se midieron las concentraciones de ICN el mismo día de la toma de muestra urinaria para extraer exosomas (ver mas adelante). En la muestra de orina se determinó sodio, potasio y creatinina urinaria.

Determinación de presión arterial y definición de hipertensión

En la etapa pretrasplante se consideró el registro de la presión arterial en 3 consultas. Definiendo como hipertensión el presentar en al menos dos consultas presión mayor o igual a 140/90mmHg o uso de antihipertensivos.

En la etapa postrasplante se tomó presión arterial en consultorio y mediante MAPA. Para el diagnóstico de hipertensión arterial, se consideró la toma de presión arterial en consultorio en al menos dos ocasiones con cifras mayores o iguales a 140/90 o prescripción de antihipertensivos por médico tratante, durante los meses 1,3,6 y 12.

Medición de presión arterial ambulatoria

La medición de presión arterial tiene el problema de una alta variabilidad. Variaciones en relación a respiración, minuto a minuto, hora a hora, día o noche. Por lo tanto, una determinación aislada en el tiempo es un pobre estimado de la presión arterial usual del paciente y por consiguiente su relación con diferentes desenlaces. La respuesta ante esta variabilidad es determinar una presión arterial en relación al tiempo, mediante la toma de presión arterial ambulatoria. Con mediciones cada 15- 30min y cada 30-60min durante la

noche. Permitiendo identificar hipertensión de bata blanca, hipertensión enmascarada y determinando la presencia/ausencia del descenso normal de presión arterial durante la noche. La toma de presión arterial mediante MAPA en pacientes trasplantados renales es un método más sensible para diagnosticar hipertensión arterial en comparación con la toma de presión arterial en consultorio, asimismo es más útil para el control de la misma.

Protocolo: La medición de presión ambulatoria automatizada se realizó a los 6 meses y al año de seguimiento. Se colocó brazalete en brazo no dominante o en el brazo con mayor presión arterial tomada de forma basal. Se descartaba uso de anticoagulantes, mastectomía con disección ganglionar o alteraciones locales en brazo. Se indicaba evitar ejercicio extenuante, manejar automóvil, por lo demás se sugería realizar actividades de la vida diaria habituales. Se programaba dispositivo para tomar presión arterial durante el día cada 15 minutos y durante la noche cada 30 minutos, con una insuflación máxima de 20mmHg por encima de la presión arterial tomada de forma basal o 180mmHg. Se daban instrucciones para retiro del dispositivo en caso de dolor intenso o presencia de equimosis.

La definición operaciónal de hipertensión arterial por medio de MAPA en el presente estudio, se cumple con cualquiera de las siguientes: Presión arterial igual o mayor a 135/85 en promedio de MAPA 24 horas (global). Presión arterial igual o mayor a 140/90 en promedio de MAPA durante el día. Presión arterial igual o mayor a 125/75 en promedio de MAPA durante la noche.

Asimismo se determinó el descenso de la presión arterial durante la noche. Clasificándose como dippers aquellos con disminución de la presión arterial igual o mayor al 10% durante la noche. Los pacientes con ausencia de este descenso esperado de la presión arterial se clasificaron como no dippers.

Análisis Estadístico

El análisis de la respuesta a la BFTZ se llevó a cabo en la siguiente forma: la excreción urinaria de 24 horas tanto de volumen como de electrolitos antes de la administración de la BFTZ fue dividida entre 12 con el fin de obtener un valor promedio para un período de

dos horas. Este valor posteriormente fue comparado con los obtenidos en la recolección de orina obtenida dos horas después de la administración del diurético.

Por otra parte, para determinar las diferencias significativas ($P<0.05$), se analizaron y graficaron el promedio ± SE. Las diferencias estadísticamente significativas entre machos y hembras o animales intactos y gonadectomizados para dos grupos fueron determinados mediante una t-Student no empatada y para tres o más grupos por una ANOVA de una vía con corrección de Dunnet.

En el caso del análisis de las variables clínicas del protocolo de *estudio de la actividad de NCC en pacientes post-trasplantados tratados con tacrolimus*, se utilizo Chi cuadrada o Exacta de Fisher para comparación de variables categóricas. T-Student o U Mann Whitney para comparación de variables continuas (dependiendo distribución). Se realizó un análisis estratificado por género y para hipertensión. Se consideró estadísticamente significativo una $P <0.05$.

Resultados

Los resultados de esta tesis ya fueron publicados en los artículos originales que se incluyen a continuación. Los resultados por lo tanto ya no serán descritos.

Lorena Rojas-Vega, *Luis A Reyes-Castro, Victoria Ramirez, Rocío Bautista-Pérez, Chloe Rafael, María Castañeda-Bueno, Patricia Meade, Paola de los Heros, Isidora Arroyo-Garza, Valérie Bernard, Nadine Binart, Norma A Bobadilla, Juliette Hadchouel, Elena Zambrano, and Gerardo Gamba. Ovarian hormones and prolactin increase renal NaCl cotransporter phosphorylation. American Journal of Physiology: Renal Physiology, 2014; 308(8), F799–F808.*

Lorena Rojas-Vega, *Aldo R Jiménez-Vega, Silvana Bazúa-Valenti, Isidora Arroyo-Garza, José Víctor Jiménez, Ruy Gomez-Ocadiz, Diego Luis Carrillo-Pérez, Erika Moreno, Luis Eduardo Morales-Buenrostro, Josefina Alberú, and Gerardo Gamba. Increased phosphorylation of the renal* Na^{+}*-*Cl^{-} *cotransporter in male kidney transplant recipient patients with hypertension: a prospective cohort .AJP renal.00326.2015. http://doi.org/10.1152/ajprenal.00326.2015*

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Ovarian hormones and prolactin increase renal NaCl cotransporter phosphorylation

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Rojas-Vega L, Reyes-Castro LA, Ramirez V, Bautista-Pérez R, Rafael C, Castañeda-Bueno M, Meade P, de los Heros P, Arroyo-Garza I, Bernard V, Binart N, Bobadilla NA, Hadchouel J, Zambrano E, Gamba G. Ovarian hormones and prolactin increase renal NaCl cotransporter phosphorylation. *Am J Physiol Renal Physiol* 308: F799–F808, 2015. First published January 13, 2015; doi:10.1152/ajprenal.00447.2014.—Unique situations in female physiology require volume retention. Accordingly, a dimorphic regulation of the thiazide-sensitive Na^+-Cl^- cotransporter (NCC) has been reported, with a higher activity in females than in males. However, little is known about the hormones and mechanisms involved. Here, we present evidence that estrogens, progesterone, and prolactin stimulate NCC expression and phosphorylation. The sex difference in NCC abundance, however, is species dependent. In rats, NCC phosphorylation is higher in females than in males, while in mice both NCC expression and phosphorylation is higher in females, and this is associated with increased expression and phosphorylation of full-length STE-20 proline-alanine-rich kinase (SPAK). Higher expression/phosphorylation of NCC was corroborated in humans by urinary exosome analysis. Ovariectomy in rats resulted in decreased expression and phosphorylation of the cotransporter and promoted the shift of SPAK isoforms toward the short inhibitory variant SPAK2. Conversely, estradiol or progesterone administration to ovariectomized rats restored NCC phosphorylation levels and shifted SPAK expression and phosphorylation towards the full-length isoform. Estradiol administration to male rats induced a significant increase in NCC phosphorylation. NCC is also modulated by prolactin. Administration of this peptide hormone to male rats induced increased phosphorylation of NCC, an effect that was observed even using the ex vivo kidney perfusion strategy. Our results indicate that estradiol, progesterone, and prolactin, the hormones that are involved in sexual cycle, pregnancy and lactation, upregulate the activity of NCC.

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distal convoluted tubule; salt transport; thiazide; WNK; hypertension

THE SEXUAL FEMALE CYCLE, PREGNANCY, and lactation are physiological states unique to women in which volume retention is required. This can be achieved, at least in part, by decreasing urinary salt and volume lost through activation of the thiazide-sensitive Na^+-Cl^- cotransporter (NCC), which is expressed in the distal convoluted tubule (DCT) and represents a key step for NaCl reabsorption (10). It is known that NCC is subjected to sexual dimorphic regulation. Chen et al. (8) showed that the urinary response to thiazides, as well as binding of the thiazide-like diuretic [^{3}H]metolazone to renal cortical homogenates, was higher in female than in male rats and that these differences were reduced by ovariectomy. Verlander et al. (52) observed by immunogold electron microscopy that ovariectomy reduced the expression of NCC in DCT cells and that administration of 17β-estradiol to ovariectomized female rats restored NCC expression.

However, ovariectomy results in a concomitant decrease in estrogens, progesterone, and prolactin (PRL) (28). Although estrogen receptors are highly expressed in the kidney, their presence in the DCT has not been reported (13). In addition, previous studies also indicate a role for the peptide hormone PRL in the stimulation of NCC. PRL is a well-known osmoregulator in lower vertebrates (38). In teleosts, PRL increases salt and water reabsorption in the urinary bladder (20), an organ in which sodium reabsorption is mediated by NCC (16, 41, 50). In zebrafish, NCC expression in the gills is modulated by PRL (4). In mammals, PRL increases renal salt reabsorption in the distal nephron by a vasopressin-independent mechanism (49) and activates the Na^+-K^+-ATPase activity in DCT in a dose-dependent fashion (6). The expression of the PRL receptor in rat DCT has been reported (12, 30). In humans, excessive PRL caused by a pituitary PRL-secreting tumor, can produce renal salt retention (22) and hyperprolactinemia in patients,

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leading to lower urine volume and solute excretion compared with control subjects (5).

Here, we present evidence that NCC expression and phosphorylation are greater in female than in male rats, mice, and humans and that NCC phosphorylation is promoted by estrogens, progesterone, and PRL. Because it is known that NCC phosphorylation is a surrogate for increased NCC activity (34, 43), these observations suggest that the three female hormones contribute to increased NCC activity during physiological states unique to women.

METHODS

All experiments involving animals were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, DC, 1996) and were approved by the Animal Care and Use Committee at our institutions.

Animals. Adult female and male rats and mice were randomly assigned to sham surgery or to gonadectomy, and when indicated, they were treated with vehicle, estradiol, progesterone, or PRL. Kidneys of $PRLR^{-/-}$ and $PRLR^{+/+}$ female and male mice were obtained for Western blot analysis (32). Hormone serum levels were measured by specific radioimmunoassay in blood samples obtained the day of the euthanasia.

Diuretic response. To study the urinary response to bendroflumethiazide (BFTZ) in metabolic cages, acclimated male and female rats were treated with a single dose of an intraperitoneal (ip) injection of a 20% DMSO solution with or without BFTZ, and urine was collected during an extra 2-h period. Urine electrolytes were measured with a NOVA4 electrolyte analyzer (NOVA Biomedical, Waltham, MA), and creatinine was measured with an autoanalyzer (Beckman Instruments, Brea, CA).

Gonadectomy. Bilateral ovariectomy (or sham operation) was performed as follows. Rats were anesthetized with an intraperitoneal injection of 30 mg/kg pentobarbital sodium and then shaved over the dorsal lumbar region and cleaned with benzil followed by an alcohol rinse. A 2-cm skin incision along the dorsal midline and through the abdominal musculature was made; the ovaries were then exposed and removed. The sham operation was performed using the above steps without removal of the ovaries.

NCC protein expression analysis. Kidney protein extracts were homogenized using a lysis buffer containing 50 mM Tris·HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyro-phosphate, 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). Protein extracts (50 μg) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1.5 h in 5% (wt/vol) nonfat milk dissolved in Tris-buffered saline (TBS)-Tween 20. Antibodies were diluted in TBS-Tween 20 containing 5% (wt/vol) nonfat milk. Membranes were incubated with primary antibodies overnight at 4°C and with horseradish peroxidase (HRP)-coupled secondary antibodies at ambient temperature for 1.5 h. Immobilized antigens were detected by chemiluminescence using the Luminata Forte Western HRP substrate (Merck Millipore).

The following antibodies were polyclonal and raised in sheep: anti-NCC [recognizing residues 906–925 of human NCC (CHTKRFEDMIAPFRLNDGFKD) S965B], anti-SPAK [residues 196–210 of mouse (QSLSVHSDQAQPNAN), S150C], anti-phosphorylated NCC at threonine 58 [residues 54–66 of human (RTFGYNT*-IDVVPTRR), S995B], and anti-phosphorylated SPAK at serine 383 [residues (RRVPGS(P)SGRLHKT), S670B]. These antibodies were produced and validated at the Medical Research Council phosphorylation unit at Dundee University and given as a kind gift by Dr. Dario Alessi (39, 43).

Western blots in Figs. 1, *A* and *B*, 2*B*, 3*A*, and 4*A* were exposed to X-ray film, and the densitometries were made on a single exposure. The rest of the Western blot signals were detected and quantitated with a C-DiGit Blot Scanner (Li-COR) and accompanying software. Values were normalized to the mean intensity measured in the male, nongonadectomized or vehicle groups defined as 1.0. All comparisons were performed between samples run on the same blot/membrane.

Immunofluorescence microscopy. Mouse kidneys were used for this analysis with the following antibodies: polyclonal anti-NCC antibody raised in sheep (recognizing residues 906–925 of human NCC, "CHTKRFEDMIAPFRLNDGFKD", S965B) (39), polyclonal anti-GPER-1 antibody, recognizing a C-terminal peptide raised in rabbit (9), monoclonal anti-parvalbumin antibody raised in mouse (Swant, PV 235), donkey anti-rabbit Alexa-Fluor 488, donkey anti-mouse Alexa-Fluor 594, and donkey anti-sheep Alexa-Fluor 488 (Life Technologies).

Exosome extraction. To assess NCC expression/phosphorylation in humans, urinary exosomes were isolated as reported previously (36, 51). The amount of sample loaded during immunoblotting was normalized by the spot urinary creatinine concentration. Urinary samples from women were taken at *day 0* of the cycle, and none of them were using contraceptives.

Hormone treatment. At 12 wk of age, rats underwent an ovariectomy. Following a month of recovery after surgery, either 60 μg/kg 17β-estradiol (Sigma-Aldrich) or 20 mg/kg progesterone (Sigma-Aldrich), both dissolved in 10% ethanol and olive oil, were given intraperitoneally (ip) every day for 3 wk until euthanasia. The same procedures were performed with males treated with 17β -estradiol. For PRL treatment, eight male rats were randomized and injected ip with 25 μg/kg body wt or vehicle as a control group every day for 2 wk. PRL (murine recombinant, Sigma-Aldrich) was dissolved in water.

Ex vivo perfused rat kidney. The right kidney of male Wistar rats was mounted in the Langerdoff system as previously described (7, 33) and perfused with vehicle or PRL (murine recombinant, Sigma-Aldrich) at a rate of 0, 10, and 40 ng/ml, which had no effect on the perfusion pressure. After 30 min of perfusion, the kidney was manually separated into the cortex and medulla, and the corresponding fragments were frozen in liquid nitrogen.

Statistical analyses. Statistical significance was defined as two-tailed ($P < 0.05$), and results are presented as means ± SE. Statistical significance between male and female or intact and gonadectomized animals for two groups was determined by a nonpaired Student's *t*-test and for three or more groups by one-way ANOVA with Dunnett correction.

RESULTS

NCC expression/phosphorylation is higher in females than in males. We compared NCC expression and phosphorylation in female and male rats. As shown in Fig. 1*A*, NCC expression was similar between female and male rats, but NCC phosphorylation at T58 was significantly higher in females. Thus the pNCC/NCC ratio was higher in female rats. This is consistent with our observations and similar to that of Fanestil and coworkers (8), who found that the diuretic and natriuretic response to a single dose of BFTZ was higher in females than in males (Fig. 1*B*). Because thiazides inhibit the activity of NCC, these data suggest that the greater activity of NCC in female rats is due to greater phosphorylation of the cotransporter. We also observed differences in NCC expression/phosphorylation in mice and humans. In mice, in addition to the stimulation of NCC phosphorylation, NCC abundance was higher in females. Thus the pNCC/NCC ratio was similar (Fig. 1*C*). Using urinary exosome analysis by Western blotting, we

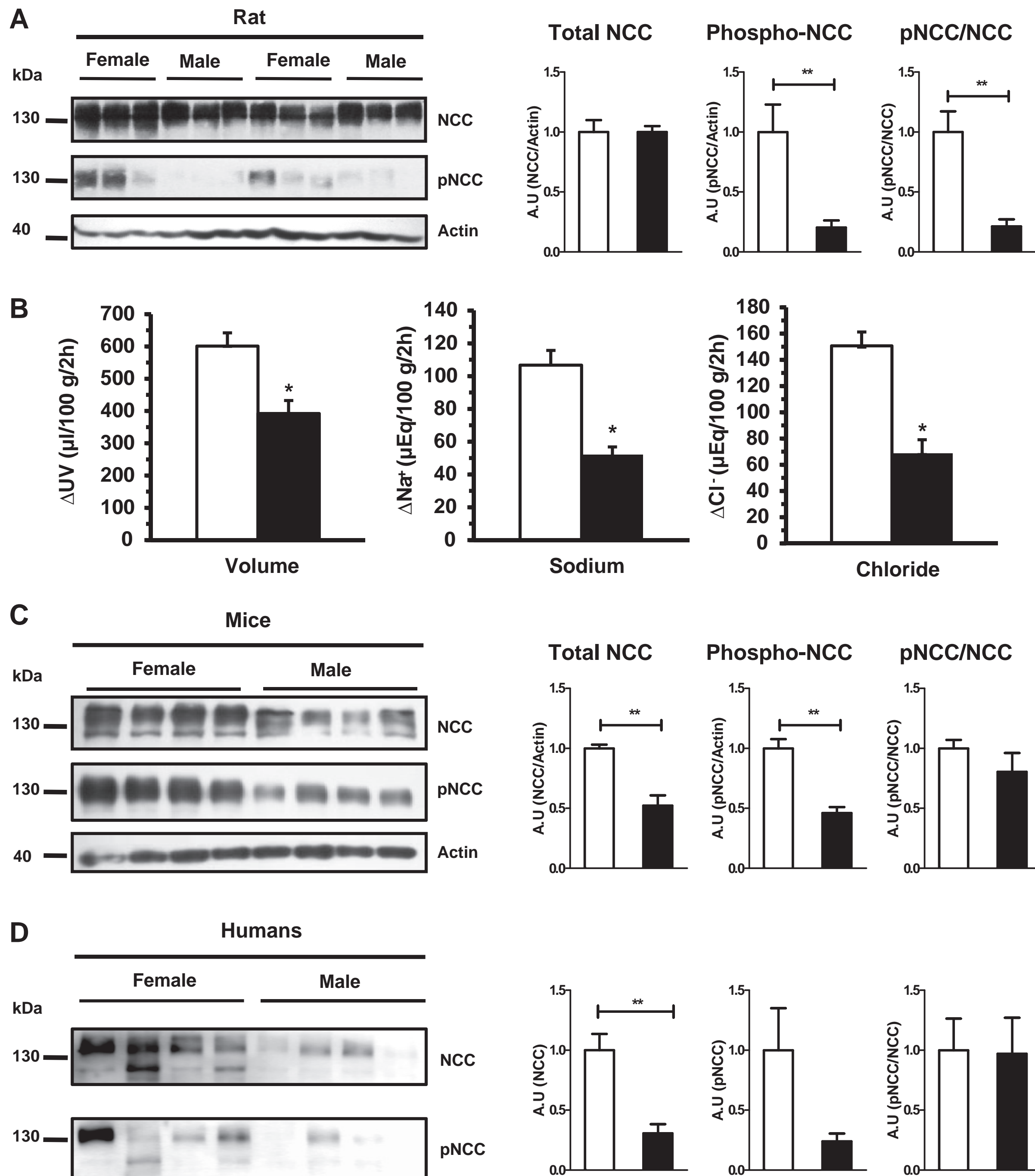

Fig. 1. Females have higher Na^+-Cl^- cotransporter (NCC) expression and phosphorylation. *A*: Western blot analysis of NCC expression and phosphorylation (T58) of female (open bars) and male (filled bars) rat proteins extracted from individual renal cortex. *B*: change in urinary volume, sodium, and chloride excretion 2 h before vs. 2 h after bendroflumethiazide (BFTZ) injection in female (open bars) and male (filled bars) rats. $^*P < 0.05$ vs. female rats; $n = 12$ rats/bar. *C*: Western blot analysis of NCC expression and phosphorylation (T58) of female and male mice total kidneys. *D*: human urine exosomes of women and men normalized to urine creatinine. The result of densitometric analysis is expressed as the fold of NCC over β-actin, pNCC (T58) over β-actin, and NCC phosphorylation overexpression. $^{**}P < 0.01$ in female rats; $n = 6$ (*A* and *B*). $^{**}P < 0.01$ in female mice; $n = 4$ (*C*). $^{**}P < 0.01$ in women; $n = 4$ (*D*).

also documented higher NCC expression and phosphorylation in humans (Fig. 1*D*).

Ovariectomy eliminates the NCC difference between female and male rats. To assess the role of the gonads in the observed NCC difference between female and male rats, we analyzed the effect of a gonadectomy on the NCC expression/phosphorylation and thiazide response. Our results show that ovariectomy reduced NCC phosphorylation in rats, whereas orquiectomy had no effect (Fig. 2, *A* and *B*). The efficiency of the gonadectomy was confirmed by the measurement of the corresponding hormone level. Estradiol was decreased by 96 and 75%, progesterone by 84 and 66%, and prolactin by 28 and 56% in males and females, respectively (Table 1). In addition, ovariectomy reduced the thiazide response in females, whereas

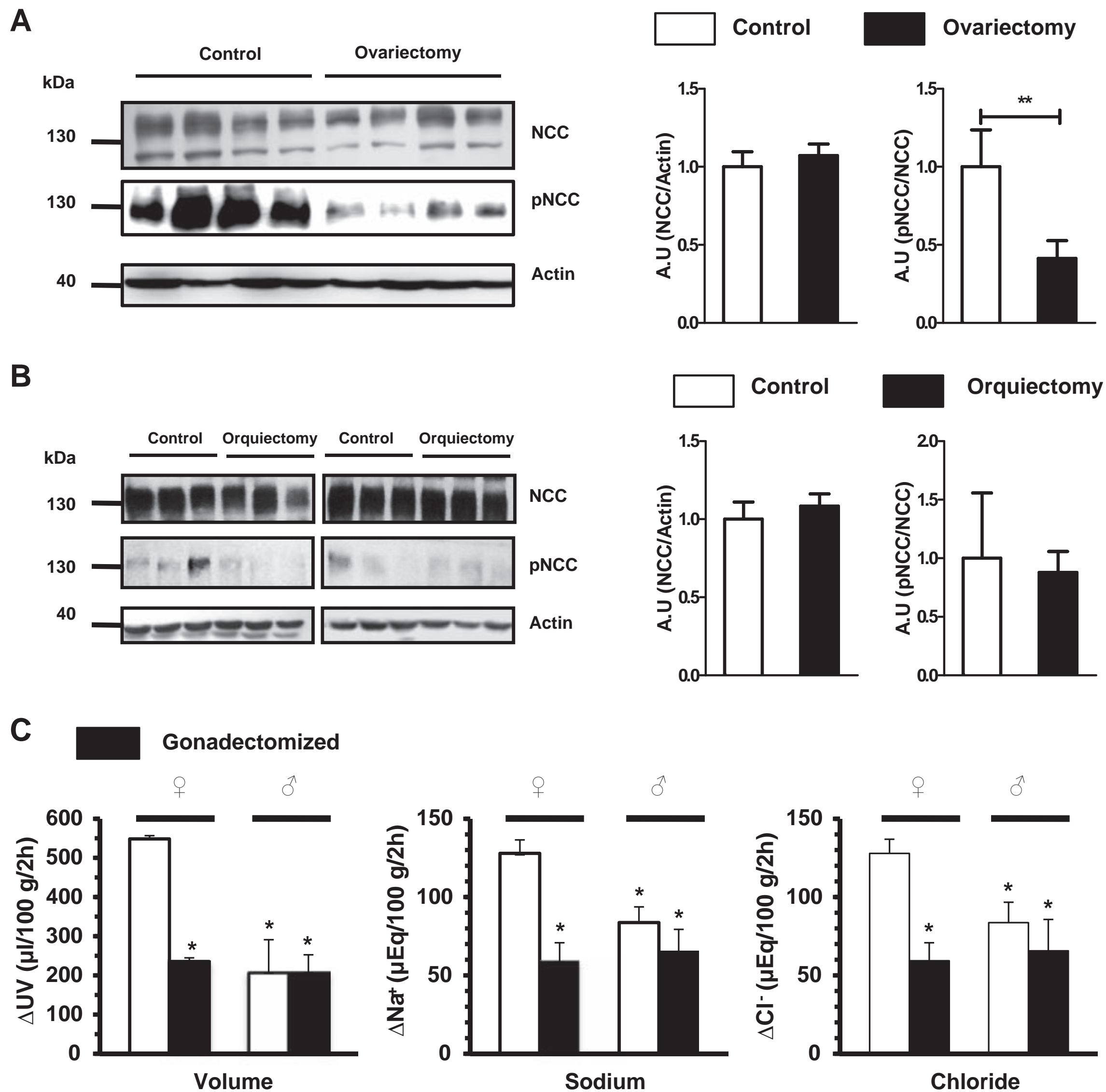

Fig. 2. Ovarian hormones regulate NCC phosphorylation. Western blot analysis of NCC expression and phosphorylation (T58) of control and ovariectomized (*A*) female rats and control and orquiectomized (*B*) male rats in proteins extracted from individual renal cortex; 2 different Western blots from different subjects are indicated by black outline. Also shown is densitometric analysis of total NCC is expressed as the fold of NCC over β-actin, and NCC phosphorylation as the fold of phosphorylated NCC over total NCC. $^{**}P < 0.01$ control vs. ovariectomized rats; $n = 6$ rats/bar. *C*: change (Δ) in urinary volume, sodium, and chloride excretion 2 h before vs. 2 h after BFTZ injection in intact (open bars) and gonadectomized (filled bars) male or female rats as stated. $^{*}P < 0.05$ vs. female rats; $n = 6$ rats/bar.

orquiectomy had no effect in males (Fig. 2*C*). These observations indicate that the sexual dimorphism observed for NCC is due to female hormones and is associated with the modulation of NCC expression/phosphorylation.

STE-20 proline-alanine-rich kinase expression and phosphorylation is higher in females. The kinase responsible for NCC activation by phosphorylation is STE-20 proline-alanine-rich kinase (SPAK) (34, 43). Therefore, we quantified the level of expression and phosphorylation of SPAK in male and female mice and rats. As shown in Fig. 3, both the expression and phosphorylation of SPAK in the kidney were higher in females than in males in both rodent species. No gender differences for OSR1 expression were observed in either mice or rats. These observations suggest that the responsible hormone for the NCC dimorphism is probably acting, at least in part, through the SPAK pathway.

Estradiol and progesterone promote NCC phosphorylation. Ovariectomy in rats significantly reduced NCC phosphorylation, suggesting that ovarian hormones are involved. By immunogold electron microscopy, 17β-estradiol administration was shown to promote increases in NCC presence in the apical membrane (52), but the effect on NCC phosphorylation is not known. In addition, the role of progesterone has not been assessed. We thus analyzed the effect of 17β-estradiol or progesterone administration on NCC expression and phosphorylation in ovariectomized rats. Estradiol or progesterone was injected daily for 3 wk. Hormone levels at the end of the steroid treatment are shown in Table 2. Consistent with observations in Fig. 1*A*, the NCC expression level was similar in the treated and control groups (Fig. 4*A*). In contrast, administration of either steroid induced a significant increase in NCC phosphorylation (Fig. 4*A*). The effect of 17β-estradiol was stronger

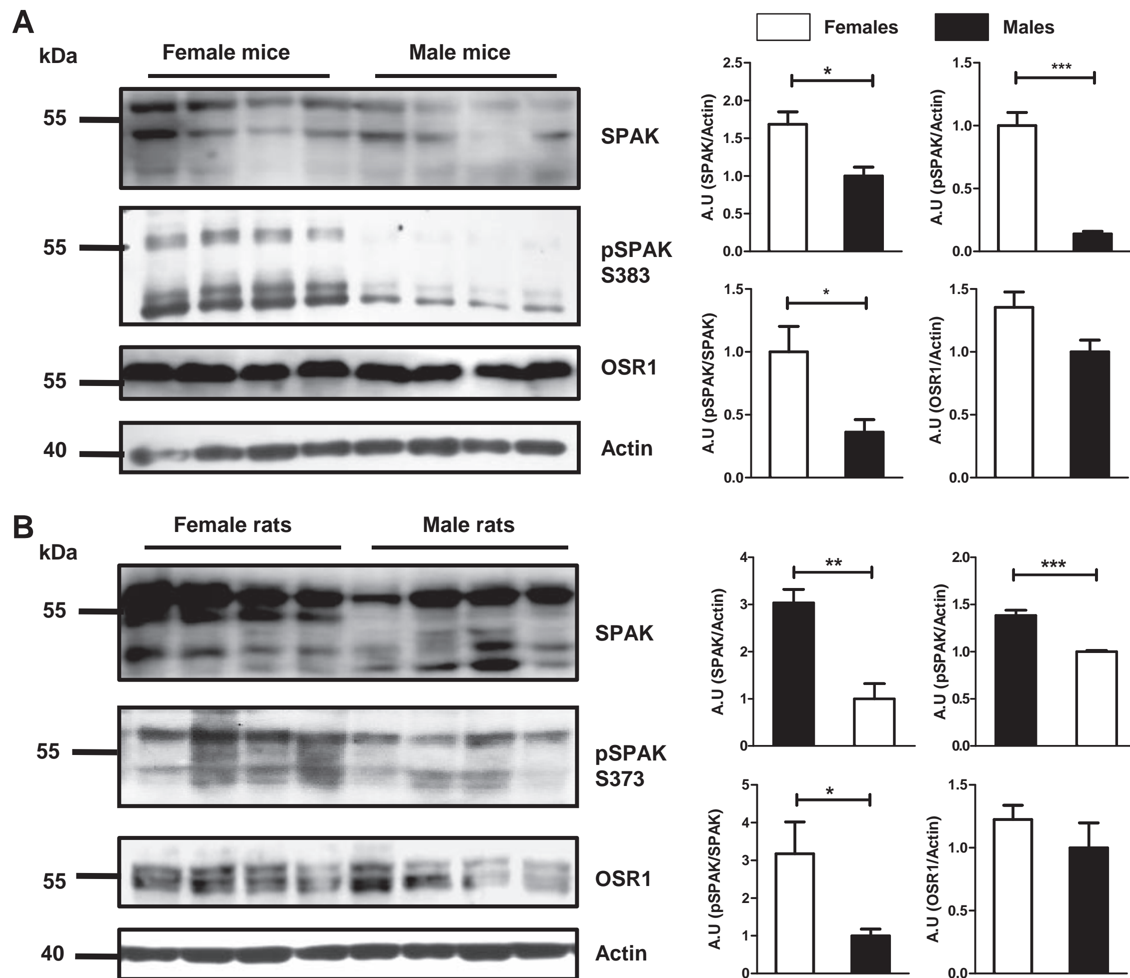

Fig. 3. Renal STE-20 proline-alanine-rich kinase (SPAK) is regulated by sexual hormones. Shown is Western blot analysis of SPAK expression and phosphorylation and OSR1 expression in control male and control female mice (*A*) and rats (*B*) in proteins extracted from whole kidney and cortex kidney, respectively. The results from densitometric analysis are expressed as the fold of total SPAK, phosphorylated SPAK, and phosphorylated SPAK over total SPAK. ***$P < 0.01$ vs. female mice. **$P < 0.01$ vs. female mice. *$P < 0.05$ vs. female mice; $n = 4$/bar.

than the effect of progesterone. We confirmed the estradiol activation/phosphorylation of NCC in male rats treated for 3 wk with the same 17β-estradiol dose as females. Hormone levels at the end of treatment are also shown in Table 2. As shown in Fig. 4*B*, estradiol administration in males is associated with increased NCC phosphorylation.

Ovarian steroids also had an effect not only on SPAK phosphorylation but also on SPAK isoform expression (Fig. 4*A*). In ovariectomized rats treated with vehicle, the most prominent band observed for SPAK and phospho-SPAK was ~45 kDa, corresponding to the SPAK2 isoform (29). The 17β-estradiol or progesterone treatment increased the abundance of the full-length SPAK (60 kDa) and a dramatic reduction in phospho-SPAK2. It is known that increased full-length SPAK together with a reduction in SPAK2 promotes NCC phosphorylation (29). Thus it is likely that ovarian steroids increased NCC phosphorylation, at least in part, through modulation of SPAK activity. We found no differences in aldosterone levels between any of the above groups: vehicle 424 ± 25 (pg/ml), ovariectomized+17β-estradiol 386 ± 32 (pg/ml), ovariectomized+progesterone 466 ± 43 (pg/ml), and sham 408 ± 59 (pg/ml).

Because the positive effect of 17β-estradiol and progesterone on NCC activity in rats is associated predominantly with increased NCC phosphorylation, rather than increased expression, it is possible that nongenomic effects are involved. Several nongenomic membrane-associated effects of classic estrogen receptors have been reported (47). A particular 33-kDa isoform of estrogen receptor α (ERα33) is highly expressed along the entire nephron (25). In addition, an estrogen 7-spanning membrane receptor, known as the G-coupled estrogen receptor 1 (GPER-1), has recently been shown to be expressed in the proximal tubule and the thick ascending limb of Henle's loop (9). Because the specific DCT localization was not shown in that study, we used the same antibody to assess the presence of GPER-1 in the basolateral membrane of DCT cells in the mammalian kidney. Figure 5 shows expression of GPER-1 at the basolateral membrane in a nephron segment that is also positive for NCC or parvalbumin, indicating that GPER-1 is expressed in DCT.

PRL promotes NCC phosphorylation. Several studies have suggested that PRL could also be involved in the regulation of NCC activity. We therefore quantified NCC expression and phosphorylation in female and male mice lacking the PRL

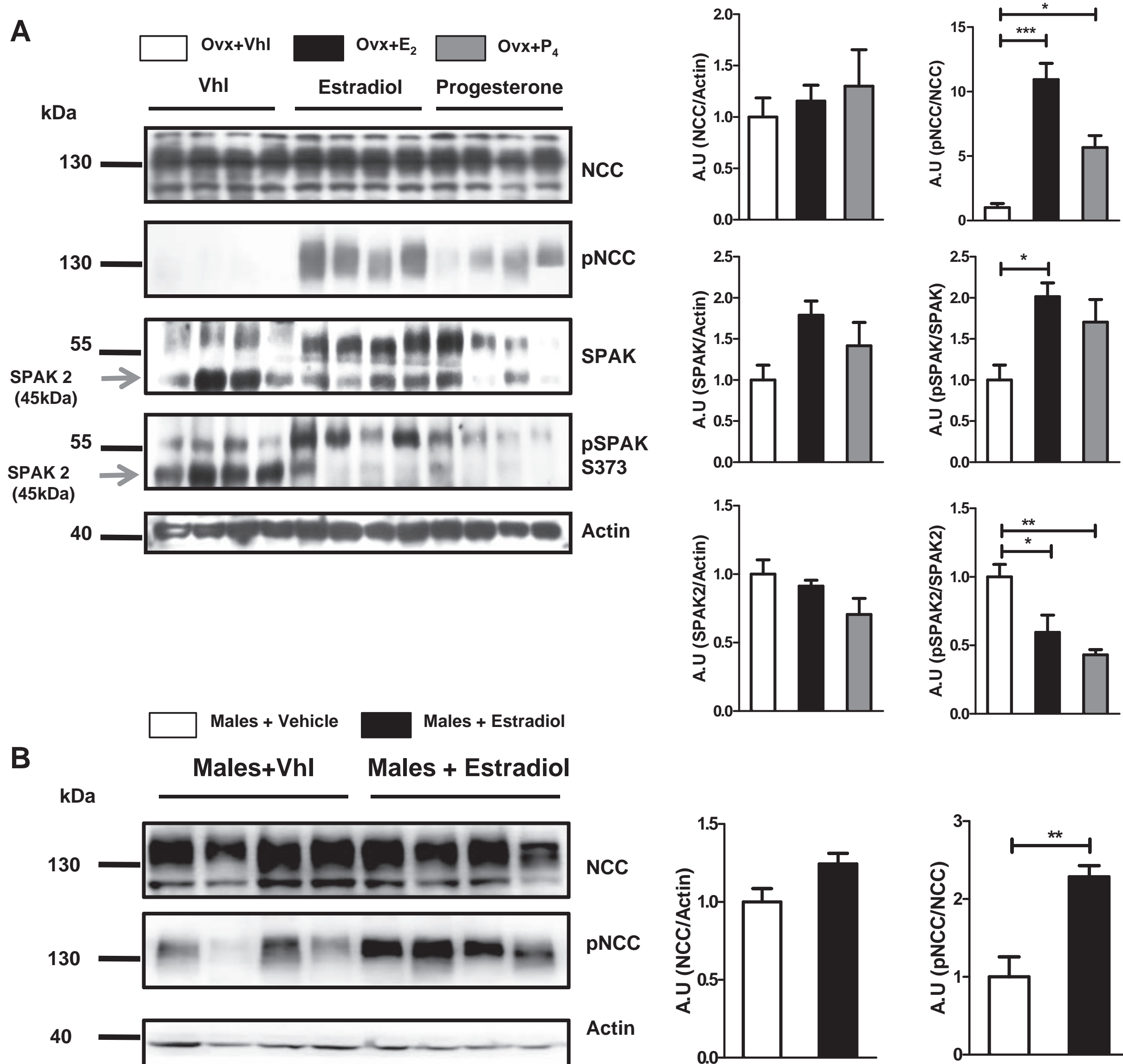

Fig. 4. Estrogens and progesterone phosphorylate NCC. *A*: Western blot analysis of NCC and SPAK expression and phosphorylation (NCC T58; SPAK S373) in ovariectomized rats treated with vehicle, 17-β-estradiol (60 μg/kg), or progesterone (100 mg/kg) for 3 wk. *B*: Western blot analysis of NCC expression and phosphorylation (NCC T58) in male rats treated with vehicle or 17-β-estradiol (60 μg/kg) for 3 wk. The results from densitometric analysis of total NCC are expressed as the fold of NCC and SPAK over β-actin and NCC and SPAK phosphorylation as the fold of phosphorylated NCC or SPAK over total NCC or SPAK, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle-treated rats; $n = 4$ rats per bar and $^{**}P < 0.01$ vs. vehicle rats; $N = 4$ rats per bar respectively.

receptor ($PRLR^{-/-}$) (3). Wild-type mice from the same colony were used as controls. The difference in NCC expression and phosphorylation between female and male mice persisted despite the presence or absence of the PRL receptors. In addition, the expression and phosphorylation of NCC were similar in $PRLR^{+/+}$ and $PRLR^{-/-}$ female mice (data not shown). We reasoned that the difference between female and male $PRLR^{-/-}$ mice persisted due to the presence of estrogens in females. The pNCC/NCC ratio tended to be lower in $PRLR^{-/-}$ male mice than in $PRLR^{+/+}$ males, but the difference did not reach significance ($P = 0.06$) (Fig. 6*A*), suggesting that prolactin may play a role in modulating NCC activity. However, the absence of the PRL effect was compensated, although not entirely. This hypothesis was tested using two different models. First, treatment of male rats with PRL for 2 wk resulted in a significant increase in the pNCC/NCC ratio (Fig. 6*B*), and second, PRL perfusion ex vivo (7, 33) resulted in a significant increase in the pNCC/NCC ratio, indicating that PRL is an activator of NCC (Fig. 6*C*).

DISCUSSION

It has been clearly demonstrated that NCC activation is associated with increased phosphorylation of certain threonine/serine residues of the amino-terminal domain, among which T58 (T60 in humans) is the key regulatory site (34, 43). The role of these sites has been corroborated in multiple in vitro and in vivo models (15). We thus analyzed the expression and phosphorylation status of NCC in female and male rats, mice, and humans. Our data show that phosphorylation of NCC was

Table 1. *Sex hormone levels in control and gonadectomized female and male rats*

	Male Control ($n = 6$)	Gnx Male ($n = 6$)	Female Control ($n = 5$)	Gnx Female ($n = 6$)
Body weight, g	443 ± 10	437 ± 12	292 ± 5	294 ± 1
Testosterone, ng/ml	2 ± 0.4	0.08 ± 0.01*	0.02 ± 0.002†	0.005 ± 0.001†
Estradiol, pg/ml	25 ± 2	4 ± 0.7*	50 ± 7	17 ± 1.8*
Prolactin, ng/ml	5 ± 0.1	3.7 ± 0.31‡	16 ± 1	7 ± 0.8*

Values are means ± SE. Gnx, gonadectomized. *$P < 0.05$ vs. same control same sex. †$P < 0.01$ vs. male control. ‡$P < 0.05$ vs. female control.

higher in female rats and mice. In rats, we did not see a difference in expression between female and male animals, but we observed an increased phosphorylation in the former. However, one study showed higher expression of NCC in females compared with male lean and obese Zucker rats (42). The difference could be strain dependent. In contrast, we did observe a difference between female and male NCC expression levels in mice and humans. The lack of difference in NCC expression levels in rats contrasted with the observation by Verlander et al. (52). In our study, we analyzed several rats and consistently found no difference in NCC expression levels between females and males (Fig. 1) or between control and ovariectomized rats (Figs. 2 and 4). The different strains of rats used (Sprague-Dawley and Wistar rats) and the different antibodies could account for this difference. However, our data show that phosphorylation of NCC was higher in female rats and mice, suggesting increased NCC activity in females.

We used urinary exosomes to quantify these parameters in humans. The analysis of urinary exosomes by Western blotting is a noninvasive strategy that allows for "molecular renal biopsies" through the quantification of the level of expression of certain proteins in the human kidney. Following the methodology developed by Knepper and coworkers (21, 36, 57), it is possible to detect a variety of proteins in urinary exosomes (17), including the apical membrane transporters (11). NCC was absent in urinary exosomes from Gitelman patients (26), whereas its abundance was increased in exosomes from patients with primary aldosteronism (51). Here, we show by comparing exosomes obtained from four young female and male subjects (aged 22–28) that there is a clear and significant increase in NCC abundance and phosphorylation in women. However, it is difficult to define with this methodology whether the primary increase in humans is at the NCC expression or phosphorylation level because exosomes mostly contain membrane proteins, where phosphorylated NCC is exclusively located (15). Phosphorylation of NCC prevents ubiquitination and thus prevents NCC retrieval from the membrane (23). Nevertheless, our data show that sexual dimorphic regulation of NCC occurs in humans.

Gonadectomy is a generally accepted tool for assessing the effect of gonadal steroids on dimorphic regulation of physiological processes. Here, we show that NCC phosphorylation in female rats is dramatically reduced 4 wk after the ovariectomy. No effect was observed after testis removal. These observations indicate that female gonadal hormones modulate the level of NCC phosphorylation. We substituted estradiol or progesterone in ovariectomized rats and observed that NCC phosphorylation is increased by administration of either steroid. Perhaps the role of progesterone is more relevant during pregnancy, in which this steroid is secreted in great amounts by the placenta. The major isoform of the classic estrogen receptor expressed in the kidney is the truncated ERα33 variant, known to be associated with the membrane and translate nongenomic effects (25, 40, 55). In addition, we observed that the G protein membrane receptor for estrogens (9, 14, 37) is heavily expressed in the basolateral membrane of DCT cells. Thus the estrogen effect on NCC could be occurring either by the classic genomic pathway or through a nongenomic membrane receptor-type effect through either the ERα33 or the GPER1 receptors. Further studies will be necessary to clarify the pathways involved.

It is known that the kinase responsible for most NCC phosphorylation in the DCT is SPAK (39, 43, 56), which in turn is known to be modulated by WNKs (53). In the kidney, in addition to full-length SPAK, several short forms resulting from proteolytic cleavage are present (27), two of which are known as SPAK-2, truncated in the amino-terminal domain, and a kidney-specific variant known as KS-SPAK, which lacks most of the kinase domain (29). These shorter forms are more apparent in physiological conditions associated with decreased NCC activity, while their present form is decreased when NCC requires activation, for instance during a low-salt diet or angiotensin II infusion (18, 29, 44). Thus the shorter forms are believed to function as dominant negative SPAK forms. In this regard, we observed that expression and phosphorylation of SPAK were higher in females than in males in both rats and mice. The higher expression of SPAK in females is eliminated by ovariectomy. In addition, the predominant expressed and phosphorylated SPAK isoform in this situation is SPAK2, whereas estradiol or progesterone promotes the expression and phosphorylation of the full-length SPAK isoform. These observations suggest that the positive effect of female hormones on NCC expression/phosphorylation is mediated, at least in part, through SPAK. Supporting these observations, a recent study showed that estradiol upregulates SPAK in the developing hypothalamus, thereby stimulating the activity of the Na^+-K^+-$2Cl^-$ cotransporter NKCC1 (31).

The increased activity/expression of NCC in females might sound counterintuitive given that blood pressure is not higher in female than in males. However, it is well known that estrogens and female hormones possess a potent vasorelaxing effect; by preventing the increase in blood pressure usually associated with salt retention, these hormones preclude the occurrence of pressure-natriuresis (1, 2, 19, 55), which could potentially explain the frequent complaint that the volume

Table 2. *Plasma estradiol and progesterone concentration in ovariectomized rats and males treated with vehicle, 17-β-estradiol, and progesterone*

	E_2, pg/ml	P_4, ng/ml
Ovx+vehicle	28 ± 6	11 ± 3
Ovx+estradiol	142 ± 40***	10 ± 1.6
Ovx+progesterone	33 ± 8	91 ± 6***
Male+vehicle	30 ± 1	16 ± 6
Male+estradiol	182 ± 19***	8 ± 3

Values are means ± SE. Ove, ovariectomized. ***$P < 0.001$ vs. vehicle-treated rats; $n = 6$ rats. ***$P < 0.001$ vs. vehicle-treated rats; $n = 4$ rats.

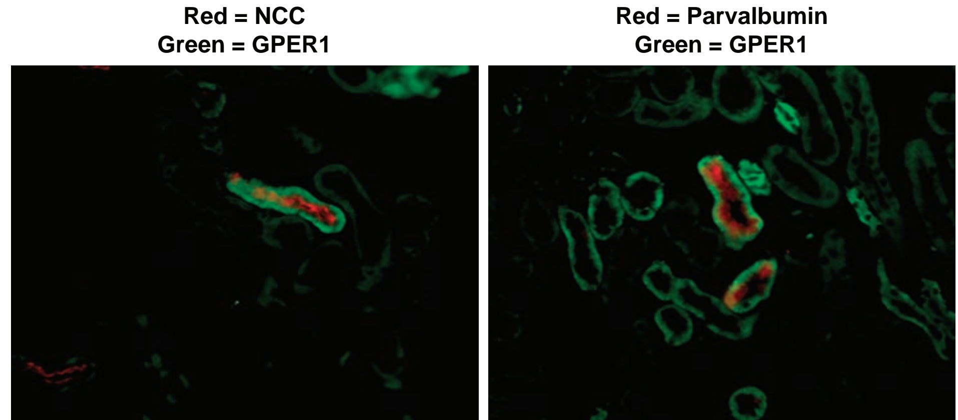

Fig. 5. G protein estrogen membrane receptor 1 (GPER-1) is expressed in the distal convoluted tubule (DCT). Distribution pattern between GPER-1, NCC, and parvalbumin is shown. Paraffin-embedded mouse kidney sections were immunostained for GPER-1 and NCC, and parvalbumin as a marker of DCT. GPER-1 and NCC were visualized with goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 594, respectively. Note: GPER-1 is localized to the basolateral membrane of epithelial cells in which NCC is apical and parvalbumin is intracellular.

retention associated with female hormones is often accompanied by edema formation (35, 48, 54).

Studies in ovariectomized females, with or without estradiol administration, cannot rule out the participation of PRL in the regulation of NCC function or expression, because its secretion is stimulated by estradiol (24, 28, 45, 46). Female rats exhibit higher levels of PRL in the blood compared with those in male rats. Ovariectomy reduces both estrogens and PRL serum levels (Table 1) (28). It is also known that PRL is an osmoregulator in lower vertebrates (20). This hormone increases salt and water reabsorption in teleost urinary bladders in which NCC mediates reabsorption (16, 50). We thus analyzed NCC expression and phosphorylation of NCC in kidneys from wild-type and PRL receptor knockout mice to compare the effects of PRL without changing the pituitary-hypothalamus axis. Female and male PRLR KO mice exhibited the gender regulation of NCC, but the presence of estrogens could be responsible for this difference. However, the positive effect of PRL was corroborated in rats treated with PRL and by using an ex vivo kidney perfusion system. Thus, consistent with the proposed role of PRL in the osmoregulation in teleosts and as a salt-retaining hormone in mammals, this peptide hormone is a positive regulator of NCC phosphorylation. The use of the ex vivo kidney perfusion system excludes the possibility that PRL administration increased NCC phosphorylation due to activation of extrarenal signals.

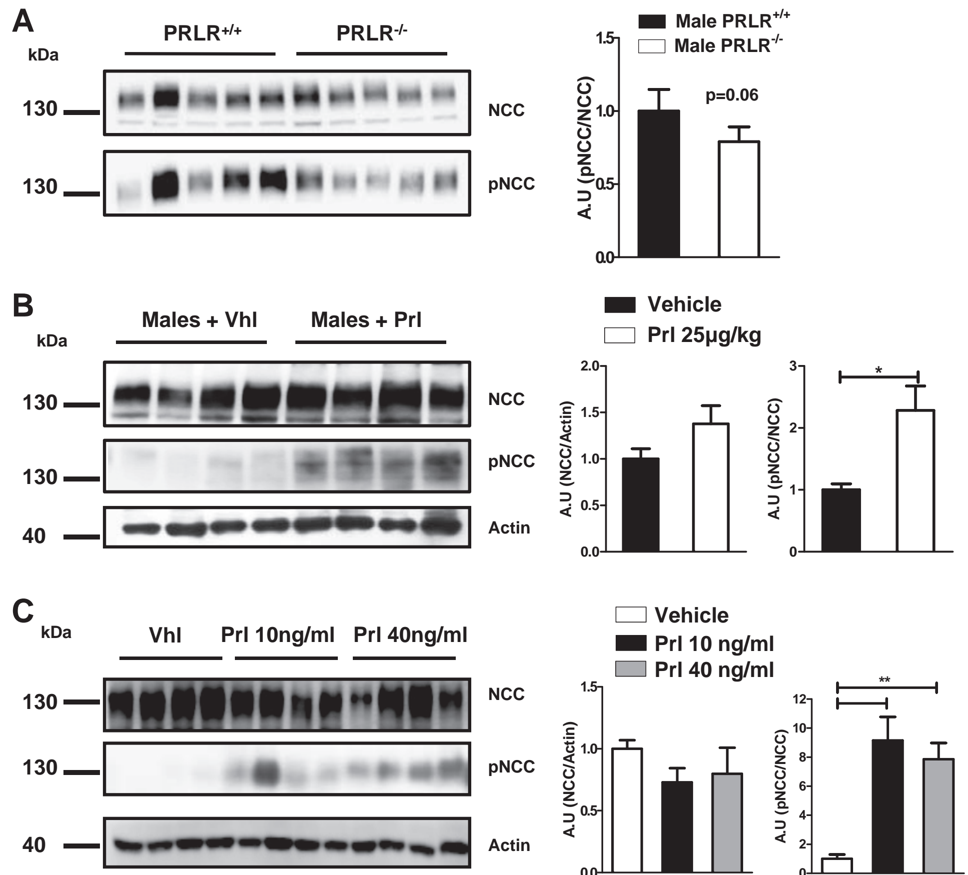

Fig. 6. NCC phosphorylation is modulated by prolactin (PRL). *A*: representative Western blot of NCC expression and phosphorylation in wild-type and PRL receptor knockout ($PRLR^{-/-}$) male mice. The results from densitometric analysis of total NCC are expressed as the fold of NCC over β-actin, and NCC phosphorylation as the fold of pNCC over total NCC. $^{*}P = 0.06$ vs. male control mice; $n = 4$–5 mice/bar. *B*: Western blot of NCC expression and phosphorylation in proteins extracted from individual renal cortex of male rats treated with 25 μg/kg PRL for 2 wk. The results from densitometric analysis of total NCC are expressed as the fold of NCC over β-actin, and NCC phosphorylation as the fold of pNCC over total NCC. $^{*}P < 0.05$ vs. vehicle-treated rats; $n = 4$ rats/bar. *C*: Western blot analysis of NCC expression and phosphorylation in kidneys from male rats perfused ex vivo with vehicle or with PRL at 10 or 40 ng/ml. The results from densitometric analysis of total NCC are expressed as the fold of NCC over β-actin, and NCC phosphorylation as the fold of pNCC over total NCC. $^{**}P < 0.01$ vs. vehicle-treated rats; $n = 4$ rats/bar.

The present study shows that physiological and molecular sex dimorphic regulation of NCC activity and expression are due to female hormones such as estrogens, progesterone, and PRL. It is known that periods in female life in which secretion of these hormones is increased are associated with the requirement of volume retention and expansion. For instance, women at the end of pregnancy had gained several kilograms due to the weight of the fetus, the placenta, the amniotic fluid, and the increased circulating blood. All of these contain considerable amounts of salt and water. In lactation, women lose several milliliters of water a day in milk production that must be promoting a salt-retaining state (54). At the end of the luteal phase, after several days of increased secretion of estrogens and progesterone, women often report the feeling of edema. Further studies will be interesting to pursue to specifically assess the role of NCC activation in each of these unique situations to female physiology.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

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Increased phosphorylation of the renal Na^{+}-Cl^{-} cotransporter in male kidney transplant recipient patients with hypertension: a prospective cohort

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Rojas-Vega L, Jiménez-Vega AR, Bazúa-Valenti S, Arroyo-Garza I, Jiménez JV, Gómez-Ocádiz R, Carrillo-Pérez DL, Moreno E, Morales-Buenrostro LE, Alberú J, Gamba G. Increased phosphorylation of the renal Na^{+}-Cl^{-} cotransporter in male kidney transplant recipient patients with hypertension: a prospective cohort. *Am J Physiol Renal Physiol* 309: F836–F842, 2015. First published September 2, 2015; doi:10.1152/ajprenal.00326.2015.—Evidence in rodents suggests that tacrolimus-induced posttransplant hypertension is due to upregulation of the thiazide-sensitive Na^{+}-Cl^{-} cotransporter NCC. Here, we analyzed whether a similar mechanism is involved in posttransplant hypertension in humans. From January 2013 to June 2014, all adult kidney transplant recipients receiving a kidney allograft were enrolled in a prospective cohort study. All patients received tacrolimus as part of the immunosuppressive therapy. Six months after surgery, we assessed general clinical and laboratory variables, tacrolimus trough blood levels, and ambulatory 24-h blood pressure monitoring. Urinary exosomes were extracted to perform Western blot analysis using total and phospho-NCC antibodies. A total of 52 patients, including 17 women and 35 men, were followed. At 6 mo after transplantation, of the 35 men, 17 developed hypertension and 18 remained normotensive, while high blood pressure was observed in only 3 of 17 women. The hypertensive patients were significantly older than the normotensive group; however, there were no significant differences in body weight, history of acute rejection, renal function, and tacrolimus trough levels. In urinary exosomes, hypertensive patients showed higher NCC expression (1.7 ± 0.19) than normotensive (1 ± 0.13) (P = 0.0096). Also, NCC phosphorylation levels were significantly higher in the hypertensive patients (1.57 ± 0.16 vs. 1 ± 0.07; P = 0.0049). Our data show that there is a positive correlation between NCC expression/phosphorylation in urinary exosomes and the development of hypertension in posttransplant male patients treated with tacrolimus. Our results are consistent with the hypothesis that NCC activation plays a major role in tacrolimus-induced hypertension.

salt transport; renal transplantation; diuretics; calcineurin inhibitors; tacrolimus

OF THE DIFFERENT MODALITIES for managing end-stage renal disease (ESRD), renal transplantation is the best treatment for the majority of patients (25, 32). However, there are various acute and chronic complications. Cardiovascular mortality risk in kidney transplant recipients (KTR) is higher than in the general population (7, 18), which could in part be due to arterial hypertension, a prevalent comorbidity in KTR (30) and a well-known risk factor for cardiovascular disease (13, 14).

The development of hypertension in KTR is multifactorial (27). Since the introduction of calcineurin inhibitors (CNIs), the acute rejection rate has decreased and the prevalence of posttransplant hypertension has significantly increased (22, 27, 29). The pathophysiological mechanism of CNI-induced hypertension was thought to be related to hemodynamic vascular effects (8); however, recent evidence in vitro and in vivo has suggested that it is associated with increased expression, phosphorylation, and therefore, NCC activity (9, 17).

NCC is the major salt reabsorption pathway in the distal convoluted tubule (DCT), particularly in the early DCT. Its role in blood pressure regulation has been clearly established by mutations in human disease affecting blood pressure regulation. Inactivating mutations of NCC result in Gitelman syndrome, which is characterized by arterial hypotension, hypokalemia, metabolic alkalosis, and hypocalciuria (24) (OMIM 263800), while NCC activation by mutant with- no-lysine kinases 1 and 4 (WNK1 and WNK4) (31) and ubiquitin-ligase complex-forming proteins Kelch3 and Cul3 (1, 11) causes pseudohypoaldosteronism type II (PHAII, OMIM 145260) featuring hypertension with hyperkalemia, metabolic acidosis, and hypercalciuria (16). It has been recognized that tacrolimus-induced hypertension may in some cases recapitulate a PHAII phenotype (9), suggesting that tacrolimus-induced NCC activation could be involved in posttransplant hypertension.

The isolation of urinary exosomes together with the availability of antibodies for studying the expression and activity of NCC (19, 21) have raised the possibility of studying NCC status in human urine. Exosomes are vesicles destined to fuse with the apical plasma membrane of the epithelial cells lining the lumen of the tubule (5, 20, 33) and are secreted towards the lumen. Exosomes can be obtained from the urine for performing Western blot analysis of apical tubular proteins. It has been shown that NCC expression is increased in exosomes from patients with primary aldosteronism and PHAII (15, 28) and decreased in Gitelman syndrome (4). Additionally, a higher expression of NCC documented in female compared with male rodents has been observed in urinary exosomes between healthy women and men (23).

Hoorn et al. (9) observed in mice that tacrolimus administration is associated with increased blood pressure (BP), accompanied by reduced urinary salt excretion and increased activity of NCC. It is not known whether in humans after renal transplantation in which several different factors may affect

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BP, this mechanism could be of relevance. However, if NCC expression and activity are increased in hypertensive patients treated with tacrolimus, the use of thiazides might offer an additional benefit for managing this condition. Thus we conducted a prospective cohort study to define the prevalence and risk factors for the development of posttransplant hypertension in patients treated with tacrolimus and the status of NCC expression/phosphorylation in hypertensive vs. normotensive patients in this cohort.

MATERIALS AND METHODS

Study population. Between January 2013 and June 2014, 62 renal transplant patients were enrolled to participate in this study. Our Institutional Review Board approved this study. All patients provided their signed informed consent to be included in the study. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul on Organ Trafficking and Transplant Tourism. In our institution, all the KTR received tacrolimus, mycophenolate mofetil, and steroids as immunosuppressive therapy. At the time of study admission, the general clinical variables recorded were age, sex, time, and cause of ESRD, arterial hypertension before transplantation, and type of donor.

The patients were evaluated 1, 3, and 6 mo after kidney transplantation. General clinical and laboratory data were collected, including BP measurements, antihypertensive therapy use, acute rejection history, and immunosuppressive therapy changes. At 6 mo, a urine sample was collected between 1200 and 1400 for urinary exosome extraction.

BP measurements. Before transplantation, BP was measured three times at the clinic. The patients were considered to be hypertensive if BP measurements exceeded 140/90 mmHg or if they had received antihypertensive medication. Twenty-four-hour ambulatory BP monitoring (ABPM) was recorded (adapt cuff monitor, ABPM 6100, Welch Allyn, Skaneateles Falls, NY) at 6 mo after transplantation. BP measurement was performed at 15-min intervals during the day and at 30-min intervals during the night; the maximum insufflation was 20 mmHg above the basal systolic BP. Additionally, office BP was recorded following European guidelines (12).

Blood and urine analyses. Serum sodium, potassium, urea, creatinine, tacrolimus trough levels, lipid profile (total cholesterol, triglycerides, HDL cholesterol, and LDL cholesterol), and glucose levels were determined using standard analytic methods. Sodium and protein levels were measured in a 24-h urine sample.

Isolation of urinary exosomes. A sample of 30 ml of urine was collected for each patient and stored immediately at −80°C with 1× protease inhibitors (protease inhibitor cocktail set V, EDTA-Free, Calbiochem, San Diego, CA).

Urinary exosomes were isolated as previously reported (20). The urine samples were thawed and centrifuged at 17,000 *g* for 15 min at 4°C to remove entire cell membranes and other high-density particles. *Supernatant 1* was held aside, and *pellet 1* was incubated with dithiothreitol for 5 min at 37°C to disrupt the Tamm-Horsfall polymeric network; the pellet was manually resuspended and centrifuged for 10 min at 17,000 *g*. *Supernatant 2* was mixed with *supernatant 1*, and together they were subjected to ultracentrifugation at 200,000 *g* for 2 h at 4°C.

The pellet that formed during the ultracentrifugation was resuspended in lysis buffer containing 50 mM Tris·HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyro-phosphate, 1 mM sodium orthovanadate, 1% (wt/vol) Nonidet P-40, 0.27 M sucrose, 0.1% (vol/vol) 2-mercaptoethanol, and protease inhibitors (complete inhibitors 20 tablets, Roche, Mannheim, Germany).

Immunoblot analysis. Gel loading of urinary exosome sodium transporters was normalized by spot urinary creatinine concentration. Thus a volume equivalent to the same creatinine concentration was used for each patient. This normalization allows us to compare subjects with different urinary flow rates.

The creatinine-normalized samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1.5 h in 5% (wt/vol) nonfat milk dissolved in Tris-buffered saline (TBS)-Tween 20. The antibodies were diluted in the same blocking solution. The membranes were incubated overnight with primary antibodies at 4°C and horseradish peroxidase (HRP)-coupled secondary antibodies at ambient temperature for 1.5 h. Immobilized antigens were detected using chemiluminescence (Luminata Forte Western HRP substrate, Merck Millipore, Billerica, MA).

The following polyclonal antibodies raised in sheep were used: anti-NCC (recognizing residues 906–925 of human NCC, CHT-KRFEDMIAPFRLNDGFKD, S965B) and anti-phosphorylated NCC at threonine 46, 50, and 55 (S990B). These antibodies were produced and validated at the Medical Research Council Phosphorylation and Ubiquitylation Unit at Dundee University and were kindly donated by Dr. Dario Alessi (3, 4, 21).

Western blots were detected and quantified using a C-DiGit Blot Scanner (Li-COR Biosciences, Lincoln, NE) and accompanying software. The values were normalized to the mean intensity, defined as 1.0, and measured in the normotensive patients. All of the comparisons were performed between samples run on the same blot/membrane.

Data presentation and statistical analysis. NCC and pNCC levels were quantified using densitometric analysis of the immunoblot bands and ImageStudio lite (Li-COR Biosciences). We compared the levels of NCC and phosphorylated NCC urinary exosomal excretion for each subject. The results were expressed as arbitrary units.

Categorical variables are shown as frequencies and percentages. The quantitative data are presented as means ± SD for clinical data and means ± SE for densitometric analysis. Statistical comparisons were performed using χ^2 for categorical variables and unpaired *t*-tests for quantitative variables. Multigroup comparisons were performed using one-way ANOVA followed by a post hoc test. A two-tailed $P < 0.05$ was considered to be statistically significant.

RESULTS

Cohort characteristics. Of the 62 patients enrolled in the study, 1 subject did not meet the inclusion criteria. Nine patients did not complete the study because of the following reasons: one died, one suspended tacrolimus treatment, four lost the renal allograft, and three were lost after follow-up. Thus a total of 52 patients, including 17 women and 35 men, remained in the study and were followed at 1, 3, and 6 mo after transplantation (Fig. 1). Table 1 shows the general data of the 62 cohort patients before transplantation. Although the mean age was slightly higher in men than in women, the difference was not significant. The frequency of pretransplant hypertension, sources of allograft, donor age, and gender, as well as the systolic, diastolic, and mean BP levels, were similar between women and men, suggesting that the cohort was homogeneous. All of the kidney recipients were treated using tacrolimus as part of the immunosuppressive therapy. One and three months after transplantation, only two and six patients, respectively, showed evidence of hypertension in the office.

Six months after transplantation, the patients were divided into hypertensive and normotensive groups according to office BP measurement and/or ABPM, a noninvasive 24-h ambulatory blood pressure monitoring system that avoids office measurement variability. Interestingly, of the 17 women enrolled and monitored for 6 mo, only 3 (∼17%) developed hyperten-

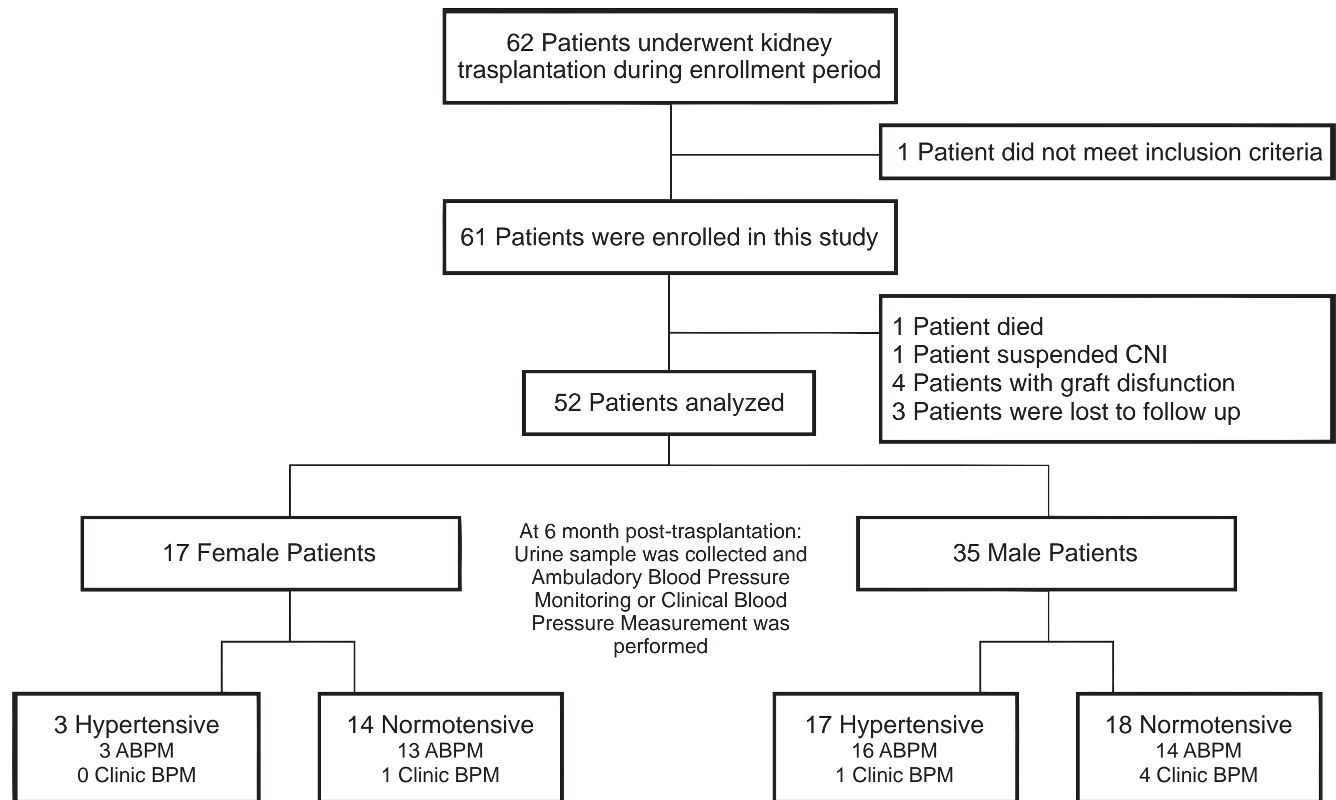


Fig. 1. Flowchart of patients enrolled in the study. CNI, calcineurin inhibitors; ABPM, ambulatory blood pressure monitoring; BPM, blood pressure measurement.

sion detected by ABPM, whereas 14 remained normotensive. In contrast, of the 35 men, 17 (~50%) developed hypertension and 18 remained normotensive. We excluded the female population from the data analysis because the percentage of hypertensive women was low compared with the male population. In addition, we have previously shown that NCC expression/phosphorylation in urinary exosomes is higher in women than in men (23). Thus mixing gender for this analysis is not appropriate.

The general data and baseline characteristics of the male cohort were divided into hypertensive and normotensive groups (Table 2). The average age of the KTR was significantly higher in the patients who developed hypertension (46.9 ± 14.1 yr) than in the normotensive patients (30.4 ± 9.7 yr). Two of the 17 patients with hypertension were diagnosed as such due to the continuous requirement of antihypertensive medication, amlodipine in one case and metoprolol in the other. None of the hypertensive patients were treated with thiazides. Table 3 shows the data for every patient in the male cohort 6 mo after renal transplantation. The etiology of the chronic kidney disease that led to renal transplantation for normotensive patients was multifactorial, without the preponderance of any particular disease. This observation was similar for patients who developed hypertension, except for a slightly higher prevalence of

Table 1. *General cohort characteristics of the 62 patients enrolled in the study*

	Total	Men	Women	
	n = 62 (100 %)	*n* = 35 (56.4 %)	*n* = 27 (43.5 %)	*P* Value
Recipient age, yr	36.7 ± 14.1	46.9 ± 1	38.7 ± 15.3	0.21
CKD etiology (100%)				
Diabetes mellitus	11 (14.5%)	9 (25.7%)	2 (7.4%)	0.06
Unknown	30 (48.3%)	14 (40%)	16 (59.2%)	0.07
Others	21 (33.8%)	12 (34.2%)	9 (33.3%)	0.93
Living donor	30 (48.3%)	19 (54.2%)	11 (40.7%)	0.19
Donor age	37.2 ± 11.6	36 ± 10	38 ± 13.1	0.21
Donor male	27 (43.5%)	14 (40%)	13 (48.1%)	0.52
Pretransplant hypertension	37 (59.6%)	23 (65.7%)	14 (51.8%)	0.26
Patients with acute rejection	22 (35.4%)	10 (28.5%)	12 (44.4%)	0.19
SBP, mmHg	128.9 ± 15.6	130.4 ± 13.7	126.6 ± 18.6	0.22
DBP, mmHg	79.5 ± 11.2	80.5 ± 10.5	78 ± 12.5	0.72
MBP, mmHg	93.7 ± 18.7	97.1 ± 10.9	87.9 ± 26.9	0.12

Values are means ± SD. The table shows the population divided into male and female patients. CKD, chronic kidney disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure.

Table 2. *General male cohort characteristics*

	Total Male Patients, n = 35 (100%)	Hypertensive, n = 17 (48.5%)	Normotensive, n = 18 (51.3%)	P Value
Recipient age, yr	37.7 ± 14.3	46.9 ± 14.0	30.4 ± 9.7	0.001
CKD etiology (100%)				
Diabetes mellitus	8 (22.8%)	7 (41%)	2 (11%)	0.04
Unknown	14 (40%)	5 (29.4%)	9 (50%)	0.21
Others	12 (34.2%)	5 (29.4%)	7 (39%)	0.55
Living donor	19 (54.2%)	7 (43.7%)	12 (60%)	0.412
Donor age, yr	36.6 ± 12.5	38.7 ± 11	34.7 ± 14	0.385
BMI, kg/m^2	23.13 ± 3.1	24 ± 3	22.4 ± 3.1	0.698
Pretransplant HTA	21 (60%)	12 (75%)	9 (45%)	0.169
Patients with acute rejection	10 (28.5%)	6 (37.5%)	4 (20%)	0.444
Tacrolimus trough levels, ng/ml	9.5 ± 3.3	9.5 ± 4.3	9.4 ± 1.3	0.914
Serum creatinine, mg/dl	1.4 ± 0.3	1.39 ± 0.33	1.4 ± 0.3	0.937
Serum Na^+, meq/l	140.5 ± 0.5	141 ± 3	139.4 ± 1.6	0.078
Serum K^+, meq/l	4.4 ± 0.08	4.4 ± 0.5	4.5 ± 0.3	0.474
Urinary Na^+, meq/l	75.6 ± 11.5	71.8 ± 54.1	81.5 ± 60	0.810
Fractional Na^+ excretion	1.2 ± 0.9	1.3 ± 1	1 ± 0.6	0.624
Arterial blood pressure				
24-h SBP (mmHg)	121.7 ± 12.08	128.4 ± 12.6	114.4 ± 5	0.0005
24-h DBP (mmHg)	72.3 ± 6	74.7 ± 6.6	69.57 ± 3.8	0.013
24-h MBP (mmHg)	88.2 ± 7.2	91.5 ± 8.1	84.4 ± 3.6	0.004
Daytime SBP, mmHg	122.7 ± 12.6	129 ± 13.8	115.4 ± 5.7	0.001
Daytime DBP, mmHg	73.2 ± 6.9	75.3 ± 8.3	70.9 ± 3.8	0.073
Daytime MBP, mmHg	89.1 ± 7.7	92 ± 9	85.7 ± 3.8	0.026
Nighttime SBP, mmHg	118.4 ± 15	127.5 ± 13.5	107.9 ± 8.5	0.0001
Nighttime, DBP mmHg	67.5 ± 7.8	71.3 ± 6.7	63.07 ± 6.6	0.002
Nighttime MBP, mmHg	83.9 ± 9.1	89.1 ± 7.6	78 ± 7	0.0003

Values are means ± SD. The table shows the male population divided into hypertensive and normotensive patient data. BMI, body mass index; HTA, hypertension; Na, K, and creatinine are serum levels.

diabetes mellitus (P = 0.04). Notably, there were no significant changes in the donor characteristics, such as body weight, and presence of hypertension previous to transplant surgery, which strongly suggests that the pathophysiological mechanism underlying the development of hypertension lies in the posttransplant period. Six months after transplantation, the body mass index, history of acute allograft rejection, serum creatinine, tacrolimus level, and electrolytes were similar between hypertensive and normotensive men.

Effect of tacrolimus on the BP of posttransplant patients. The systolic, diastolic, and mean arterial pressures (MAPs) using 24-h total BP, nighttime total BP, and daytime total BP measure by ABPM in hypertensive and normotensive male patients 6 mo after transplantation surgery are shown in Table 1. Except for the daytime diastolic BP, all the BP measurements were significantly different between normotensive and hypertensive men. Five patients were classified as hypertensive because of their medication use.

Table 3. *Individual male cohort data*

Hypertensive Patients, n = 17								Normotensive Patients, n = 18							
ID	Age, yr	CKD Origin	BMI, kg/m^2	Na, meq/l	K, meq/l	Creatinine, mg/dl	Tacrolimus levels, ng/ml	ID	Age, yr	CKD Origin	BMI, kg/m^2	Na, meq/l	K, meq/l	Creatinine, mg/dl	Tacrolimus, levels, ng/ml
4	64	DM	27.9	139	4.29	0.96	10.1	*2*	24	Unknown	18.8	141	4.9	1.17	6
7	45	Polycystic kidney disease	23.1	141	4.43	1.15	6.5	*13*	21	Unknown	17.5	138	4.65	1.37	9.3
10	26	DM	19.6	140	4.6	1.1	11.5	*14*	18	Unknown	17.2	142	4.89	1.41	9
11	59	Unknown	24.2	145	4.86	2	8.4	*23*	23	Fabry	21.1	137	4.86	1.56	10
15	43	Glomerulonephritis	24.7	147	4.86	1.44	8.3	*27*	34	Fabry	19.8	137	4.5	1.13	9.8
19	19	DM	21.4	138	4.5	1.37	12.6	*32*	25	Unknown	22.8	139	3.8	1.34	9.8
20	54	Unknown	22.4	139	4.31	1.28	21	*36*	22	Urological	24.8	141	4.1	1.6	11
21	39	DM	24.2	143	3.4	1.33	9.6	*44*	31	Kidney Qx	27	139	4.6	1.74	9.4
25	53	DM	26.1	142	4.1	1	15.5	*51*	20	Unknown	22	139	5	2.21	11
28	56	IgA nephropathy	30.7	140	4.3	2	4.8	*54*	29	Unknown	20.4	141	4.6	1.16	8.6
31	50	DM	19.5	139	5	1.5	8.6	*58*	36	Unknown	21.3	140	4.6	0.79	10.1
35	50	Unknown	24	145	3.7	1.6	7.6	*34*	28	DM	19.6	144	5.3	1	3.6
38	28	Unknown	22.9	142	4.8	1.8	9.7	*48*	53	DM	23.8	138	4.53	1.4	9.3
47	69	Amyloidosis	23.7	136	4	1.41	6	*45*	45	Polycystic kidney disease	25.8	146	4.5	1.4	8.3
81	65	Systemic lupus erythematosus	23.6	137	4.4	1.34	4.9	*78*	21	Unknown	26.1	140	4.4	1.35	7.4
55	49	DM	26.1	139	4.5	1.33	10.7	*71*	40	Unknown	27.6	142	4.62	1.61	11.5
72	31	Unknown	23	142	4.31	1.36	13.6	*70*	44	Systemic lupus erythematosus	22.2	141	4.55	0.91	7
								74	33	Renal hypoplasia	25.2	142	4.4	1.13	9.6

Data are divided into hypertensive and normotensive, and patients are identified by an assigned ID number. DM, diabetes mellitus.

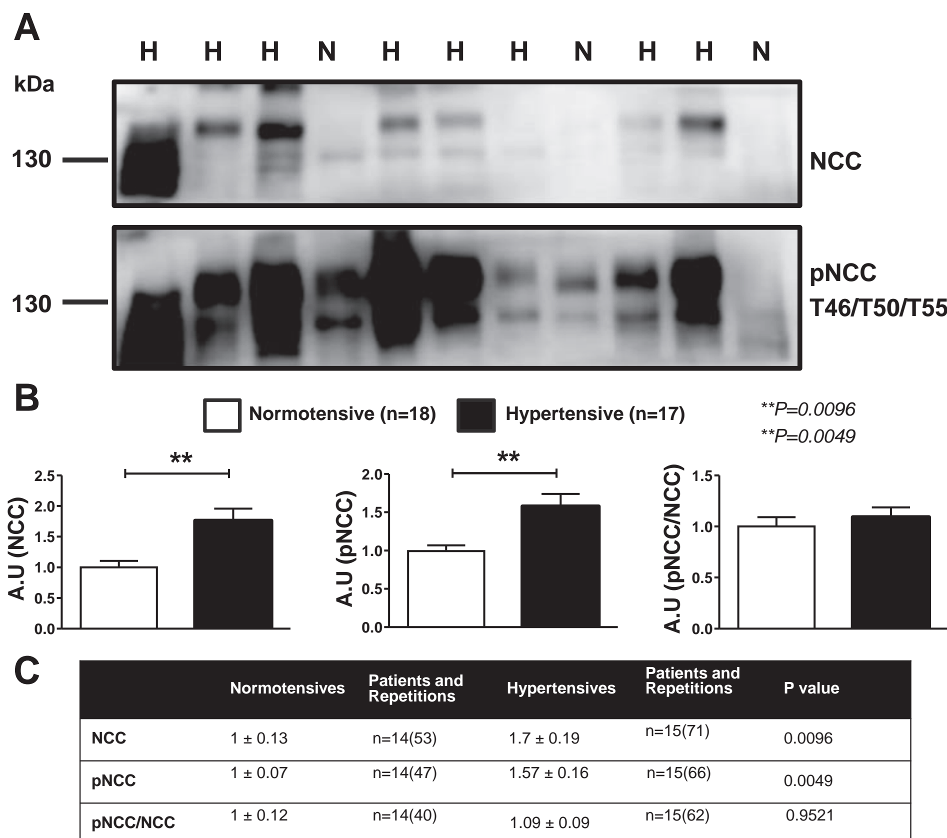
NCC expression and pNCC levels. The total expression and phosphorylation of NCC were assessed using Western blot analysis of urinary exosomes from normotensive vs. hypertensive male patients. Figure 2*A* shows a representative blot with intercalated samples from hypertensive and normotensive patients. The exosomes of each patient were studied in three different blots. The values observed in the normotensive patients were obtained as 100% and were normalized accordingly in the hypertensive patients. Figure 2*B* shows the densitometric analysis results. The hypertensive male patients showed higher NCC expression (1.7 ± 0.19) than the normotensive patients (1 ± 0.13) ($P = 0.009$). Additionally, NCC phosphorylation levels were significantly higher in the hypertensive patients (1.57 ± 0.16 vs. 1 ± 0.07; $P = 0.004$). Because the differences in the total protein and the phosphorylated state of NCC were both higher in the hypertensive group, the pNCC/NCC ratio was not significant.

DISCUSSION

In this prospective cohort study, we found a positive correlation between NCC expression and phosphorylation in urinary exosomes with the development of hypertension in posttransplant male patients treated with tacrolimus. Although all of the patients in the cohort were treated with tacrolimus, expression and phosphorylation of NCC were significantly higher in the patients who developed hypertension 6 mo after transplantation. Thus our results are consistent with the hypothesis that NCC activation plays a major role in tacrolimus-induced hypertension (9, 17).

We prospectively monitored transplant patients for 1, 3, and 6 mo after they received the renal allograft. We observed that at 6 mo the prevalence of posttransplant hypertension was lower in women (3 of 17, 17%) than in men (17 of 35, 49%). The low prevalence in women was likely due to the young age of our cohort (38 ± 15 yr), at which women are protected against hypertension. Because of the low prevalence of hypertension in women and our previous observation that NCC expression in urinary exosomes is higher in women compared with men (23), we focused on the analysis of NCC expression/phosphorylation in the male cohort only.

Prevalence of hypertension after renal transplantation has been reported to be as high as 80–90%. In our cohort of men, the prevalence is 50% probably because patients were studied at 6 mo and thus not all have yet developed hypertension. We think that this is a strength of our study because factors such as hemodynamics and chronic rejection that could be associated with the development of hypertension are not present. In this regard, in our male cohort the hypertensive group was older than the normotensive and with a higher incidence of diabetes mellitus, suggesting that even as early as 6 mo hypertension in this group is multifactorial, because increased age or the presence of diabetes in some cases can influence the development of hypertension. Nevertheless, the expression/phosphorylation of NCC in urinary exosomes was higher in the group of patients with high blood pressure, suggesting that hypertension in tacrolimus-treated patients is associated with activation of the cotransporter. Notably, the tacrolimus levels and allograft function were similar between normotensive and hypertensive men. Additionally, body mass index and history of acute rejection were similar. Thus other factors associated with hypertension, such as tacrolimus intoxication, renal dysfunction, or obesity were excluded.



	Normotensives	Patients and Repetitions	Hypertensives	Patients and Repetitions	P value
NCC	1 ± 0.13	n=14(53)	1.7 ± 0.19	n=15(71)	0.0096
pNCC	1 ± 0.07	n=14(47)	1.57 ± 0.16	n=15(66)	0.0049
pNCC/NCC	1 ± 0.12	n=14(40)	1.09 ± 0.09	n=15(62)	0.9521

Fig. 2. Thiazide-sensitive Na^+-Cl^- cotransporter (NCC) expression and phosphorylation are increased in tacrolimus-treated hypertensive men. *A*: representative Western blot of total NCC (*top*) and its phosphorylated state (pNCC; *bottom*) in urinary exosomes from the normotensive (N) and hypertensive (H) men, normalized by creatinine at 6 mo after renal transplantation. A phospho-antibody recognizing threonine residues in the amino terminal of NCC (T46, T50, and T55) was developed and characterized by Richardson et al. (21). *B*: comparison of the densitometric analysis of compiled data from 14 normotensive and 15 hypertensive patients. Samples where analyzed by Western blotting more than twice. Total NCC protein was arbitrarily set to 1, and pNCC was normalized accordingly. Data are expressed as fold-increase in total NCC (*left*), pNCC (*middle*), and pNCC/NCC ratio (*right*). All graphs are expressed in arbitrary units (A.U). *C*: statistical analysis of the male cohort of the comparison of total NCC, pNCC, and pNCC/NCC ratio, expressed as means ± SE by an unpaired Student's *t*-test, with a significance of $P < 0.05$. The total number of repetitions per group is indicated in parenthesis.

One limitation of our study is that being observational, causality between tacrolimus, NCC activation, and hypertension cannot be established. Tacrolimus administration in mice induced the activation of NCC, together with the development of hypertension that was associated with decreased renal sodium and potassium excretion and high sensitivity to thiazides (9). The authors observed that calcineurin A-α is expressed in DCT and that increased NCC activity was accompanied by increased expression of WNK4 and WNK3, as well as the downstream kinase SPAK. Similar observations were observed by Melnikov et al. (17) in rats treated with cyclosporine. Preliminary observation indicates that tacrolimus-induced hypertension requires the specific expression of the FKBP12 in renal epithelial cells since a kidney-specific knockout mouse for this protein is protected against tacrolimus-induced hypertension, thus suggesting that vascular effects of calcineurin inhibitors are not enough to induce hypertension, in the absence of renal-specific effects (10). Additionally, Hoorn et al. (9) observed that posttransplanted hypertensive patients receiving tacrolimus exhibited higher fractions of chloride excretion than healthy controls in response to a single administration of bendroflumethiazide, suggesting a higher sensibility to thiazide diuretics in this population. Finally, in renal biopsies of patients with tacrolimus and hypertension, expression and phosphorylation of NCC by immunohistochemistry were higher than in patients treated only with azathioprine. Thus, in animal models causation associated between calcineurin inhibitors, activation of NCC, and hypertension has been established. Our data in a prospective follow-up study support that in humans who received a renal allograft, it is likely that the mechanism established by the previous observations of Hoorn et al. (9) and Melnikov et al. (17) may occur.

Although the typical clinical presentation of a hyperactive NCC state involves the development of hyperkalemia and metabolic acidosis (16) and this has been observed in some tacrolimus-treated patients with hypertension (9), we did not observe these characteristics in our population. A likely explanation for this observation could be that tacrolimus levels are rigorously adjusted for minimal toxicity. Therefore, the lack of development of clinical characteristics of PHAII does not discard the activation of NCC as a pathological player in the tacrolimus-induced hypertension noted in this population.

Another limiting factor to be considered in our study is that we did not have access to the control patients who did not receive tacrolimus because all of the transplanted patients in our institution receive this agent as part of the immunosuppression therapy. A recent study in humans demonstrated a correlation between cyclosporine administration and NCC expression in urinary exosomes (6), but no correlation was observed between NCC expression and the development of hypertension in cyclosporine-treated patients. That analysis, however, was based on retrospective data using samples from patients with a mean of 10 yr after transplantation, with a range from 2.5 to 25 yr, thus allowing multiple factors to intervene in the development of hypertension. In our study, all of the patients were prospectively monitored; the urinary sample was obtained at approximately the same time of the day, was immediately mixed with protease inhibitors, and was processed several days after storage. Additionally, all patients were studied at 6 mo posttransplantation with similar and conserved allograft function and were still at an early stage of transplantation, before other confounding factors for the development of hypertension could appear. In fact, hypertension in some of our patients was at an early stage, only detected by ABPM. A recent report demonstrated that there is a circadian rhythm in the presence of NCC-containing exosomes in urine, with the highest expression between noon and 1400 (2). Although this information was not known during the recollection period of this study, fortunately all samples were collected within this time frame for all patients because it is exactly the period of time during which the posttransplant outpatient clinic operates. Thus it is unlikely that the circadian rhythm of NCC exosome excretion affected our cohort.

In conclusion, our data support an association between NCC activation and the development of hypertension in posttransplant tacrolimus-treated male patients. Because the prevalence of hypertension in women was very low in our cohort, it is not known whether this conclusion can be applied to women. Given that the use of thiazides has been sidelined as the first-line treatment of hypertension in this population and its safety has been established in this context (26), the likely role of NCC activity in the pathogenesis of the disease presents the possibility for its pharmacological inhibition. The latter highlights that the need for a clinical, randomized, controlled trial for the use of thiazides in the treatment of tacrolimus-induced hypertension is warranted.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

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Discusión y Conclusiones

Regulación por hormonas femeninas

Se ha demostrado que la activación de NCC está asociada con incrementos en la fosforilación del residuo de treonina T58 (T60 en humanos), sitio que al ser fosforilado es sinónimo de la activación del cotransportador (149, 171). Dicho sitio ha sido relacionado en diversos estudios en modelos *in vitro* e *in vivo* (62). Los anticuerpos creados para estos sitos nos han permitido estudiar de una manera más exacta y rápida a NCC. De esta manera, utilizando estas herramientas en este estudio, se analizó el estado de fosforilación/activación de NCC en ratas, ratones y humanos (hembras y machos) y los resultados mostraron que NCC tiene una mayor actividad y/o expresión en hembras en comparación con los machos y ésta es dependiente de las hormonas sexuales femeninas.

Al analizar la expresión/fosforilación de NCC, en específico en las ratas, no se observó una diferencia en la expresión total del transportador entre las hembras y los machos, pero sí un incremento en la fosforilación de las hembras. Aunque en un estudio realizado en ratas Zucker Obese y su control Zucker Lean[14], tanto en hembras como en machos, se observó una mayor expresión de NCC en hembras vs machos (169). Las diferencias halladas en trabajos previos en comparación con nuestros resultados se pueden deber tanto a las diferencias en las cepas de ratas como en la sensibilidad del anticuerpo utilizado, ya que eran los primeros anticuerpos desarrollados para reconocer a NCC. Por otra parte, en las ratas, encontramos una diferencia entre la expresión de NCC en machos y hembras en ratones y en exosomas urinarios de humano.

La falta de diferencia en los niveles de expresión de NCC en ratas contrasta con la observación de *Verlander*; sin embargo ese estudio tiene algunas diferencias en comparación con el nuestro: 1) Se utilizaron pocos animales, 2) Usaron una técnica que

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[14] Un modelo de ratas con síndrome metabólico, genéticamente modificadas por selección y cruzamiento.

sólo puede evaluar cualitativamente a NCC y 3) Emplearon un anticuerpo poco sensible al transportador (201), mientras que en nuestro estudio se analizaron muchas ratas y consistentemente no encontramos diferencias en los niveles de expresión de NCC entre hembras y machos, o entre los controles y las ratas ovariectomizadas.

Se observó que la fosforilación de NCC fue mayor en ratas así como en ratones hembras, sugiriendo un incremento en la actividad de NCC en las hembras.

Adicionalmente, para poder confirmar la observación de *Chen* (34) decidimos realizar estudios de sensibilidad a BFTZ en ratas hembra y machos, y hallamos resultados similares, es decir la excreción de Na^+ y Cl^- en ratas hembras es mayor a la de los machos al tratarlas con BFTZ, sugiriendo que NCC se encuentra más activo en las hembras, lo que puede ser debido a una mayor cantidad del transportador o a una mayor activación del mismo. Los resultados hallados a través de w.b. indican que, en el caso particular de las ratas, es a través de la activación y no de la expresión.

Durante el desarrollo de este proyecto, se montó un nuevo protocolo en el laboratorio para poder analizar las proteínas expresadas en exosomas excretados en orina humana. Al observar los hallazgos en la regulación dimórfica sexual en rata y ratón, la curiosidad de verificarlo en humanos nació naturalmente. Aprovechando la técnica, se analizó la orina de voluntarios, mujeres y hombres, en un rango de edad similar. En el caso de las mujeres la muestra fue tomada en el día 0 del ciclo menstrual y sólo se recolectó muestras de mujeres que no estuvieran utilizando anticonceptivos hormonales, para normalizar lo más posible las condiciones de los voluntarios. Los resultados siempre deben ser interpretados a la conciencia de la dificultad operativa en el control de variables tan importantes como la ingesta de sal y de los niveles de hidratación en lo sujetos de observación, en este sentido se reconoce universalmente la normalización por creatinina para lidiar con esta dificultad. El análisis de exosomas urinarios por w.b. es entonces, una estrategia que permite tomar “biopsias moleculares” del riñón a través de la cuantificación de los niveles de expresión y modificaciones postraduccionales asociadas en cambios en el estado de activación (mientras se tengan los anticuerpos adecuados).

El análisis de NCC en orina ya ha sido utilizado en otros trabajos: en pacientes con síndrome Gitelman la presencia de NCC en orina es menor a los pacientes control (89), en pacientes con hiperaldosteronismo la presencia de NCC es mayor que en pacientes control sanos (197) y en pacientes con síndrome de Gordon (115); todas las enfermedades anteriores se conoce por estudios en modelos *in vitro* e *in vivo* que afectan a NCC. Por lo tanto la excreción de NCC y su análisis en exosomas parece ser una manera valida de estudiar al cotransportador en pacientes arrojando resultados valiosos nunca antes explorados debido a la dificultad que representa obtener una biopsia del riñón de un persona sana. De esta manera en este trabajo se muestra, al comparar la expresión y fosforilación de NCC de exosomas obtenidos de mujeres y hombres jóvenes (22-28 años), que hay una mayor cantidad/fosforilación en las mujeres. Sin embargo es difícil definir con esta metodología si el incremento de los niveles de NCC en los exosomas se deba a una mayor expresión y/o fosforilación del cotransportador (62).

La gonadectomía es aceptada como la mejor herramienta para realizar un primer acercamiento al efecto de las hormonas sexuales sobre alguna función fisiológica en ambos sexos. Debido al mejor control y acceso, decidimos practicar Gdx en ratas adultas hembras y machos. Demostramos que la fosforilación de NCC en las ratas hembras se reduce dramáticamente 4 semanas después de la ovariectomía mientras que no se observó efecto alguno al remover los testículos. Además se realizaron estudios de sensibilidad a BFTZ y se encontró que sólo la ovariectomía tuvo efectos en la excreción de orina y sal tras la administración del diurético, mientras que la orquidectomía no alteró la respuesta de NCC a la BFTZ. Estos resultados fueron parcialmente similares a los encontrados por *Chen* en donde sí observaron un aumento en la excreción de sodio en las ratas machos Gdx (33). Como hemos mencionado antes si bien es de notar esta discrepancia, lo que es consistente es la relación de la respuesta a BFTZ con el estado del transportador en el análisis de western blot.

Por lo anterior hallamos una relación entre la expresión/fosforilación dimórfica de NCC y la presencia de las hormonas femeninas producidas por los ovarios. Para analizar el efecto de las hormonas ováricas, E2 y P4, se ovarectomizaron ratas (debido a la evidencia de inactividad de la testosterona sobre NCC se decidió no analizar machos) y

se les reemplazó con E2 y P4 durante tres semanas de manera individual para poder analizar por separado el efecto sobre NCC. Lo que observamos fue que nuevamente la ovariectomía disminuyó la fosforilación de NCC y el tratamiento hormonal restableció los niveles de fosforilación, siendo el efecto más potente por parte del E2 que del de la P4. Dicha observación se puede deber a las funciones que realizan las hormonas a lo largo de la vida sexual de las hembras. Es bien sabido que el papel de la P4 es más relevante durante el embarazo, que es cuando el esteroide se secreta en grandes cantidades por parte de la placenta. En el tratamiento con P4 no alcanzamos tiempos y concentraciones tan altas como durante el embarazo, por lo que es probable que el efecto de la P4 debe requerir más tiempo de exposición y probablemente sea un complemento en la reprogramación hormonal y fisiológico del balance hidroelectrolítico de la hembra gestante(30, 205). Por otra parte, el efecto de la P4 debe ser analizado a la luz de las evidencias que muestran que es un antagonista del MR, donde su unión supera a la de la aldosterona pues es más afín y además bloquea la función del MR (137, 162, 163, 176, 191) en relación se sabe que durante el embarazo, las mujeres con Addison[15](98) deben aumentar su tratamiento de reemplazo hormonal y de manera opuesta las mujeres con hiperaldosteronismo tienen niveles normales de PA a lo largo de su embarazo (135), de esta manera pese a que la PA pudiese tener un efecto directo sobre la activación de NCC, podría ser cubierto por la falta de activación del MR y por lo tanto de los efectos de la aldosterona.

Por el contrario el E2 es una hormona que esta más involucrada en la regulación de los ciclos ovárico-menstruales y a la cual la hembra está expuesta constantemente a lo largo de su vida reproductiva. Es probable entonces que el E2 sea la hormona esteroidea ovárica con más efecto sobre NCC para poder producir cambios agudos en la reabsorción de Na^+ y por lo tanto de agua durante los picos de E2, momentos bien conocidos durante el ciclo sexual por tener efectos secundarios como la retención de agua, reflejado en un aumento en la masa corporal y una disminución en la excreción de Na^+:Cl^- por parte del riñón (36, 70, 153-155, 185).

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[15] Trastorno que ocurre cuando las glándulas suprarrenales no producen suficiente cantidad de hormonas incluyendo a la aldosterona.

Como se describió en el planteamiento, una limitante para estos hallazgos al iniciar este estudio, era la falta de una clara evidencia de la expresión de los receptores de E2 en el TCD, pese a que sí se tenían datos de la expresión del ARNm de ERα y de que los genes regulados por E2 están ampliamente distribuidos en el riñón (88) no existía evidencia concluyente de la expresión de la proteína del ERα en el TCD. Durante el estudio del efecto de la hormonas ováricas, salió oportunamente un reporte por el grupo de Filardo de la expresión del GPER-1 a lo largo de la nefrona, hecho a través de inmunofluoresencias y marcadores específicos de los segmentos de la nefrona, sin embargo el único lugar que no lo estudiaron fue el TCD (35). A través de una colaboración solicitamos el anticuerpo al grupo de Filardo, y realizamos inmunofluoresencias para el TCD en ratones, utilizando NCC como marcador. Lo que encontramos es que el GPER-1 se expresa ampliamente en el TCD. Este hallazgo, en conjunto con el efecto del E2 sobre la actividad de NCC y *no* sobre su expresión observado en machos tratados con E2, nos hace pensar que es más probable que la regulación sea a través de una vía no genómica, como lo puede ser a través de la señalización aguda por de un receptor acoplado a proteínas G como el GPER-1. Sin embargo, dichos resultados no descartan la posibilidad que el ERα esté involucrado. Sobre dichos hallazgos es necesario realizar más estudios para clarificar las vías y/o mecanismos de acción.

Se sabe que la cinasa responsable de la activación/fosforilación de NCC (71, 164, 213) es SPAK, la cual a su vez es modulada por las WNK's (202). En el riñón, además de la isoforma completa de SPAK (full lenght), se expresan isoformas cortas resultado de cortes proteolíticos (110), dos de las cuales son conocidas como SPAK-2, una isoforma truncada en el amino-terminal, y KS-SPAK a la que le falta el dominio cinasa (117). Las isoformas cortas están asociadas a un efecto inhibidor regulador sobre NCC, mientras que la actividad/expresión de las mismas es disminuida cuando NCC requiere activación, por ejemplo durante la ingesta de una dieta baja en Na^+ o ante infusiones de Ang II (68, 117, 180). Se cree que las isoformas cortas funcionan como dominantes negativas de SPAK (full lenght). En este proyecto observamos que la expresión y fosforilación de SPAK fue mayor en las ratas y ratones hembras en comparación con los machos y no se observo un

efecto similar con OSR1. Dichas diferencias son reguladas por el efecto de los ovarios ya que la Gdx de ratas hembras elimino dicha diferencia. Al explorar el efecto de E2 y P4 sobre SPAK se encontró que en las ratas Gdx la isoforma predominante fue SPAK2 y el tratamiento hormonal promovió la expresión/fosforilación de la isoforma SPAK (full-lenght). Lo anterior sugiere que el efecto positivo de las hormonas femeninas sobre NCC es mediada, en parte por SPAK. Apoyando nuestras observaciones un estudio reciente muestra que el E2 regula a SPAK en el hipotálamo en desarrollo al estimular la actividad de NKCC1 (144), por lo tanto SPAK ya era conocida como una cinasa regulada por los estrógenos.

El incremento en la actividad y cantidad de NCC en hembras puede sonar controversial dado que la PA es más alta en hombres en comparación con las mujeres durante el período reproductivo de la mujer y la disminución de las hormonas circulantes, como ocurre en la menopausia, tiene como efecto la pérdida del efecto protector contra el desarrollo de hipertensión arterial en las mujeres (141, 146, 205). Sin embargo, es bien conocido que el E2 tiene potentes efectos vasorelajantes pudiendo prevenir el incremento en la PA, usualmente asociada con la retención de sodio, favoreciendo la natriuresis de presión lo que puede explicar potencialmente los frecuentes reportes del aumento de la retención de líquidos asociado con los picos de liberación de las hormonas sexuales femeninas que es frecuentemente acompañada de la formación de edema (153, 185, 205).

Los estudios en ratas ovariectomizadas, con o sin la administración de E2 no descartan la participación de la PRL en la regulación de NCC, ya sea en su expresión o función debido a que al menos en la rata el estradiol tiene un efecto estimulatorio sobre la secreción de PRL en la hipófisis anterior (82, 182). De esta manera en el animal ovariectomizado no sólo están disminuidas las concentraciones de E2 sino también de PRL y en el caso del reemplazo hormonal con E2 también se está estimulando la producción de PRL por lo que esos modelos son insuficientes para poder aislar el efecto de la PRL sobre NCC.

La PRL tiene efectos osmoreguladores principalmente estudiados en vertebrados inferiores como los peces (76, 107), pero también existen reportes de dicha regulación

sobre mamíferos, aunque son pocos (21, 132, 159). La PRL incrementa la reabsorción de Na^+:Cl^- y agua en la vejiga urinaria de teleósteos, que es donde NCC regula la reabsorción de sal (189). De hecho recientemente salió un reporte donde, en el pez Mozambique, la PRL regula al gen de NCC en la vejiga urinaria (19, 20).

En este proyecto analizamos el papel de la PRL sobre la expresión/fosforilación de NCC en riñones de ratones knockout para el PRLR (PRLR KO) para poder ver sus efectos sin alterar al EHH. Como ya se había observado los ratones PRLR KO hembras y machos presentan regulación dimórfica en la expresión/fosforilación de NCC, sin embargo, los efectos sólo fueron vistos en los machos KO en comparación con sus controles; esto se puedo deber a que los ratones PRLR KO aun tiene E2 lo que en las hembras enmascara el efecto de la ausencia del efecto de la PRL sobre NCC. Para ello se corroboró el efecto de PRL en ratas macho tratadas con PRL y en riñones de rata machos, en un sistema de perfusión *ex vivo*, de esta manera pudimos excluir tanto los efectos de del E2 circulante como del efecto de la presencia del EEH. Así y de forma consistente con el papel de la PRL en la osmoregulación en teleósteos y como una hormona que favorece la retención de Na^+ en mamíferos, encontramos que la PRL es un regulador positivo de la fosforilación de NCC.

En resumen, el presente estudio muestra que la regulación dimórfica sexual de la actividad y expresión de NCC se debe a las hormonas femeninas: estrógenos, progesterona y PRL. Dicho hallazgo tiene implicaciones fisiológicas importantes ya que hay períodos en la vida de las hembras que la secreción de estas hormonas se asocia con estados donde se requiere de la expansión y/o incremento del volumen del líquido extracelular circulante. Por ejemplo, como cuando en las mujeres al final del embarazo hay una ganancia de varios kilogramos, debido al peso del feto, la placenta y el líquido amniótico, lo que provoca un mayor requerimiento en el volumen circulante en sangre, en la lactancia, las mujeres pueden producir varios litros de agua en la generación diaria de leche, que también requiere de la retención de sal (205), y al final de la fase lútea, las mujeres reportan una sensación de edema que puede relacionarse en parte a un aumento en la reabsorción de Na^+ por parte del riñón.

Sabemos que son necesarios más estudios para explorar las vías de señalización y el perfil de activación de NCC a lo largo del período menstrual, la lactancia y el embarazo, pero nuestro estudio es el primero en analizar el efecto de las *tres* hormonas en *diversos modelos experimentales* y en *distintas especies* sobre una proteína vital para la reabsorción de sal y con repercusión sobre la PA como lo es NCC.

Por último, este proyecto deja una enseñanza invaluable, ya que al inicio del estudio se perseguía el efecto de la PRL como el regulador *único* del efecto dimórfico positivo sobre NCC. Sin embargo, al realizar los experimentos y analizar los resultados nos sorprendió ver que en realidad son las tres hormonas las activadoras del cotransportador. Y, aunque a primer vista, pareciera redundante y pleiotrópica la activación, lo que nunca se debe perder es el contexto en el cual cada hormona estimula la función de NCC, el cual puede ser único temporalmente e incluso puede establecer cruzamiento entre las vías de señalización intracelular creando respuestas aditivas, integrativas y más complejas. La utilidad de un sistema de activación multi-hormonal de NCC recae en que el TCD es un sitio de regulación altamente fina de la reabsorción de sal ya que su actividad escapa a la retroalimentación tubuloglomerular de la MD convirtiéndolo en un sitio ideal para ser modulado con precisión por las hormonas.

Papel en la hipertensión arterial inducida por tacrolimus en pacientes con trasplante renal

En este proyecto se encontró una relación positiva en la expresión/fosforilación de NCC en exosomas urinarios de pacientes masculinos con HTA 6 meses después del trasplante renal y cuyo tratamiento inmunosupresivo fue el tacrolimus. Pese a que todos los pacientes en esta cohorte fueron tratados con tacrolimus, la expresión/fosforilación de NCC fue significativamente mayor en los pacientes que desarrollaron hipertensión. Los resultados son consistentes con la hipótesis de que la activación de NCC tiene un papel importante en el desarrollo de la hipertensión inducida por tacrolimus, como fue sugerido en los trabajos previos hechos en ratas (121) y ratones (78).

Este es el primer estudio de este tipo realizado en humanos, en una población de pacientes seguidos desde el momento del trasplante renal. En este seguimiento se combinó la experimentación básica mediante el aislamiento y análisis de exosomas urinarios en humanos dentro del contexto clínico en un estudio prospectivo con seguimiento estrecho en pacientes incidentes de trasplante renal. La posibilidad de determinar simultáneamente variables bioquímicas, clínicas y el muestreo para extracción de exososomas urinarios permitió una mejor asociación entre estas variables.

Uno de los principales aciertos es el método para montar la técnica para extracción de exosomas en orina y normalizar con creatinina urinaria su cuantificación. Permitiendo caracterizar la asociación de los inhibidores de calcineurina (ICN) en el paciente postransplantado con su acción a nivel del túbulo distal a través del análisis de los exosomas. Cabe aclarar que gracias a esto, se añadió una herramienta en el laboratorio para poder analizar el estado de NCC en pacientes o voluntarios sanos en distintos contextos, como se hizo para el caso del análisis dimórfico sexual de NCC.

Por otro lado, el método de detección de presión arterial mediante MAPA, que es el método por excelencia para un análisis exacto de la PA, permitió clasificar de manera certera a los individuos con hipertensión y a su vez mejorar la predicción de daño a órgano blanco, riesgo cardiovascular y progresión de daño renal. Con capacidad de distinguir de la hipertensión por bata blanca y/o hipertensión enmascarada. Así como la

detección del paciente con hipertensión de predominio nocturno con la pérdida del descenso normal de la presión arterial por la noche (non-dipper).

Una característica que puede ligar este estudio al de la regulación dimórfica sexual de NCC, es la confirmación que la mujeres tienen “protección” en contra de la HTA, sin embargo debido al número pequeño de mujeres hipertensas, se excluyeron de este análisis . Una debilidad de este tipo de estudios es la perdida de seguimiento de los pacientes por cuestiones sociales y económicas, así como la ausencia de un grupo control de sujetos sanos o postrasplantados renales sin un ICN como esquema base inmunosupresor. Siendo difícil esto último debido a que es el esquema de inmunosupresion estándar en la actualidad.

En conclusión, nuestros datos sugieren una asociación entre la activación de NCC y el desarrollo de hipertensión en hombres post-trasplantados tratados con tacrolimus. Dado que el uso de tiazdias ha sido marginado, como tratamiento de primera línea contra la hipertensión en los pacientes con hipertensión con trasplante renal, este estudio podría sentar las bases para realizar un estudio clínico, aleatorio del uso de tiazidas en pacientes con trasplante renal e hipertensión resultado del tratamiento con ICN.

Lista de conclusiones

Regulación por hormonas femeninas

1. **La sensibilidad a los diuréticos tipo tiazida es mayor en hembras en comparación con los machos y está asociada con una mayor fosforilación de NCC.** Dichos resultados confirmaron observaciones previas realizadas en ratas tratadas con bendroflumetiazida, nuestros datos muestran que dicha diferencia se debe a un aumento en la actividad basal del cotransportador. Estos resultados fueron consistentes con un incremento en la expresión y fosforilación de SPAK, tanto en ratas como en ratones, sugiriendo que SPAK es parte de la vía de señalización regulada por las hormonas sexuales.

2. **La ovariectomía reduce la sensibilidad a tiazidas y la fosforilación de NCC. No hubo efecto con la orquidectomía en machos.** Lo cual sugirió que NCC y SPAK es regulado directamente por las hormonas ovarícas de manera positiva.

3. **La administración de estradiol y progesterona recupera la fosforilación de NCC en ratas ovariectomizadas.** A través de aislar los efectos de progesterona y de estrogenos, se pudo verificar que ambos favorecen la actividad del transportador. Dicho efecto activador no depende completamente del ciclo estral de las hembras, ya que la admisnitración de E2 en ratas machos también aumento la fosforilación de NCC.

4. **La prolactina también regula la actividad/fosforilación de NCC.** Este fue el primer reporte del efecto activador de la prolactina sobre el transportador, estos efectos fueron confirmados en ditintos modelos. Las implicaciones fisiológicas de este hallazgo, podrían estar relacionadas con el efecto osmorregulador que tienen la PRL en vertebrados inferiores, sin embargo los mamiferos han desarrollado sistemas osmoreguladores hormonales más finos que enmascaran el de la PRL sobre NCC.

Papel en la hipertensión arterial inducida por tacrolimus en pacientes con trasplante renal

5. **Seis meses tras el trasplante renal, la HTA fue más prevalente en hombres en comparación con las mujeres.** Dichos resultados son congruentes con los datos que indican que las mujeres tienen menor factor de riesgo a desarrollar HTA en comparación con los hombres.

6. **Los hombres con HTA son mayores y tienen una mayor incidencia de diabetes en comparación con los pacientes normotensos.** Lo cual sugiere que la muestra de nuestros pacietes es similar al resto de la población, en cuanto a que el desarrollo de HTA está asociada a la presencia de diabetes tipo II, así como factores intrinsecos relacionados al envejecimiento.

7. **Seis meses después del trasplante renal, los pacientes masculinos con hipertensión presentaron una mayor expresión y fosforilación de NCC en exosomas urinarios**. De esta manera nuestros resultados confirman hallazgos en modelos animales extrapolados hacia los humanos, abriendo una vetana de oportunidad al diágnostico temprano de HTA mediante biopsias moleculares, como lo son los exosomas, así como a la posibilidad de utilizar a las tiazidas como fármacos de primera línea para tratar la HTA en los pacientes con trasplante renal.

Estas observaciones resaltan el papel de NCC en la retención de sal y volumen que ocurre en estados fisiológicos en los que se eleva la producción y secreción de estas hormonas así como el desarrollo de enfermedades como la HTA.

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Apéndices

Artículos

1. **Rojas-Vega L**, Gamba G. Mini-review: regulation of the renal NaCl cotransporter by hormones. *Am J Physiol Renal Physiol.*1;310(1):F10-4, 2016.

2. **Rojas-Vega L**, Jiménez-Vega AR, Bazúa-Valenti S, Arroyo-Garza I, Jiménez JV, Gómez-Ocádiz R, Carrillo-Pérez DL, Moreno E, Morales-Buenrostro LE, Alberú J, Gamba G. Increased phosphorylation of the renal Na^{+}-Cl^{-} cotransporter in male kidney transplant recipient patients with hypertension: a prospective cohort. *Am J Physiol Renal Physiol.*15;309(10):F836-42, 2015.

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Capítulo

1. Capítulo 29. MANEJO RENAL DE CALCIO, FÓSFORO Y MAGNESIO; Fisiología cardiovascular, renal y respiratoria; Editores: DR. JOSÉ LUIS MORALES SAAVEDRA, LIC. VANESSA BERENICE TORRES; MANUAL MODERNO, Paginas: 277, 2014. Rojas-Vega Lorena, Gamba Gerardo.

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Mini-review: regulation of the renal NaCl cotransporter by hormones

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Rojas-Vega L, Gamba G. Mini-review: regulation of the renal NaCl cotransporter by hormones. *Am J Physiol Renal Physiol* 310: F10–F14, 2016. First published October 28, 2015; doi:10.1152/ajprenal.00354.2015.—The renal thiazide-sensitive NaCl cotransporter, NCC, is the major pathway for salt reabsorption in the distal convoluted tubule. The activity of this cotransporter is critical for regulation of several physiological variables such as blood pressure, serum potassium, acid base metabolism, and urinary calcium excretion. Therefore, it is not surprising that numerous hormone-signaling pathways regulate NCC activity to maintain homeostasis. In this review, we will provide an overview of the most recent evidence on NCC modulation by aldosterone, angiotensin II, vasopressin, glucocorticoids, insulin, norepinephrine, estradiol, progesterone, prolactin, and parathyroid hormone.

angiotensin II; NCC; norepinephrine; prolactin; PTH

IN THE DISTAL CONVOLUTED TUBULE (DCT), Na^+ and Cl^- reabsorption is fine tuned by the electroneutral Na^+-Cl^- cotransporter (NCC) (13) expressed in the apical membrane and target of the thiazide-type diuretics (11). Thiazides are the first-line pharmacological therapy for the management of arterial hypertension (10, 34). Inactivating mutations of the SLC12A3 gene (NCC) result in Gitelman syndrome, featuring arterial hypotension with hypokalemia, metabolic alkalosis, and hypocalciuria (48), while NCC activation by mutant kinases (56) or ubiquitin ligases (3, 25) produce pseudohypoaldosteronism type II, a mirror image disease featuring hypertension, hyperkalemia, metabolic acidosis, and hypercalciuria. Thus NCC activity is not only critical for salt balance and blood pressure regulation, but also for potassium, calcium, and acid-base handling in the kidney (30).

It is known that expression/activity of NCC is affected by two known posttranslational mechanisms (14). Phosphorylation by the kinase STE-20 proline-alanine-rich (SPAK) in the NCC amino-terminal domain (38) [which in turn is modulated by the "with no lysine kinases" (WNKs)] is associated with the activation of the cotransporter (35, 39), and ubiquitylation by either a direct effect of the HECT-type E3 ubiquitin ligase complex, Nedd4-2, which reduces the amount or activity of NCC in the plasma membrane (2, 41), or an indirect effect of the RING-type ubiquitin ligase complex, KLHL3 and Cul3, that modulates the ubiquitylation of WNKs (3, 25).

Regulation of NCC activity by the master rheostats of the body, the hormones, has been extensively studied over the years. It has been established that NCC is a pleiotropic protein in the sense that many different stimuli can modulate its activity. The goal of this short review is to provide a glimpse of the most recent evidences of NCC control by hormones (Table 1) (Fig. 1).

Aldosterone

Aldosterone was the first hormone found to have an effect on NCC activity. This was demonstrated in rats by direct aldosterone infusion and as a response to a low-sodium diet (LSD) (18). Increased activity of NCC in response to aldosterone is due to increased expression and phosphorylation of the cotransporter (1, 16, 27) by a mineralocorticoid receptor-dependent SPAK-mediated phosphorylation of NCC (9, 22). The mechanism has not been elucidated yet. Some evidence suggests that it could be through modulation of WNK4 activity and/or SGK1 activation (43, 54), and a recent study showed that WNK1 is a target of Nedd4-2, an effect of the ligase that is inhibited by SGK1 (42). Inhibition of Nedd4-2 ligase by SGK1 phosphorylation could also explain the increased expression of NCC since it has been observed that Nedd4-2 promotes NCC ubiquitylation in a SGK1-dependent fashion (2, 29). Supporting this possibility, inducible and nephron-specific knockout of Nedd4-2 in mice results in overexpression of NCC, suggesting a tonic reduction of the cotransporter by Nedd4-2 (41). Additionally, WNK3, an activator of NCC, also inhibits Nedd4-2 by a mechanism that appears to be different to SGK1 (24).

ANG II

It was first reported in 2007 that administration of captopril, an ANG II-receptor antagonist, in rats acutely promoted the redistribution of apical NCC to cytoplasmic vesicles and that the coadministration of ANG II reversed this effect (45). In *Xenopus laevis* oocytes ANG II promoted NCC activation by a WNK4- and SPAK-dependent mechanism (44). Later, it was observed that in rats exposed to a LSD, NCC apical expression increased in an aldosterone-independent fashion (12). Supporting this, another work showed that chronic ANG II infusion

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Table 1. *Summary of the hormones that regulates NCC*

Hormone	Receptor	Activity	Proposed pathway	Reference(s)
Aldosterone	MR (nuclear receptor)	↑	SGK1/Nedd4-2 SGK1/WNK4/SPAK	18, 9, 2, 1, 22, 29, 16, 43, 41, 27, 54, 24.
Angiotensin II	AT_1 (metabotropic)	↑	WNK4/SPAK	45, 12, 53, 51, 44, 57, 5, 53, 47, 21.
Vasopressin	V2R (metabotropic)	↑	SPAK	32, 36, 28, 33, 38, 46.
Glucocorticoids	GR (nuclear receptor)	↑	Unknown	7, 54, 17.
Insulin	IR (tyrosine kinase receptors)	↑	PI3K/Akt	50, 49, 23, 6.
Norepinephrine	β_2-AR (metabotropic)	↑	PKA/histone acetylation/WNK4	31, 52.
Estradiol	Unknown	↑	Unknown	55, 40.
Prolactin	PrlR (class 1 cytokine receptors)	↑	Unknown	40.
Progesterone	Unknown	↑	Unknown	40.
PTH	PTH1R (metabotropic)	↓	RasGRP1/ERK1/2	19, 26, 20, 51.

PTH, parathyroid hormone.

increased the thiazide-sensitive distal salt reabsorption that was not prevented by spironolactone (57). The aldosterone-independent action of ANG II was demonstrated by the ANG II-induced NCC phosphorylation in previously adrenalectomized rats (53). The positive effect of ANG II on SPAK and NCC is lost in the total WNK4 knockout mice, confirming a WNK4-dependent mechanism (5). In addition, the WNK4-dependent effect of ANG II has been observed also in mDCT15 and mpkDCT cells (21, 51, 53). Finally, another mechanism by which ANG II promotes NCC activity is by preventing the KLHL3 recognition of WNK4, thus promoting WNK4 accumulation, which in turn can increase the activity of NCC (47).

Vasopressin

It has been known for a long time that vasopressin receptors are expressed in the DCT (32), but the demonstration of a direct effect on NCC was not obtained until 2010 by two independent groups that reported that vasopressin increased NCC activity. NCC was activated in Brattleboro rats infused with an agonist of vasopressin receptors (32, 36). This effect was also observed in isolated cells from the DCT through what appeared to be a WNK-SPAK-related pathway (36) (Fig. 1). Later, it was also observed that switching the expression of SPAK isoforms (28) by vasopressin had differential stimulatory effects along the nephron, being the full-length isoform the responsible of phosphorylating NCC (33, 38, 46).

Glucocorticoids

The evidence of NCC activation by adrenal steroids was initially reported before the cloning of NCC cDNA, when the specific binding of [^{3}H]metolazone to renal cortex proteins was used as an index of NCC expression/activity. It was observed that the binding decreased after rat adrenalectomy and that

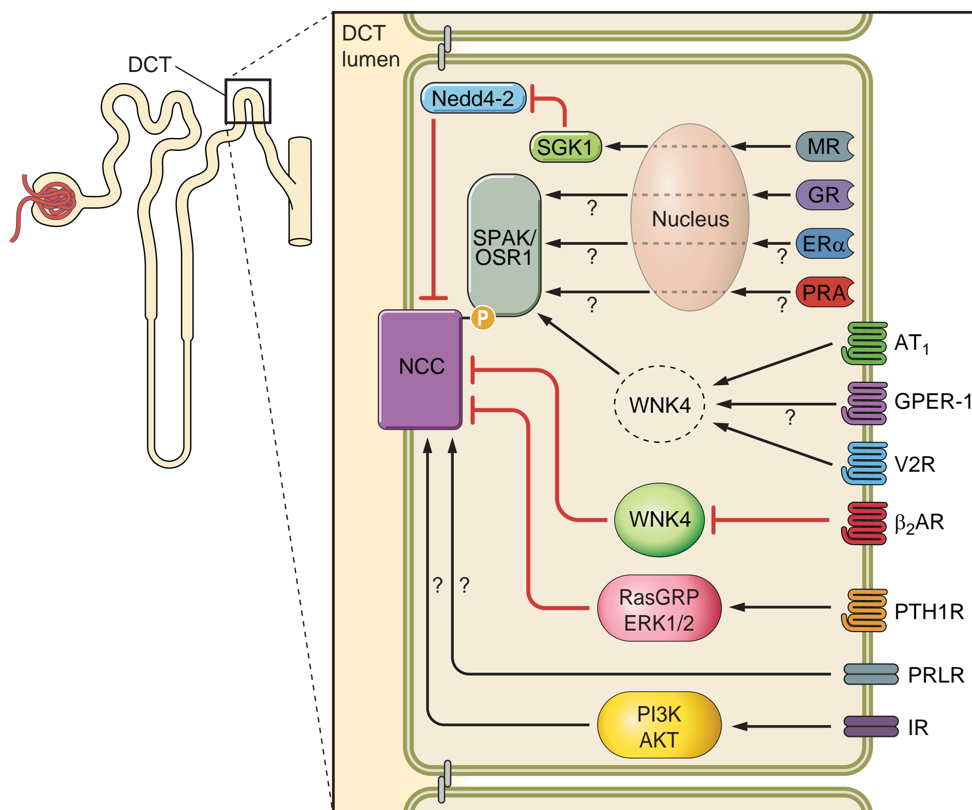

Fig. 1. Illustration of a transverse view of a distal convoluted tubule segment (DCT2) with all the hormones known to regulate the Na^+-Cl^- cotransporter (NCC). See text for additional details. The hormone receptors are located in the basolateral membrane. PRLR, prolactin receptor; PRA, progesterone receptor A; GPER-1, G protein estrogen receptor 1; ER-α, estrogen receptor α; α1-AR, α1-adrenergic receptor; V2R, vasopressin receptor 2; IR, insulin receptor; PTH1R, parathyroid hormone receptor 1; GR, glucocorticoid receptor; AT_1, angiotensin receptor 1; MR, mineralocorticoid receptor.

replacement therapy with glucocorticoids restored NCC density back to control levels (7). A few years later, it was reported that both aldosterone and dexamethasone increased NCC expression and activity in adrenalectomized rats (54). However, the latter did not prove an aldosterone-independent effect of glucocorticoids on NCC. A more recent preliminary report showed glucocorticoid-positive regulation of NCC activity is dependent on diurnal rhythm (17).

Insulin

It is known that obesity, diabetes, and metabolic syndrome are often associated with hypertension, and it was hypothesized that one potential mechanism could be that insulin stimulates NCC activity (50). Three groups reported this type of modulation. In 2011, Sohara et al. (49) provided evidence that insulin indeed activates NCC and SPAK by a phosphoinositol 3-kinase (PI3K)-dependent pathway in mpKDCT cells. Koomers et al. (23) reported that NCC phosphorylation is increased in a metabolic syndrome animal model (Zucker rats) and that these animals have a higher hydrochlorothiazide sensitivity than control rats. More recently, NCC activation and phosphorylation induced by insulin were confirmed in *X. laevis* oocytes (6). Moreover, supporting this hypothesis there is evidence showing that the effect of insulin is direct and not mediated by another hormonal system in kidney ex vivo perfusion techniques (6).

Norepinephrine

In 2011, a study showed that chronic adrenergic activation by β_2-adrenergic agonists causes salt-sensitive hypertension associated with activation of NCC, which in turn was due to an isoproterenol-induced epigenetic effect on the glucocorticoid receptor that modulated WNK expression (31). The positive effect of norepinephrine on NCC activity through stimulation of β-adrenergic receptors was confirmed by another group showing that OSR1 kinase, but not SPAK, is required for such an effect (52).

Female Hormones

Sexual dimorphic regulation of NCC has been studied intermittently over the years. In 1994, experiments in rats showed that the sensitivity to thiazides and [^{3}H]metolazone binding to renal proteins were higher in female than in male rats (8). This difference was reversed in gonadectomized female rats. Later on, a positive effect of estradiol on NCC expression was observed by immunogold electron microscopy (55). In 2006, a study in lean and obese Zucker rats showed increased NCC expression in the kidney of female vs. male rats (38). Recent evidences from our group demonstrated higher NCC and SPAK activity/phosphorylation in female than in male rats and mice. This increase is reduced after ovariectomy, and it is due to positive effects of estrogens, progesterone, and also prolactin on NCC (40). The estradiol activation of NCC was related to changes in SPAK isoform expression (40). The prolactin effect was demonstrated using ex vivo kidney perfusion, thus excluding other hormonal systems that could be implicated (40). Interestingly, these findings were confirmed in humans since expression/phosphorylation of NCC in urinary exosomes was higher in women than in men (40).

Parathyroid Hormone

Evidence of NCC suppression by diacylglycerol (DAG) through activation of Ras guanyl-releasing protein 1 (RasGRP1) suggested a physiological regulation of NCC by DAG (20). In this regard, Ko et al. (19) observed that parathyroid hormone (PTH) suppresses NCC function and surface expression through DAG activation of RasGRP1 and the ERK1/2 MAPK pathway. The expression of PTH receptors in DCT have been documented (26). Thus it is likely that PTH-induced reduction in NCC activity results in diuresis seen with elevated levels of PTH (19). This could potentially explain increased calcium reabsorption induced by PTH, since it is known that the lower the NCC activity, the higher the calcium reabsorption (15).

In summary at first glance the pleiotropy in the activation/phosphorylation of NCC seems redundant, but the context in which every hormone stimulates its activation is unique, even when the cross talk between the networks could lead to integrative and additive responses. The utility of the multihormone NCC activation system exists in the point that distal reabsorption is not subjected to the tubuloglomerular feedback, and despite the low level of salt reabsorption by NCC important changes in urinary sodium excretion can be produced (4). Next, studies must be centered on the development and integration of new models to study the effect of two or more hormones on NCC dynamics in the same period of time.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: L.R.-V. and G.G. edited and revised manuscript; L.R.-V. and G.G. approved final version of manuscript; G.G. drafted manuscript.

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

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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.

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

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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 $I \cdot ml^{-1} \cdot h^{-1}$				
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

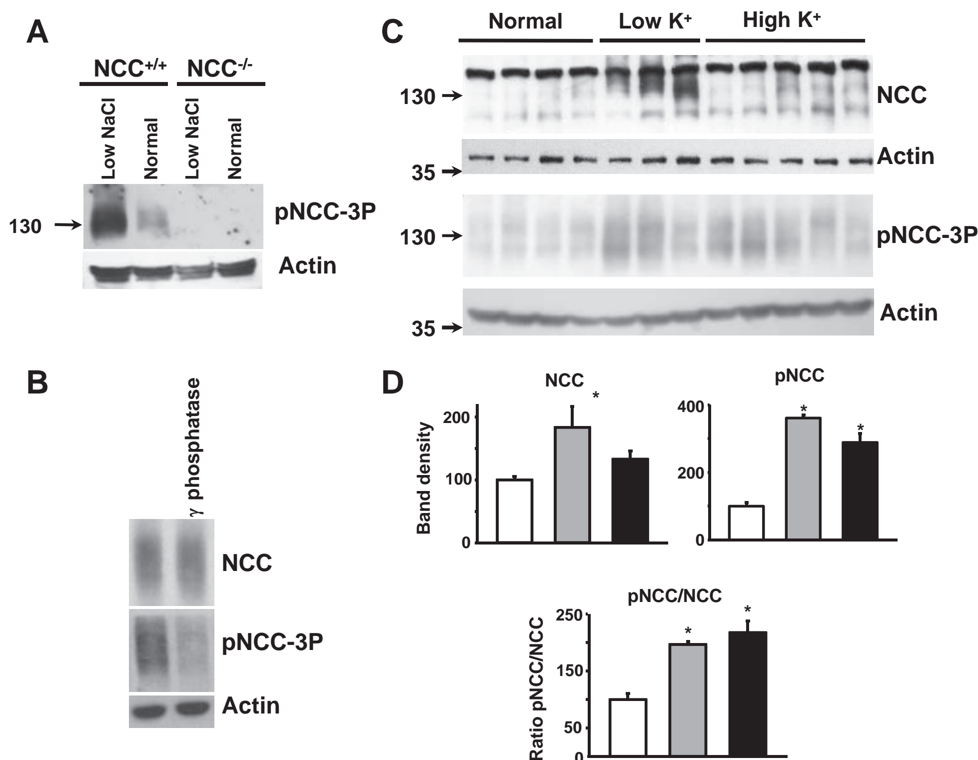

Fig. 1. Effects of varying dietary K^+ on Na^+-Cl^- cotransporter (NCC) expression and phosphorylation. *A*: specificity of the NCC phospho-antibody used in this work. The antibody recognizes three phosphorylated residues in NCC ($Thr^{44}/Thr^{48}/Thr^{53}$) [phosphorylated (p)NCC-3P]. Samples from 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

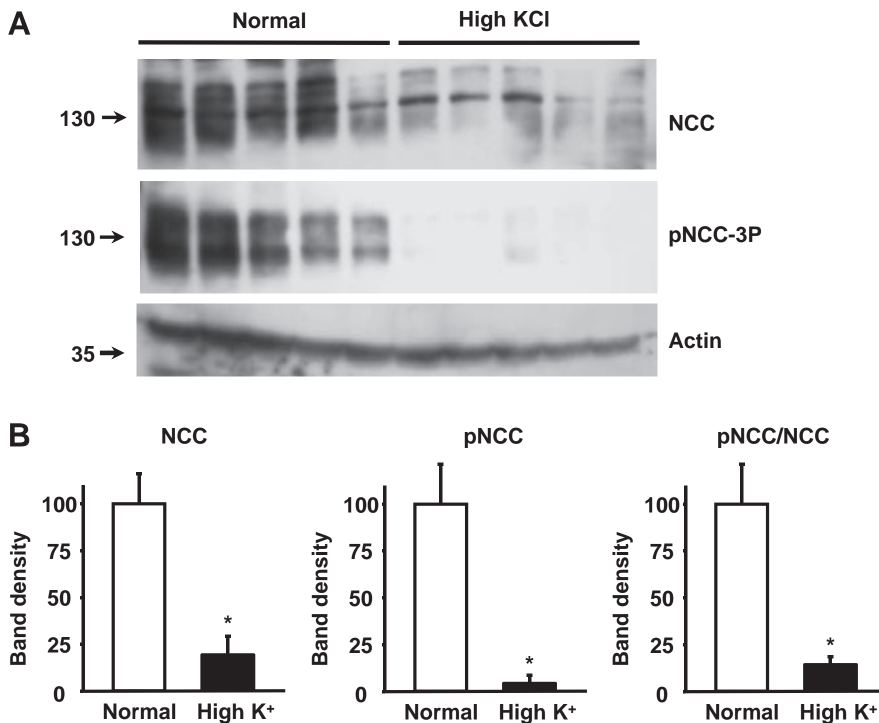

Fig. 2. Effect of the high-KCl diet on the expression and phosphorylation of NCC. *A*: representative Western blot analysis of total kidney protein samples of wild-type mice maintained on diets with normal or high KCl content. *B*: densitometric analysis of blots shown in *A*. Results are expressed as 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

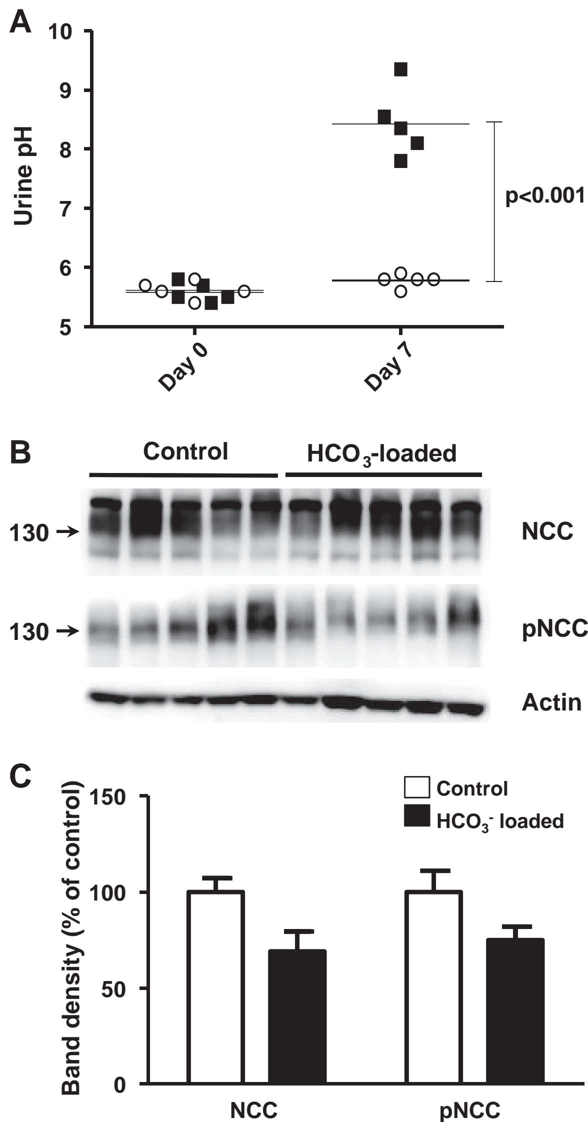

Fig. 3. Effect of HCO_3^- loading in expression and phosphorylation levels of NCC. *A*: urine pH of control mice (○) and HCO_3^--loaded mice (□) on the previous day to the beginning of treatment (*day 0*) and on *day 7* of high HCO_3^- intake. The mean urine pH for each group is indicated by the horizontal 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

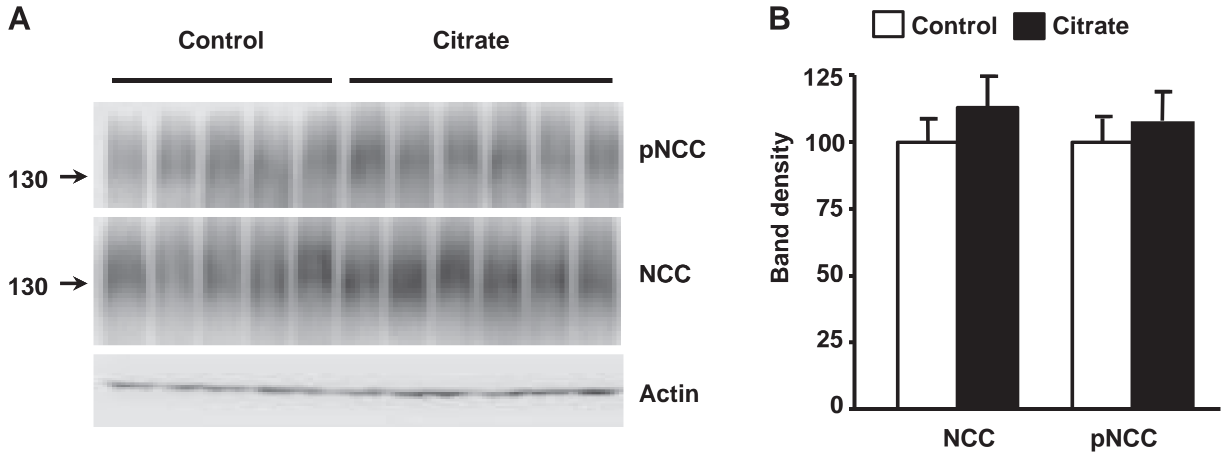

Fig. 4. Effect of high citrate intake on NCC phosphorylation. *A*: Western blot analysis of renal proteins of wild-type mice fed with a high-citrate diet. Citrate was administered in the drinking water. Both groups consumed a similar amount of water (5.16 ± 1.6 ml for the control group vs. 5 ± 1.4 ml 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,

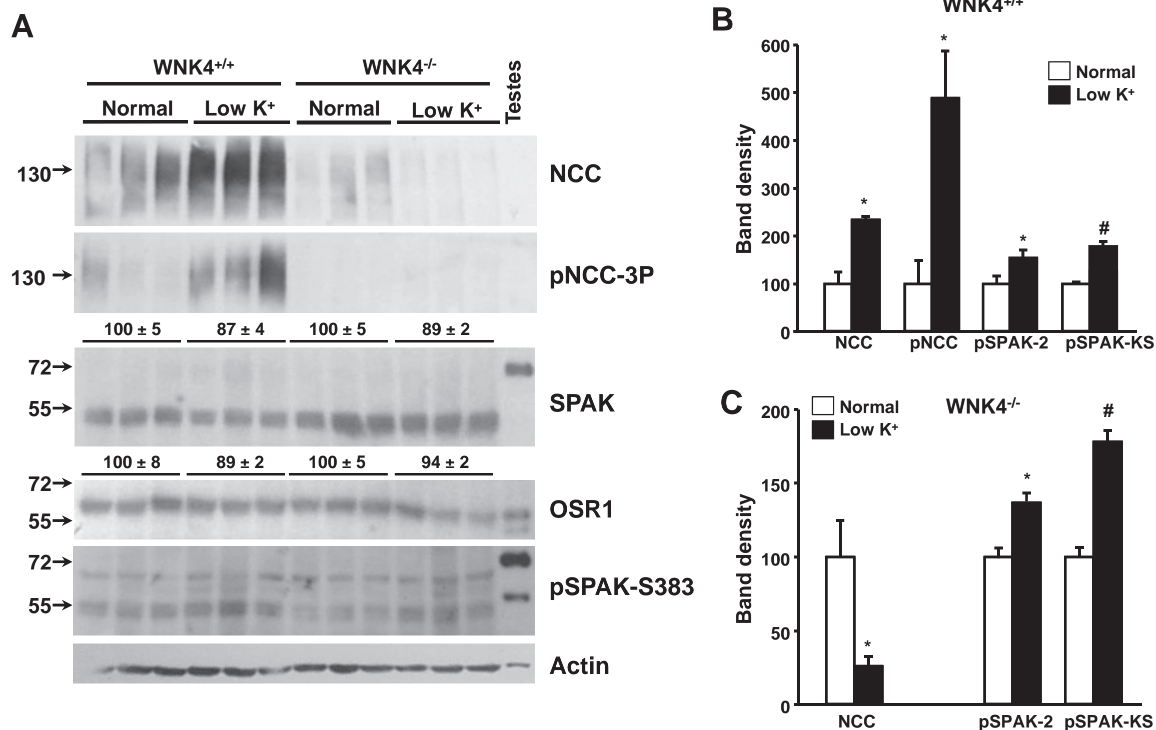

Fig. 5. Effects of low-K^+ diet on the expression and phosphorylation of Ste20-related proline-alanine-rich kinase (SPAK) and NCC in the renal cortex of with-no-lysine kinase 4 (WNK4)$^{+/+}$ and WNK4$^{-/-}$ mice. *A*: Western blot analysis of renal cortex protein samples of WNK4$^{+/+}$ or WNK4$^{-/-}$ 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.

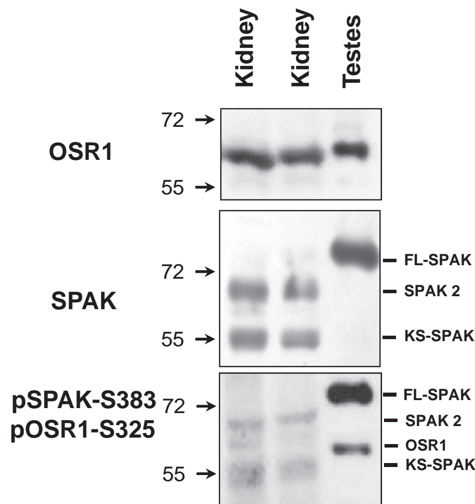

Fig. 6. Characterization of the SPAK Ser[383]/OSR1 Ser[325] phospho-antibody. Proteins extracted from mouse kidneys or testes were subjected to Western blot analysis with SPAK- and OSR1-specific antibodies and with a SPAK/OSR1 pSer[375]/Ser[325] (S-motif) antibody. Labels on the right indicate to which 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).

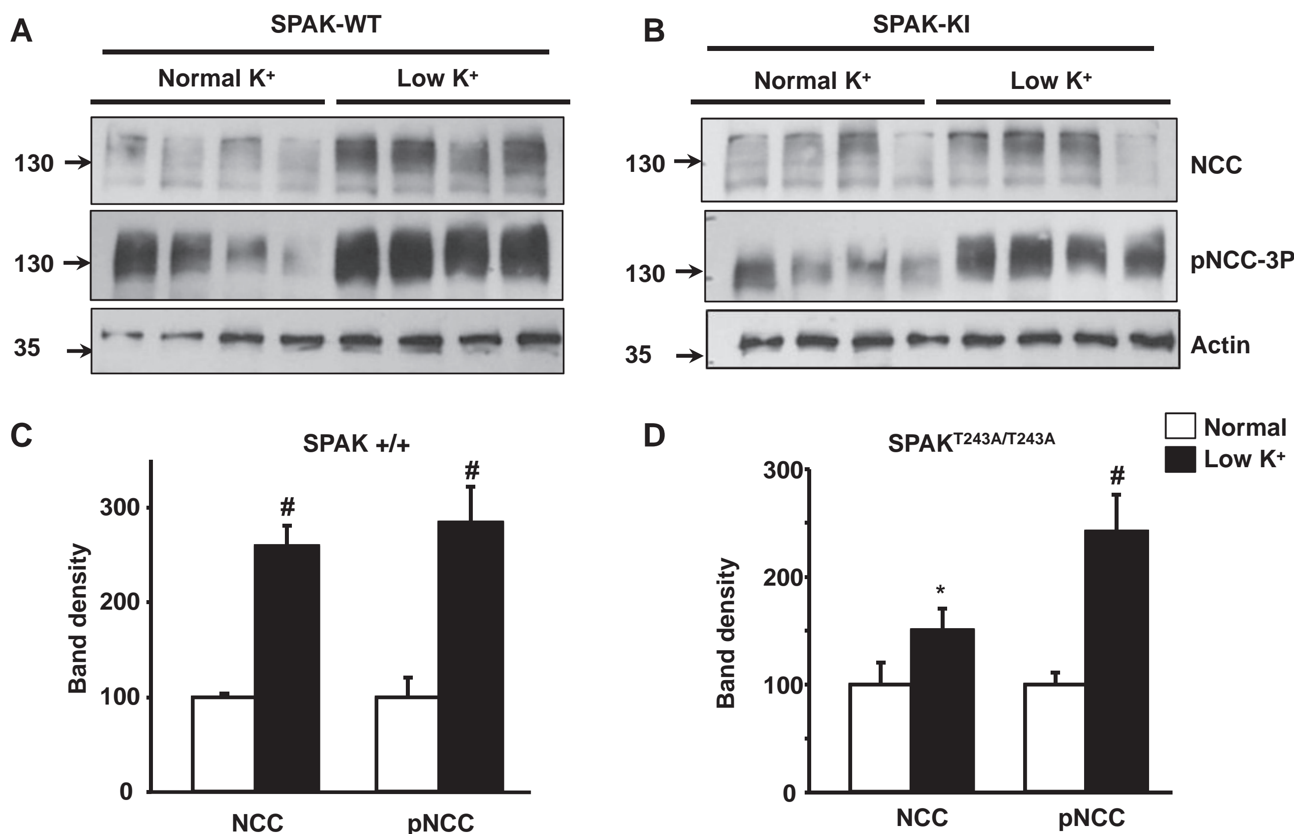

Fig. 7. Effects of low-K^+ diet on the expression and phosphorylation of NCC in $SPAK^{T243A/T243A}$ mice. *A* and *B*: Western blot analyses of total kidney protein samples of $SPAK^{+/+}$ [wild type (WT); *A*] and $SPAK^{T243A/T243A}$ [knockin (KI); *B*] mice kept on normal- or low-K^+ diets. Representative 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

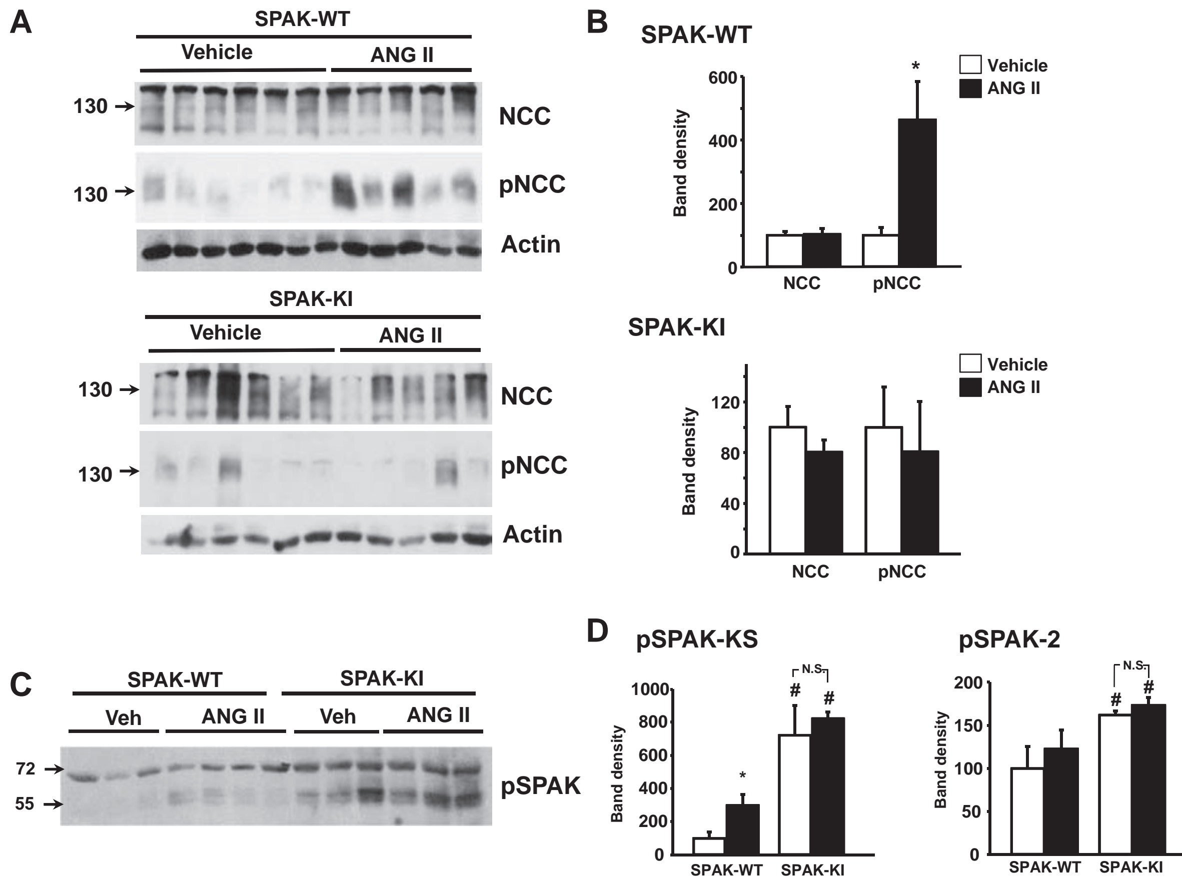

Fig. 8. Effects of ANG II infusion on the expression and phosphorylation of SPAK and NCC in SPAK$^{T243A/T243A}$ mice. *A*: Western blot analysis of NCC and pNCC in total kidney protein samples of wild-type SPAK and SPAK$^{T243A/T243A}$ mice infused with vehicle or ANG II. *B*: densitometric analyses 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

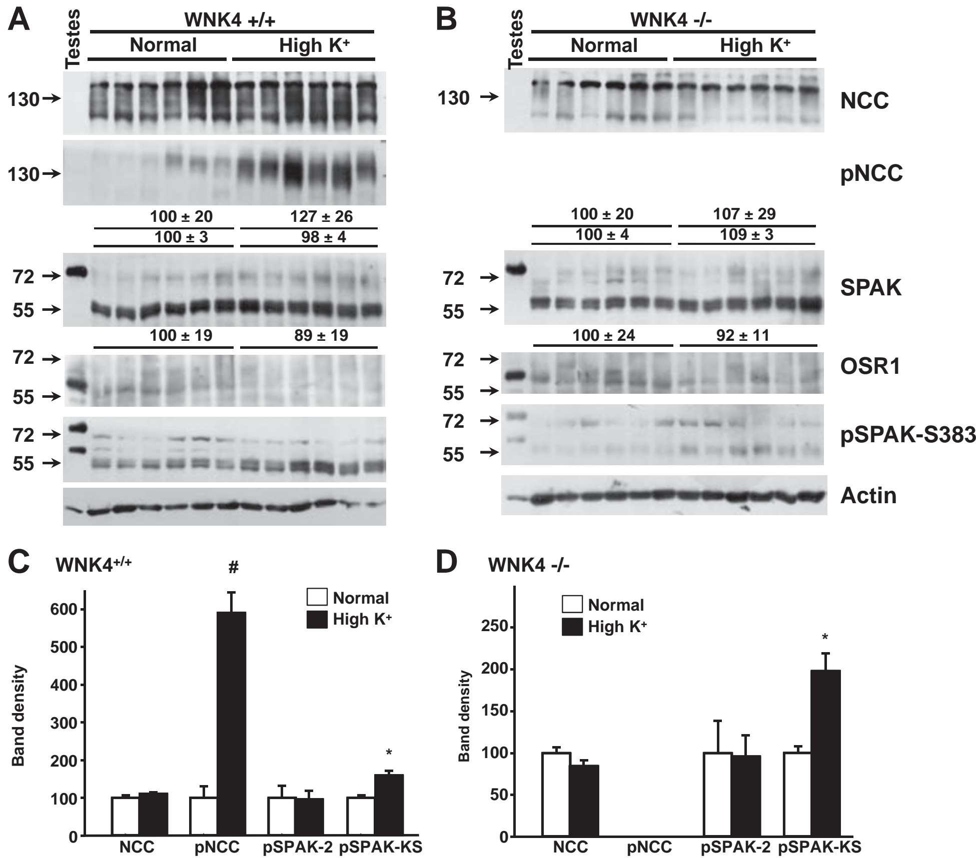

Fig. 9. Effects of high-K^+ diet on the expression and phosphorylation of SPAK and NCC in $WNK4^{-/-}$ mice. *A* and *B*: Western blot analyses of total kidney protein samples of $WNK4^{+/+}$ (*A*) or $WNK4^{-/-}$ mice (*B*) kept on normal- or high-K^+ diets. Representative blots are shown. *C* and *D*: 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

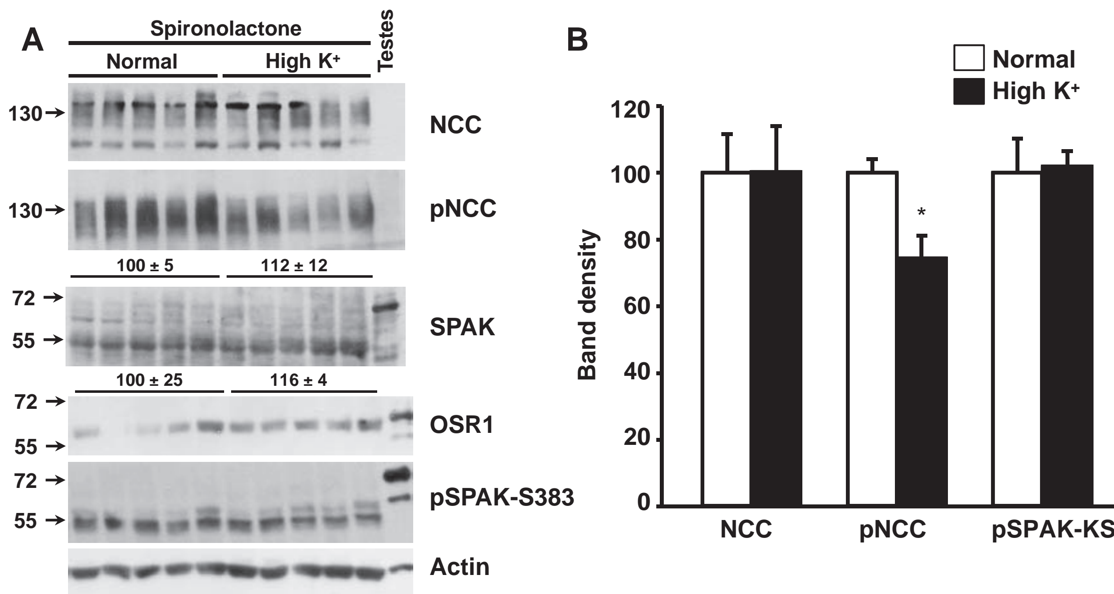

Fig. 10. Effects of high-K^+ diet on NCC and SPAK phosphorylation are blocked by spironolactone. Wild-type mice were kept on normal or high-K^+ diets. Ethanol-dissolved spironolactone or vehicle (ethanol) was added to the drinking water. The calculated dose was 40 $mg \cdot kg^{-1} \cdot day^{-1}$. *A*: 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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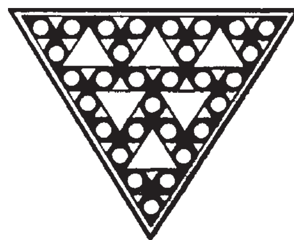

WNK-SPAK-NCC Cascade Revisited

WNK1 Stimulates the Activity of the Na-Cl Cotransporter via SPAK, an Effect Antagonized by WNK4

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Abstract—The with-no-lysine (K) kinases, WNK1 and WNK4, are key regulators of blood pressure. Their mutations lead to familial hyperkalemic hypertension (FHHt), associated with an activation of the Na-Cl cotransporter (NCC). Although it is clear that WNK4 mutants activate NCC via Ste20 proline-alanine–rich kinase, the mechanisms responsible for WNK1-related FHHt and alterations in NCC activity are not as clear. We tested whether WNK1 modulates NCC through WNK4, as predicted by some models, by crossing our recently developed WNK1-FHHt mice ($WNK1^{+/FHHt}$) with $WNK4^{-/-}$ mice. Surprisingly, the activated NCC, hypertension, and hyperkalemia of $WNK1^{+/FHHt}$ mice remain in the absence of WNK4. We demonstrate that WNK1 powerfully stimulates NCC in a WNK4-independent and Ste20 proline-alanine–rich kinase–dependent manner. Moreover, WNK4 decreases the WNK1 and WNK3-mediated activation of NCC. Finally, the formation of oligomers of WNK kinases through their C-terminal coiled-coil domain is essential for their activity toward NCC. In conclusion, WNK kinases form a network in which WNK4 associates with WNK1 and WNK3 to regulate NCC. **(*Hypertension*. 2014;64:1047-1053.)** • **Online Data Supplement**

Key Words: familial hypertensive hyperkalemia ■ hypertension, renal ■ kidney tubules, distal ■ mice, knockout ■ pseudohypoaldosteronism, type II ■ water-electrolyte balance ■ *Xenopus laevis*

Arterial hypertension is now the largest contributor to premature morbidity and mortality worldwide.[1] Although increased renal salt reabsorption is recognized as a crucial pathogenic factor, the underlying pathophysiological mechanisms remain unknown in most patients. To identify pathways, the disturbance of which could cause hypertension, one of the strategies used is the genetic analysis of rare monogenic hypertensive or salt-losing disorders, such as familial hyperkalemic hypertension (FHHt; OMIM 145260; Gordon syndrome or pseudohypoaldosteronism type II). FHHt is a rare disease of hypertension, hyperkalemia, and metabolic hyperchloremic acidosis.[2] Genetic analysis of patients with FHHt showed that the disease results from mutations in genes encoding the serine/threonine kinases, WNK1 or WNK4 (with-no-lysine [K] kinases 1 and 4),[3] and components of an ubiquitin–ligase complex, KLHL3 (Kelch-like 3) and Cullin3.[4] These proteins seem to belong to the same blood pressure regulating pathway, as WNK1 and WNK4 are recruited by KLHL3 as substrates for ubiquitination by the Cul3-based E3 RING-type ubiquitin–ligase complex.[5]

The high sensitivity of patients to thiazides suggested that FHHt was caused by an activation of NCC, the Na^+-Cl^- transporter of the distal convoluted tubule (DCT) inhibited by this class of diuretics. Subsequent to the discovery of WNK kinases, the efforts of several groups focused on characterizing

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the mechanisms underlying the regulation of NCC by WNK1 and WNK4. The results were, however, often contradictory and did not allow the establishment of a clear and unifying model to explain how WNKs regulate the expression and activity of the cotransporter.

We first reported that WNK4 inhibits NCC activity in *Xenopus laevis* oocytes,[6,7] an effect reversed by angiotensin II.[8] This inhibitory effect was also observed in mouse DCT cells,[9] endogenously expressing WNK4 and NCC, suggesting that it is not an overexpression artifact. The mechanisms by which WNK1 regulates NCC are less well defined. Biochemical studies showed that the catalytically active WNK1 isoform, Long-WNK1 (L-WNK1), activates the Ste20 proline-alanine–rich kinase (SPAK),[10,11] responsible for phosphorylating and activating NCC directly.[12] However, these data were not supported by studies performed in *Xenopus* oocytes. In this system, L-WNK1 failed to activate NCC but relieved NCC from WNK4-mediated inhibition,[7] suggesting that L-WNK1 lies upstream of WNK4 to activate NCC. The apparent discrepancies between results obtained using different model systems have generated substantial confusion concerning WNK effects.

To identify the pathway(s) involved in vivo in WNK1-dependent activation of NCC, we generated a mouse model of FHHt caused by *WNK1* mutations (*WNK1*$^{+/FHHt}$ mice).[13] This model exhibited a full FHHt phenotype, resulting from an increased expression of L-WNK1 in the DCT and, to a lesser extent, in the connecting tubule. As expected, the abundance of total and phosphorylated NCC at the apical membrane of DCT cells was enhanced. We also showed that phosphorylated SPAK abundance is increased near the apical membrane of DCT cells of mutant mice. These data support the existence of a WNK1/SPAK/NCC cascade but do not indicate whether SPAK/NCC is stimulated directly by WNK1 or whether the effects result from relief of WNK4 inhibition.

The present study therefore tested the role of WNK1–WNK4 interaction in vitro and in vivo. Our results provide evidence that WNK1 is a powerful activator of NCC, independent of WNK4. In addition, we show that WNK4 antagonizes the activating effect of WNK1 on the cotransporter. Together, these results help resolve existing controversies and elucidate the mechanisms by which L-WNK1 and WNK4 signal to NCC.

Methods

A detailed Methods section is available in the online-only Data Supplement.

Results

WNK1-FHHt Mice Retain the Full FHHt Phenotype in the Absence of WNK4

Mutations identified at the *WNK1* locus in patients with FHHt are large deletions of the first intron.[3] We recently developed a mouse model for *WNK1*-FHHt by deleting the first intron of mouse *WNK1* (*WNK1*$^{+/FHHt}$ mice).[13] This model recapitulates the FHHt phenotype, with elevated arterial pressure, hyperkalemia, and hyperchloremic metabolic acidosis. We used these mice to test the interaction between WNK1 and WNK4 in vivo, by crossing the *WNK1*$^{+/FHHt}$ animals with *WNK4*$^{-/-}$ mice. The latter displayed decreased abundance of total and phosphorylated NCC, associated with hypochloremic metabolic alkalosis and increased renin and hematocrit.[14,15] If WNK1 can activate SPAK and NCC, independent of WNK4, as suggested by biochemical studies, the phenotype of *WNK1*$^{+/FHHt}$ might persist when WNK4 is inactivated. As shown in the Table and Figure 1, *WNK1*$^{+/FHHt}$:*WNK4*$^{-/-}$ animals retained the FHHt phenotype, including elevated systolic blood pressure (*WNK1*$^{+/i1lox}$, 98±2.6; *WNK1*$^{+/FHHt}$, 109±3; and *WNK1*$^{+/FHHt}$:*WNK4*$^{-/-}$, 112±8; $P<0.05$). The increased extracellular volume was also evidenced by a decreased renin expression in both *WNK1*$^{+/FHHt}$ and *WNK1*$^{+/FHHt}$:*WNK4*$^{-/-}$. Moreover, the increased NCC expression and phosphorylation seen in *WNK1*$^{+/FHHt}$ mice were still present in the double mutants (Figure 1B).

We did not measure the blood pressure of *WNK4*$^{-/-}$ mice as previous studies, including ours, reported that it was not different from that of control mice.[14,15] However, we measured renin expression and found that it was increased in *WNK4*$^{-/-}$ mice, similar to what we showed previously.[14] Finally, it is important to note that we used the tail-cuff method to measure the blood pressure of all mice. Although methods using tail cuff are validated and approved, such methods are less sensitive than telemetric ones. Thus, small differences in arterial pressure may have gone undetected in the current study.[16]

These data show that *WNK1* mutations cause FHHt by increasing the level of expression of L-WNK1 in the distal nephron, which then activates NCC independently of WNK4. As prior studies by others and ourselves[7,17] found that L-WNK1 did not regulate NCC directly in *Xenopus* oocytes, we decided to revisit the effects of L-WNK1 in this system and in cells.

Kidney-Enriched L-WNK1 Isoform Activates NCC in a SPAK-Dependent Manner

Several isoforms of WNK1 are generated by alternative splicing of 8 exons in humans and mice, and we showed that the isoform lacking only exon 11 (L-WNK1-Δ11) is the major variant in the nephron (≈70% of all WNK1 isoforms).[18] This isoform was, however, not the one used in the studies aimed at characterizing the regulation of NCC by L-WNK1,[7] which used the isoform lacking both exons 11 and 12.[19] Therefore, the lack of L-WNK1 effect on NCC in expression systems could be because of the use of this isoform. We tested the effect of the L-WNK1 variants on NCC activity in *Xenopus* oocytes and human embryonic kidney 293 (HEK293) cells. L-WNK1-Δ11 induced a 3.25-fold

Table. ***WNK1*$^{+/FHHt}$:*WNK4*$^{-/-}$ Mice Still Exhibit FHHt Metabolic Abnormalities**

Plasma	*WNK4*$^{-/-}$ (n=4–5)	Controls (n=14–22)	*WNK1*$^{+/FHHt}$ (n=15–29)	*WNK1*$^{+/FHHt}$:*WNK4*$^{-/-}$ (n=13)
Na^+, mmol/L	144.5±0.5	147.4±0.4	147.7±0.3	149.0±0.4*
K^+, mmol/L	3.9±0.1	4.1±0.1	4.9±0.1†	5.0±0.1†
Cl^-, mmol/L	107.0±0.7†	111.1±0.4	114.6±0.3†	115.3±0.5†
pH	7.39±0.01‡	7.32±0.01	7.28±0.01‡	7.28±0.02*
HCO_3^-, mmol/L	27.2±1.5‡	24.6±0.4	22.7±0.4*	23.6±0.6
Hematocrit	47.2±0.5‡	42.8±0.6	39.7±0.5†	39.3±0.6‡

Blood was sampled from the retro-orbital sinus and blood gas and electrolytes were measured using a Radiometer ABL80 Flex analyzer. FHHt indicates familial hyperkalemic hypertension; and WNK, with-no-lysine (K) kinase.

*$P<0.05$, †$P<0.001$, and ‡$P<0.01$ compared with controls (1-way ANOVA followed by Dunnet multiple comparisons test).

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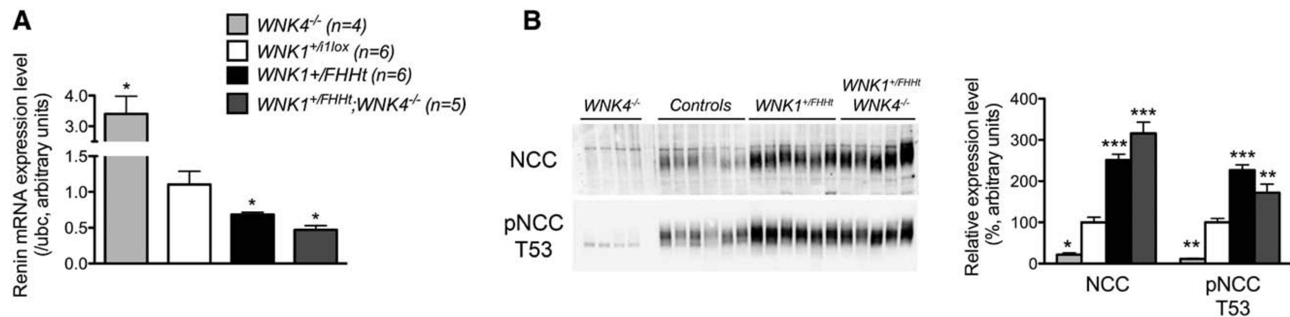

Figure 1. Long–with-no-lysine (K) kinase 1 (L-WNK1) induces hyperkalemic hypertension and Na-Cl cotransporter (NCC) phosphorylation independently of WNK4 in mice. **A**, Renin transcripts level was measured by quantitative real-time polymerase chain reaction in the kidney cortex of *WNK4*$^{-/-}$ (n=4), *WNK1*$^{+/i1lox}$ (n=5), *WNK1*$^{+/FHHt}$ (n=6), and *WNK1*$^{+/FHHt}$*:WNK4*$^{-/-}$ (n=3) males. Results are expressed in arbitrary units relative to the expression of ubiquitin c (ubc). The control group (*WNK1*$^{+/i1lox}$ mice) has been arbitrarily set to 1. Data are mean±SEM **P*<0.05 compared with the control group. **B**, **Left**, Immunoblots performed on membrane-enriched fraction of renal cortex dissected from *WNK4*$^{-/-}$ (n=4), *WNK1*$^{+/i1lox}$ (n=6), *WNK1*$^{+/FHHt}$ (n=6), and *WNK1*$^{+/FHHt}$*:WNK4*$^{-/-}$ (n=5) males, incubated with antibodies against anti-NCC or its T53 phosphorylated residue (pNCC-T53). **Right**, Densitometric analysis of the immunoblots. Data, expressed as a percentage of the average signal intensity measured in the control group, set arbitrarily to 100%, are mean±SEM **P*<0.05, ***P*<0.01, and ****P*<0.001 compared with the control group (Student unpaired *t* test).

increase in NCC activity (Figure 2A), associated with increased membrane abundance of total and phosphorylated NCC (Figure S1 in the online-only Data Supplement). With the exception of L-WNK1-Δ9, variants containing all exons or a combination of the alternatively spliced exons 9, 11, and 12 also activated NCC, but significantly less than L-WNK1-Δ11 (Figure 2A). This effect was kinase dependent as it was absent when WNK1 D368A, known to lack kinase activity,[19] was used (Figure 2B). Previous studies did not observe the direct activation of NCC by the rat L-WNK1-Δ11 to 12 variant first because it is less potent than L-WNK1-Δ11 (Figure 2A) and, second, because it carries a previously unsuspected mutation in the C-terminal domain, identified by sequencing (G2120S; Figure S2A). This mutation reduced the effect of L-WNK1 on the cotransporter. Replacing the serine residue by a glycine in the rat L-WNK1-Δ11 to 12 cDNA restored NCC activation (Figure S2B), whereas mutating the glycine into a serine in the human L-WNK1-Δ11 cDNA prevented NCC activation (Figure S2C).

To test the requirement for SPAK in L-WNK1–mediated activation of NCC, we disrupted the L-WNK1/SPAK interaction by eliminating a SPAK-binding motif in L-WNK1-Δ11. Disruption of a similar motif in WNK3 blocks its ability to activate NCC.[20] This mutant (L-WNK1-Δ11-F316A) not only failed to stimulate NCC but also inhibited the cotransporter (Figure 2B). Because oocytes express L-WNK1[21] and L-WNK1 oligomerizes,[22] we hypothesized that L-WNK1-Δ11-F316A exerted this effect through interactions with endogenous L-WNK1. Thastrup et al[23] showed that a small HQ motif (HIQEVVSLQT), located in the C-terminal coiled-coil domain, is essential for the formation of homo and heteromers of WNK kinases. The replacement of the HIQEVVSLQT sequence by AIQEVVSLAT impairs the interaction of WNK kinases.[23] Introduction of these mutations into L-WNK1-Δ11 (Δ11HQ/AA) prevented the activation of NCC. Moreover, mutation of the HQ motif of L-WNK1-Δ11-F316A abrogated its inhibition (Δ11-F136A-HQ/AA; Figure 2B). This suggests that basal NCC activity in vitro is dependent on endogenous L-WNK1 and that L-WNK1-Δ11-F316A acts as dominant negative form. To confirm that basal NCC activity in vitro requires endogenous L-WNK1 activity, we used NCC-transfected mammalian kidney cells, which express, glycosylate, and phosphorylate NCC in a manner that resembles mammalian kidney (Figure S3). Overexpression of human L-WNK1-Δ11 increased the abundance of total and phosphorylated NCC (Figure 2C). Conversely, L-WNK1 small interfering RNA–mediated knockdown (Figure 2D) reduced phosphorylated NCC abundance. These results demonstrate that kidney-enriched isoform of L-WNK1 is a powerful activator of NCC through a WNK4-independent mechanism because WNK4 is not expressed in HEK293 cells (Figure S3).

L-WNK1 Effect on NCC Is Antagonized by WNK4

WNK4 inhibits NCC in *Xenopus* oocytes,[6,7] mammalian cells,[24] and mice.[25] In addition, WNK4 prevents the positive effect exerted by WNK3 on NCC[26] (Figure 3A). We observed the same effect when WNK4 and L-WNK1-Δ11 were coexpressed, in *Xenopus* oocytes and HEK293 cells (Figure 3A and 3B). The decreased NCC activity observed when WNK4 and L-WNK1-Δ11 are coexpressed could result from the inhibition of L-WNK1-Δ11 by WNK4 or from the parallel action of an activator (L-WNK1-Δ11) and an inhibitor (WNK4). The former could be caused by a reduced L-WNK1-Δ11 abundance mediated by WNK4. Figure 3C and 3D shows that WNK4 significantly reduced the abundance of L-WNK1-Δ11 in both oocytes and HEK293 cells. The effects to reduce L-WNK1 were not simply the result of an overexpression artifact. Indeed, overexpression of WNK3 did not modify L-WNK1-Δ11 (Figure S4A) and overexpression of WNK4 did not affect SPAK or pSPAK abundance in HEK293 cells (Figure S4B).

We next assessed the requirement for the formation of WNK4 oligomers by mutating the C-terminal HQ motif.[23] The resulting WNK4-HQ/AA mutant lost the capacity to inhibit NCC (Figure 3E) and precluded the ability of WNK4 to inhibit L-WNK1–induced or WNK3-induced activation of NCC in oocytes (Figure 3E). These functional observations indicate that interactions of WNKs kinases via the HQ motif are necessary for their effects on NCC.

Discussion

Since the discovery of WNK kinases and their relation to FHHt, an inherited disease featuring arterial hypertension, a great deal

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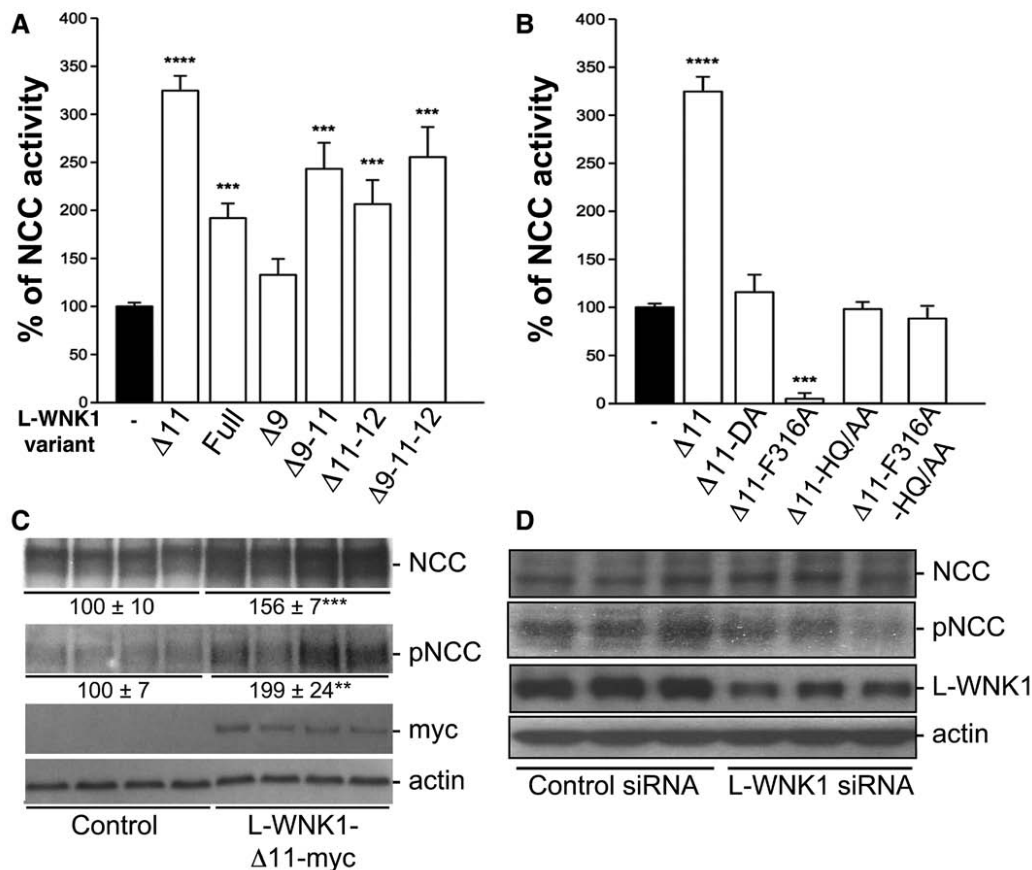

Figure 2. Long–with-no-lysine (K) kinase 1 (L-WNK1) is a powerful activator of Na-Cl cotransporter (NCC) activity in *Xenopus laevis* oocytes. **A**, NCC activity was measured in *Xenopus* oocytes overexpressing NCC alone or in combination with different L-WNK1 splicing variants. All L-WNK1 variants, except the one deleted from exon 9 (L-WNK1-Δ9), stimulated NCC activity. **B**, Effect of L-WNK1-Δ11 point mutations affecting its kinase activity (Δ11-DA), Ste20 proline-alanine–rich kinase binding (Δ11-F316A), dimer formation (Δ11-HQ), or both (Δ11-F316A-HQ) on NCC activity. For **A** and **B**, NCC activity in oocytes injected with NCC cDNA alone (black bar) was arbitrarily set to 100%. Data are mean±SEM. ****P*<0.001 and *****P*<0.0001 vs NCC alone (Student unpaired *t* test). **C**, Immunoblots from NCC–human embryonic kidney 293 (HEK293) cells transiently transfected with NCC and human L-WNK1-Δ11-myc constructs and probed with antibodies indicated. Data, indicated below the blots, are mean±SEM. ***P*<0.01 and ****P*<0.001 vs the control (Student unpaired *t* test). **D**, The inhibition of L-WNK1 expression by RNA-interference decreased NCC phosphorylation. Immunoblots from NCC-HEK293 cells transfected with control and WNK1-specific small interfering RNA (siRNA), probed with the antibodies indicated. pNCC indicates phosphorylated NCC.

of effort has been directed at understanding how WNK kinase mutations produce the disease. As all FHHt symptoms are corrected by a thiazide treatment and as the expression of the Na-Cl cotransporter NCC is increased in FHHt mouse models, several groups aimed at understanding how the WNK kinases modulate NCC activity. However, the results thus obtained failed to generate an unifying model. In the present study, we show data supporting a revised model of WNK kinase action in signaling to NCC, in which WNK1 and WNK3 mediate activation of NCC through SPAK, and WNK4 antagonizes WNK1 and WNK3 (Figure 4).

This model can explain several contradictory observations. First, the demonstration that L-WNK1 is a strong stimulator of NCC activity clarifies the pathophysiology of FHHt (Figure S5) because increased L-WNK1 expression in the DCT is sufficient to promote the development of the FHHt phenotype.[13] Second, it fits with the in vitro observations that L-WNK1 is a more powerful activator of SPAK than WNK4.[11] Third, the new model provides a plausible explanation for the discrepancy between the mild phenotype consequent to WNK3 deletion in vivo, and the powerful ability of WNK3 to activate NCC in vitro.[26,27] Because both WNK1 and WNK3 can activate NCC, the increased WNK1 expression observed in *WNK3*$^{-/-}$ mice likely compensates for the loss of WNK3.[28,29]

Previous studies failed to demonstrate the WNK1 effect on NCC for 2 reasons. First, those studies did not use the L-WNK1 isoform lacking exon 11, the most abundant isoform along the nephron[18] and the most powerful NCC activator. Instead, the studies used an isoform lacking both exons 11 and 12, which represents only 20% of WNK1 isoforms in the kidney.[18] Second, the previously used rat L-WNK1-Δ11 to 12 cDNA carries a mutation in the C-terminal region of the protein that reduces its effect on NCC. This cDNA corresponds to the first isolated L-WNK1 cDNA, which was subcloned from a rat brain library,[19] where the variant missing both exons 11 and 12 is the predominant variant.[18]

Coexpression of L-WNK1-Δ11 and WNK4 results in a decreased NCC activity compared with L-WNK1-Δ11 alone. This could result from the inhibition of L-WNK1-Δ11 by WNK4 or from the parallel action of an activator (L-WNK1-Δ11) and an inhibitor (WNK4). WNK kinases can form homo or heteromers via a conserved carboxyl-terminal domain.[22,30] Coimmunoprecipitation experiments indicated that

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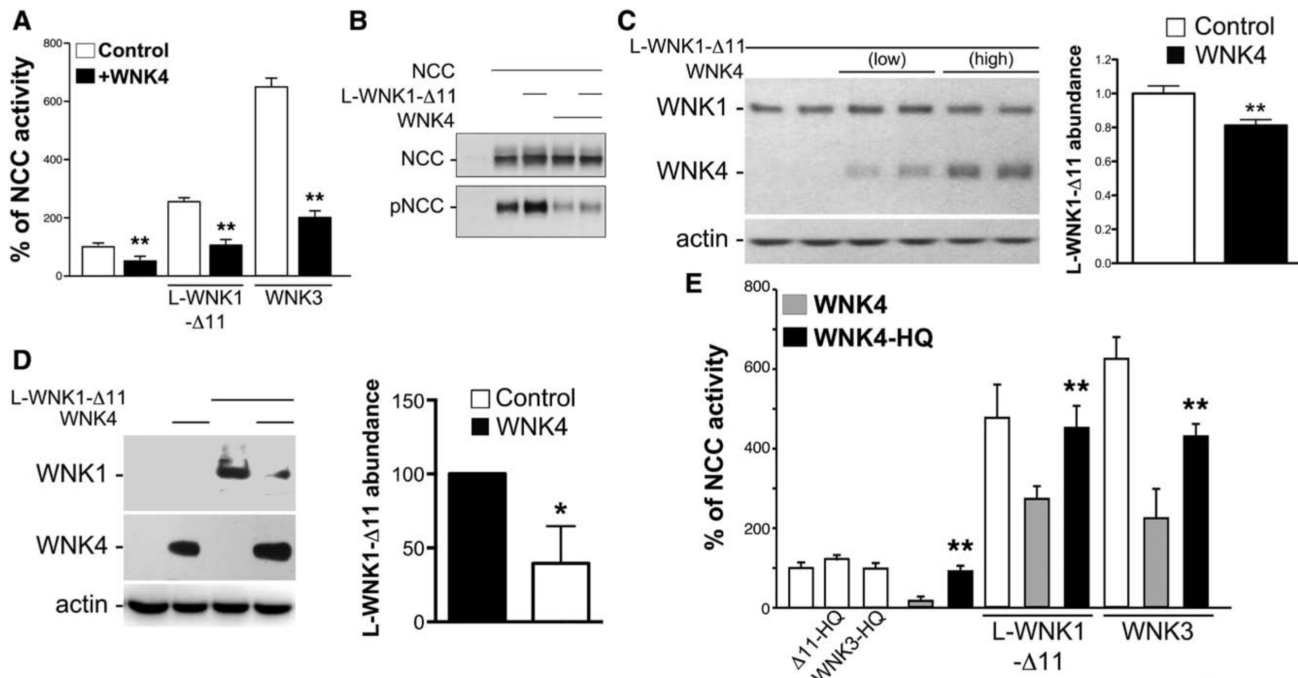

Figure 3. With-no-lysine kinase 4 (WNK4) inhibits the activation of Na-Cl cotransporter (NCC) by Long-WNK1 (L-WNK1) and WNK3. **A**, NCC activity was measured in *Xenopus* oocytes injected alone or in combination with L-WNK1-Δ11 (WNK1), WNK3 and WNK4 as stated. WNK4 not only inhibited NCC activity but also prevented the L-WNK1-Δ11 or WNK3-dependent stimulation of NCC (n=15 independent experiments per combination). **B**, Immunoblots from NCC–human embryonic kidney 293 (HEK293) cells transiently overexpressing L-WNK1-Δ11 and WNK4. WNK4 overexpression prevented the activation of NCC by L-WNK1-Δ11. **C** and **D**, Immunoblots from NCC-HEK293 cells transiently transfected with myc-tagged L-WNK1-Δ11 and WNK4 constructs (**C**) or from *Xenopus* oocytes injected with myc-tagged L-WNK1-Δ11 alone or in combination with WNK4 (**D**). WNK4 overexpression significantly decreased abundance of L-WNK1-Δ11 in both systems. *P<0.05 and **P<0.01 for the comparison between L-WNK1-Δ11 alone and L-WNK1-Δ11+WNK4 (Student unpaired *t* test). **E**, NCC activity alone or in combination of wild-type or mutant-HQ WNKs as stated. The inhibition of the formation of WNKs dimers by mutating the C-terminal HQ motif relieved L-WNK1-Δ11 or WNK3 from WNK4-mediated inhibition (Δ11-HQ: L-WNK1-Δ11-HQ). For oocytes experiments, the activity measured in oocytes injected with NCC alone was arbitrarily set to 100% and data are mean±SEM. **P<0.01 for the comparison between wild-type WNK4 and WNK4-HQ (Student unpaired *t* test). pNCC indicates phosphorylated NCC.

L-WNK1 interacts with both WNK4 and WNK3 in HEK293 cells through a C-terminal conserved HQ motif.[23] We show here that the interaction among L-WNK1-Δ11, WNK3, and WNK4 is essential for the regulation of NCC, as the mutation of their HQ motif abrogates the effect of all 3 kinases. Our study does not, however, allow us to determine which homo or heteromers are required for NCC regulation. The disruption of the interaction by mutating the HQ motif results in a decreased capacity of WNK3 to autophosphorylate and phosphorylate WNK1 on the T-loop residue (Ser382),[23] required for WNK1 activation. Inhibiting the formation of activating WNK3 homomers and L-WNK1/WNK3 heteromers could therefore prevent the activation of NCC by L-WNK1 or WNK3 in oocytes. Similarly, preventing the formation of WNK4 homomers reduced its autophosphorylation. Interestingly, Thastrup et al[23] showed that, although WNK4 can interact with WNK1, it is unable to phosphorylate its T-loop residue. The expression of WNK4 in oocytes could thus result in the formation of inhibitory WNK4 homomers and L-WNK1/WNK4 or WNK3/WNK4 heteromers, in which L-WNK1 or WNK3 is not activated (Figure 4). The expression of WNK4-HQ mutant in *Xenopus* oocytes would then restore NCC activity by preventing the formation of these inhibitory homomers and heteromers.

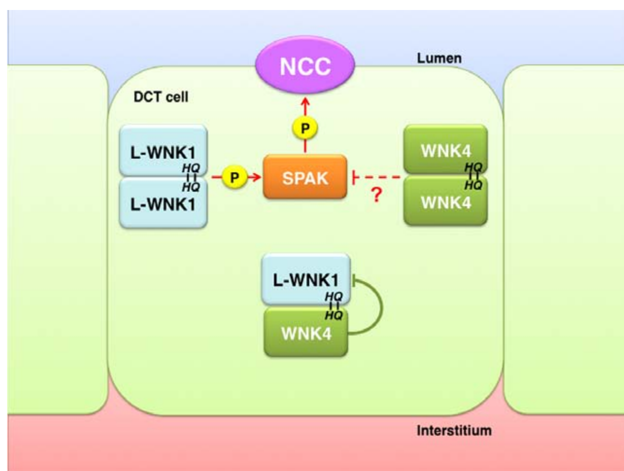

Figure 4. Proposed new model for the regulation of Na-Cl cotransporter (NCC) by with-no-lysine kinases (WNKs). Note that WNK3 is not shown and that Long-WNK1 (L-WNK1) is indicated instead of L-WNK1-Δ11 to simplify the figure. At baseline, L-WNK1-Δ11 forms homomers that activate NCC in a Ste20 proline-alanine–rich kinase (SPAK)–dependent manner. WNK4 could antagonize the effect of L-WNK11-Δ11 on NCC by either forming inactive WNK4–WNK1 heteromers or inhibitory WNK4 homomers. DCT indicates distal convoluted tubule.

The present study reconciles in vivo and in vitro observations about the mode of L-WNK1 action toward NCC, but controversy still remains concerning intrinsic WNK4 activity. Most in vitro studies, including the present one, concluded that WNK4 is an inhibitor of NCC, either when overexpressed in *Xenopus* oocytes and mammalian cells[6,7,24] or when

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endogenous WNK4 is knocked down in mDCT15 cells.[9] These results are supported by some but not all in vivo studies. Lalioti et al[25] first reported that WNK4 overexpression in transgenic mice results in a decreased NCC expression, again suggesting that WNK4 inhibits the cotransporter. This conclusion is however not supported by 2 subsequent studies. First, WNK4 inactivation in mice causes a dramatic reduction of NCC abundance and phosphorylation.[14,15] Second, Wakabayashi et al[31] observed that WNK4 overexpression caused FHHt in mice, although they used the same transgene than the one used in Lalioti's study. These 2 studies suggest that WNK4 is an activator of NCC, in vivo.

How could we reconcile the WNK4 inhibitory effect observed in vitro and in vivo with the activating effect observed in vivo? NCC inhibition by WNK4 in *Xenopus* can be abrogated by angiotensin II.[8] Similarly, the phosphorylation of SPAK by WNK4 is stimulated by an increased Ca^{2+} concentration.[32] One could therefore speculate that WNK4 inhibitory and stimulatory actions coexist and can be modulated by intracellular signals and that the environment of in situ DCT cells at baseline favors the activating mode of WNK4 , whereas the culture conditions of oocytes or mammalian cells mostly favors its inhibitory mode.

Perspectives

We present evidence that the kidney-enriched isoform of L-WNK1 stimulates NCC expression and activity in a SPAK-dependent manner and not through modulation of an inhibitory effect of WNK4, as previously reported. In addition, we show that WNK4 antagonizes WNK1- or WNK3-mediated activation of the cotransporter. A lot of questions, however, remain open before we can fully understand how the WNK kinases interact with each other to regulate the activity of the Na-Cl cotransporter. We think that one of the most important issues is to determine the WNK's relative expression level in the DCT and the characterization of the oligomers truly formed by WNK1, WNK3, and WNK4 in vivo.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Long–no-lysine (K) kinase 1 (L-WNK1) activates Na-Cl cotransporter in a WNK4-independent manner.
- WNK4 antagonizes L-WNK1 action.

What Is Significant?

- The WNKs C-terminal HQ domain is required for the regulation of Na-Cl cotransporter by the kinases.

Summary

We propose a revised model of WNK kinase action in signaling to Na-Cl cotransporter, in which WNK1 and WNK3 activate Na-Cl cotransporter through Ste20 proline-alanine–rich kinase, and WNK4 antagonizes WNK1 and WNK3.

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The Effect of WNK4 on the Na^{+}–Cl^{-} Cotransporter Is Modulated by Intracellular Chloride

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ABSTRACT

It is widely recognized that the phenotype of familial hyperkalemic hypertension is mainly a consequence of increased activity of the renal Na^{+}–Cl^{-} cotransporter (NCC) because of altered regulation by with no–lysine–kinase 1 (WNK1) or WNK4. The effect of WNK4 on NCC, however, has been controversial because both inhibition and activation have been reported. It has been recently shown that the long isoform of WNK1 (L-WNK1) is a chloride-sensitive kinase activated by a low Cl^{-} concentration. Therefore, we hypothesized that WNK4 effects on NCC could be modulated by intracellular chloride concentration ($[Cl^{-}]_i$), and we tested this hypothesis in oocytes injected with NCC cRNA with or without WNK4 cRNA. At baseline in oocytes, $[Cl^{-}]_i$ was near 50 mM, autophosphorylation of WNK4 was undetectable, and NCC activity was either decreased or unaffected by WNK4. A reduction of $[Cl^{-}]_i$, either by low chloride hypotonic stress or coinjection of oocytes with the solute carrier family 26 (anion exchanger)-member 9 (SLC26A9) cRNA, promoted WNK4 autophosphorylation and increased NCC-dependent Na^{+} transport in a WNK4-dependent manner. Substitution of the leucine with phenylalanine at residue 322 of WNK4, homologous to the chloride-binding pocket in L-WNK1, converted WNK4 into a constitutively autophosphorylated kinase that activated NCC, even without chloride depletion. Elimination of the catalytic activity (D321A or D321K-K186D) or the autophosphorylation site (S335A) in mutant WNK4-L322F abrogated the positive effect on NCC. These observations suggest that WNK4 can exert differential effects on NCC, depending on the intracellular chloride concentration.

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Essential hypertension is a major risk factor for mortality worldwide. Salt reabsorption by the kidney plays a central role in its development. All monogenic diseases featuring high or low blood pressure are caused by mutations in genes encoding the renal salt transporters or their regulatory pathways.[1] Among those are disorders affecting the activity of the renal Na^{+}:Cl^{-} cotransporter (NCC). Inactivating mutations of NCC result in Gitelman's syndrome (OMIM 263800) featuring arterial hypotension. In contrast, NCC activation triggers familiar hyperkalemic hypertension (OMIM 145260). This syndrome is caused in some patients by mutations in with no lysine (K) kinase 4 (WNK4). The understanding of NCC regulation by WNK4 has been complicated by contradictory findings. Many groups have reported that WNK4 reduces activity, phosphorylation, and half-life of NCC *in vitro* and *in vivo*.[2–7] However, evidence for WNK4-induced NCC activation has also been documented.[8,9] In this regard, the WNK4 inhibitory effect could be reversed by angiotensin II,[10] suggesting that WNK4 inhibitory and activating effects may coexist and be modulated by the renin-angiotensin system.[8,10] A reduction of $[Cl^{-}]_i$ is associated with increased activity and phosphorylation of NCC[11] by Ste20-related proline-alanine rich kinase (SPAK),[12] which in turn is modulated by WNKs.[13] It has been suggested that WNKs behave as chloride-sensitive kinases.[14] Therefore, we hypothesized that

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$[Cl^-]_i$ could modulate the effect of WNK4 toward NCC, potentially explaining the dual effect of the kinase.

To test this hypothesis, the functional activity of NCC was assessed in oocytes exposed to control conditions or low chloride hypotonic stress (LCHS) that is known to increase NCC phosphorylation and activity.[11,12] Coinjection of NCC and mouse WNK4 cRNA resulted in a slight but significant decrease in NCC-mediated Na^+ uptake. LCHS induced a significant increase in NCC activity, which was further enhanced by coexpression with either mouse or human WNK4 (Figure 1, A and B).

The $[Cl^-]_i$ in control oocytes was 49.8±0.7 mM, whereas it was 57.45±0.8 and 58.58±1.4 mM in oocytes injected with NCC or NCC plus WNK4 cRNAs, respectively. NCC probably caused this approximately 15% increase. In contrast, $[Cl^-]_i$ was significantly reduced by LCHS to a value of approximately 35 mM in all groups, representing a 30%–40% decrease (Figure 1C). The reduction of $[Cl^-]_i$ observed in this study is similar to that reported previously by Bertram *et al.*,[15] using an identical LCHS protocol.[16]

We next assessed how time of exposure to LCHS altered the regulation of NCC by WNK4 because previous observations have shown that $[Cl^-]_i$ slowly decreases under these conditions.[16] One hour of LCHS had no effect on Na^+ uptake in oocytes injected with NCC alone, whereas 16 hours resulted in a significant increase (Figure 1D). In contrast, WNK4 expression led to a significant activation of NCC

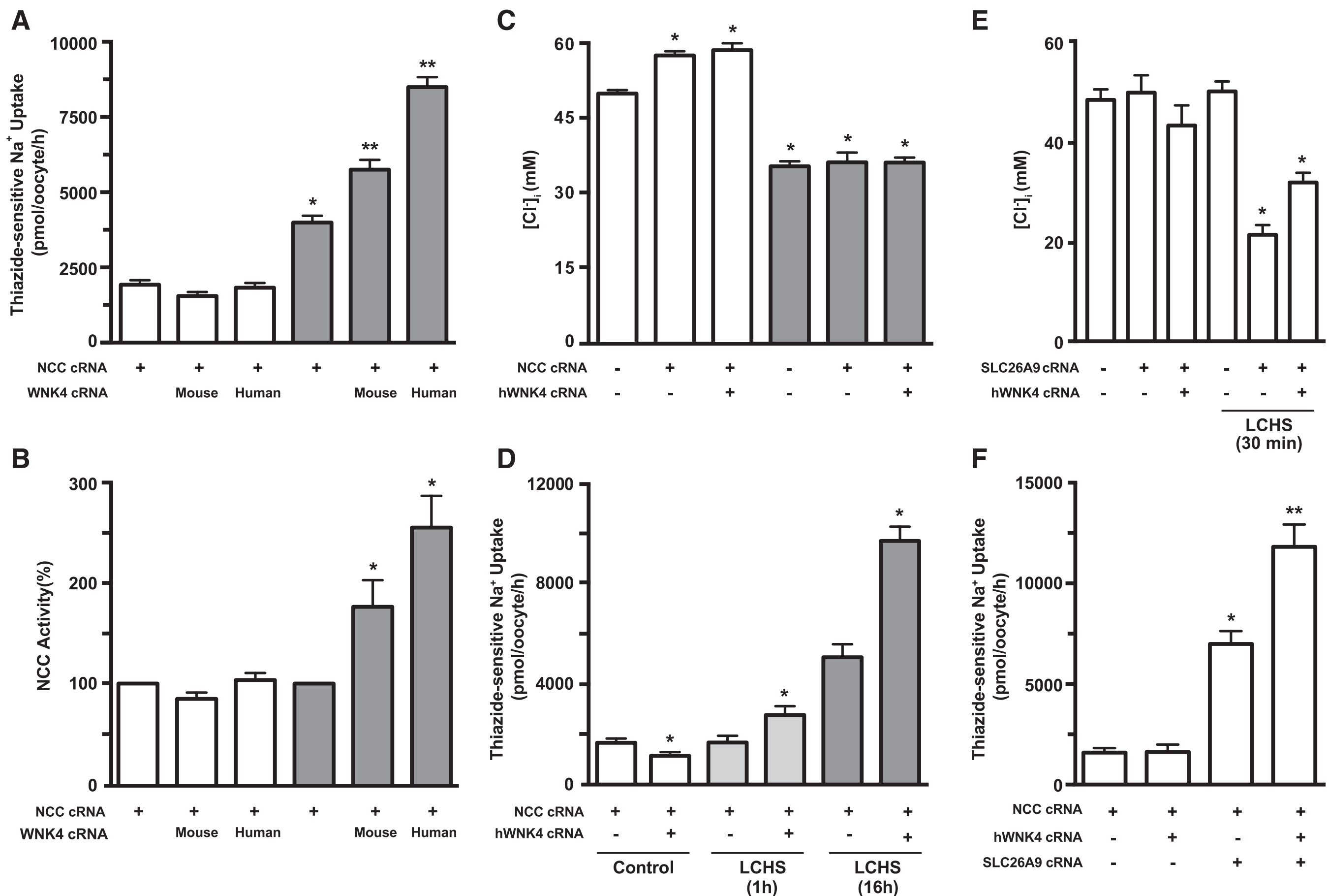

Figure 1. WNK4 effect on NCC is modulated by $[Cl^-]_i$. (A) Functional expression assay shows the thiazide-sensitive Na^+ uptake in groups of oocytes injected with NCC cRNA alone or together with mouse WNK4 or human WNK4 cRNA, as stated. Uptake was performed in control conditions (white bars) or after 16 hours of LCHS (gray bars) (see Supplemental Material). *$P<0.01$ versus same group in control conditions. **$P<0.05$ versus NCC in LCHS. n=13. (B) Uptake in oocytes injected with NCC alone, in control conditions (white bars) or LCHS (gray bars), was arbitrarily set to 100%, and the corresponding groups were normalized accordingly. *$P<0.01$ versus any group in control conditions and versus NCC in LCHS. (C) The $[Cl^-]_i$ in oocytes was assessed with custom-made glass capillary ion selective microelectrodes 48 hours after injection of water, NCC, or NCC plus WNK4 cRNA, as stated, in control conditions (white bars) or after 16 hours of LCHS (gray bars). *$P<0.05$ versus water injected in control conditions. n=2 (six oocytes per group, per experiment). (D) Uptake was performed in control conditions (white bars) or in LCHS in 1 hour (light gray bars) and 16 hours (dark gray bars) in groups injected with NCC alone or NCC plus WNK4 group, as stated. *$P<0.05$ versus own control. n=3. (E) $[Cl^-]_i$ in oocytes injected with water, SLC26A9, or SLC26A9 plus WNK4 cRNA, as stated, after 48 hours of incubation in ND96 or after 30 minutes of LCHS. *$P<0.05$ versus water in control conditions. n=3. (F) Thiazide-sensitive Na^+ uptake in oocytes injected with NCC cRNA alone and NCC plus WNK4 cRNA in the absence or presence of SLC26A9 cRNA. *$P<0.01$ versus NCC or NCC plus WNK4 groups. **$P<0.05$ versus NCC plus SLC26A9 cRNA injection.

after only 1 hour and was further increased after 16 hours. Therefore, the presence of WNK4 accelerated the effect of LCHS on NCC.

Because LCHS may have several effects on oocytes, we used an alternative method to reduce $[Cl^-]_i$ by coinjecting SLC26A9 cRNA, which in the absence of extracellular Cl^- performs as a chloride efflux pathway.[17] Indeed, 2 days after injection, the $[Cl^-]_i$ was similar in water, SLC26A9, or SLC26A9 plus WNK4 injected oocytes, whereas LCHS for only 30 minutes significantly reduced the $[Cl^-]_i$ in SLC26A9 or SLC26A9 plus WNK4, but not in water oocytes (Figure 1E). As a consequence, NCC activity was increased, and this effect was dramatically potentiated by WNK4 (Figure 1F).

Activation of NCC under LCHS is associated with increased phosphorylation of key residues in the amino-terminal domain.[11,12] To determine if the catalytic activity of WNK4 is required for NCC activation in these conditions, we used the catalytically inactive mutant WNK4-D321A.[13] This mutant not only prevented the activation of NCC by WNK4, but it also decreased its activity below the level of oocytes injected with NCC alone in LCHS (Figure 2A). Because our previous experiments suggested basal NCC activity relies on the endogenous long isoform of WNK1 (L-WNK1),[18] these results suggest WNK4-D321A may prevent the effect of endogenous L-WNK1 during LCHS.

Piala *et al.*[19] showed that L-WNK1 autophosphorylation and activity is inhibited by $[Cl^-]_i$, therefore demonstrating that L-WNK1 is a chloride-sensitive kinase. They identified two leucine residues, conserved among WNKs, in the catalytic domain of L-WNK1 (L369 and L371) that serve as the Cl^- binding site. To test whether these residues display the same function in WNK4, we replaced the homologous residues (L322 and L324) by phenylalanine (WNK4-L322F, WNK4-L324F, and the double mutant WNK4-LL-FF). WNK4-LL-FF induced a remarkable activation of NCC in control conditions (Figure 2B). Mutation of L322 alone resulted in a greater increase in NCC activity, similar to what was observed for WNK4 in LCHS. Moreover, the activation

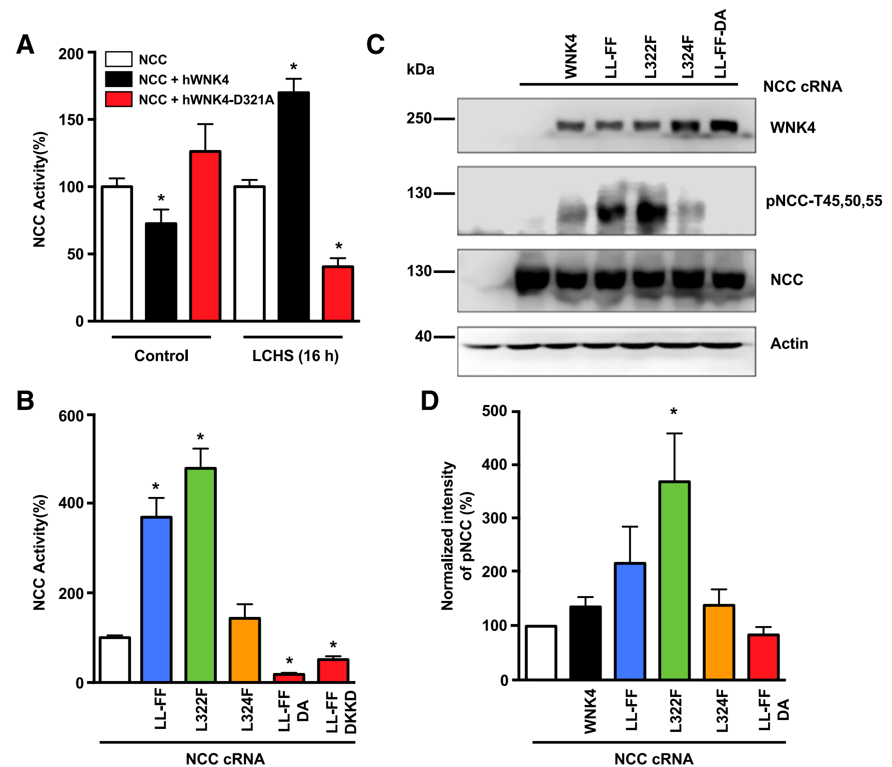

Figure 2. Elimination of the putative Cl^--binding pocket turns WNK4 into a constitutive activator of NCC. (A) NCC activity in oocytes injected with NCC cRNA (white bars), NCC plus wild-type WNK4 (black bars), or NCC plus catalytically inactive WNK4-D321A (red bars), as stated. Oocytes were incubated in the control solution or LCHS for 16 hours before the measurement of Na^+ uptake. Uptake in oocytes injected with NCC alone and incubated in control or LCHS was arbitrarily set to 100%, and the NCC plus WNK4 or NCC plus WNK4-D321A groups were normalized accordingly. *$P<0.05$ versus NCC corresponding control. $n=3$. (B) Uptake in oocytes injected with NCC alone was arbitrarily set to 100% (white bar), and the rest of the groups were normalized accordingly (as stated, WNK4-LL-FF is the blue bar, WNK4-L322F is the green bar, WNK4-L324F is the orange bar, and WNK4-LL-FF-DA and WNK4-LL-FF-DKKD are the red bars). These series of experiments were performed in control conditions only. *$P<0.01$ versus NCC alone. $n=6$. (C) Representative Western blot showing the effect of wild-type or mutant WNK4, as stated, on NCC expression and phosphorylation in control conditions. Blot shows WNK4, pNCC, NCC, and actin expression, as stated. WNK4 is present only in oocytes injected with wild-type or mutant WNK4. (D) Densitometric analysis of data compiled from five different experiments. NCC basal phosphorylation (white bar) was arbitrarily set to 100% and the corresponding groups were normalized accordingly (as stated, WNK4 wild-type (black bar), WNK4-LL-FF (blue bar), WNK4-L322F (green bar), WNK4-L324F (orange bar) and catalytically inactive WNK4-LL-FF-DA (red bar). *$P<0.05$ versus NCC.

of NCC by WNK4-LL-FF is kinase-dependent. The introduction of the D321A or the double-charge D321K-K186D mutations in WNK4-LL-FF not only prevented the activation of NCC, but also abolished most of its activity (Figure 2B). Therefore, elimination of the Cl^- binding site turned WNK4 into a constitutive activator of NCC, in a kinase-dependent manner. This was further confirmed by analysis of NCC phosphorylation.[12] WNK4-LL-FF and WNK4-L322F stimulated NCC phosphorylation on all three residues tested, whereas WNK4-LL-FF-DA completely prevented it (Figure 2, C and D).

To analyze the effect of $[Cl^-]_i$ on WNK4 activity, we determined the autophosphorylation status of serine residue S335, known to be required for WNK activation. According to Piala *et al.*,[19] phosphorylation of L-WNK1 homologous serine

(S382) is modulated by chloride. No phosphorylation of WNK4 was observed in control conditions, whereas it became evident in oocytes challenged with LCHS (Figure 3, A and B). The phosphorylation level of L-WNK1 S382 was also increased by LCHS, whereas it was similar in both conditions for WNK3 S308 (Figure 3C). Interestingly, WNK4-L322F was strong in basal conditions and was not affected by LCHS (Figure 3, D and E). These findings suggest WNK3 and WNK4-L322F are less sensitive to $[Cl^-]_i$ than L-WNK1 and WNK4. Supporting that phosphorylation of WNK4-S335 is required for NCC activation; mutation of this residue to alanine in WNK4-LL-FF and WNK4-L322F abrogated their effect on NCC (Figure 3F).

Soon after the recognition that mutations in WNK4 cause familiar hyperkalemic hypertension,[20] it was reported that WNK4 reduces the activity of NCC in *Xenopus laevis* oocytes and mammalian cells.[2–6,21] Animal models supported the *in vitro* observations because overexpression of WNK4 in transgenic mice resulted in decreased expression and phosphorylation of NCC.[7] However, one study reported increased NCC activity by human WNK4 in *Xenopus* oocytes,[22] and inactivation of WNK4 in mice resulted in a reduced NCC phosphorylation and expression.[8] Moreover, both parameters were increased in a new model of WNK4 overexpression in mice.[9] Therefore, there is evidence *in vitro* and *in vivo* for both inhibition and activation of NCC by WNK4.

Here we present data indicating that WNK4 is a chloride-sensitive kinase, potentially explaining the dual effects of WNK4 on NCC. In the presence of high Cl^- concentrations, WNK4 is inactive and may reduce basal activity of NCC by its inhibiting interaction with L-WNK1 and WNK3.[18,21] When $[Cl^-]_i$ is low, WNK4 phosphorylation, and therefore activity, is stimulated, thereby activating the cotransporter (Figure 4). Our results suggest a similar mechanism to the one proposed by Piala *et al.*[19] is probably involved in the switch of WNK4 behavior.

The apparent median inhibitory concentration of Cl^- (IC_{50}) for L-WNK1 is 20 mM. In oocytes and HEK293 cells, $[Cl^-]_i$ is around 40–50 mM,[16,17,23] therefore allowing partial activity of L-WNK1. This is consistent with our recent observation that basal NCC activity in oocytes and HEK293 cells depends on endogenous L-WNK1.[18] The increased NCC activity induced by LCHS in oocytes injected only with NCC is most likely caused by enhanced endogenous L-WNK1 activity. Coinjection of oocytes with SLC26A9 reduces $[Cl^-]_i$ to approximately 30 mM,[17] leading to activation of NCC without LCHS. In oocytes in basal conditions, the most potent activator of NCC is WNK3,[24] followed by L-WNK1,[18] whereas WNK4 may inhibit or have no effect on its basal activity. This difference in WNKs activity toward NCC could be a function of their sensitivity to chloride.[18]

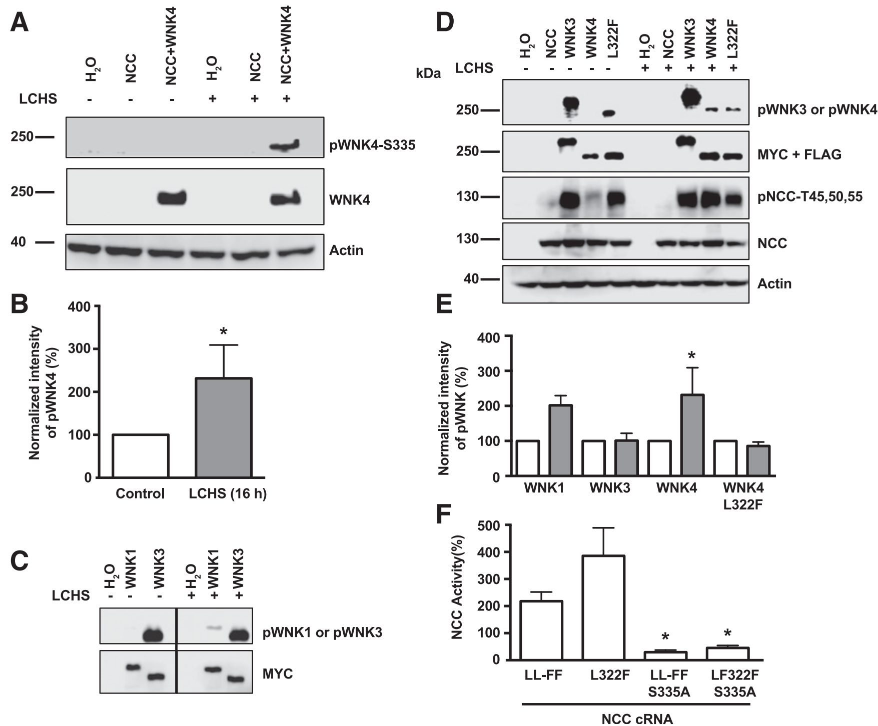

Figure 3. Modulation of WNK autophosphorylation by decrease of $[Cl^-]_i$ induced by LCHS. (A) Representative Western blot showing the effect of LCHS on wild-type WNK4 autophosphorylation, as stated, in control conditions or LCHS. Blot shows pWNK4, WNK4, and actin expression, as stated. Phosphoantibody recognizing S335 in WNK4 was developed and characterized by Thastrup *et al.*[30] (B) Densitometric analysis of compiled results from six different experiments. Phosphorylation level of WNK4 in control conditions was arbitrary set to 100% (white bar), and the phosphorylation status in LCHS was normalized accordingly (gray bar). *$P<0.05$ versus control (Wilcoxon signed-rank test). (C) L-WNK1 and WNK3 autophosphorylation at S382 and S308, respectively, in control and LCHS conditions, as stated. The upper blot depicts phospho-WNK, and the lower blot shows the total amount of L-WNK1 or WNK3 as detected using anti-Myc antibody. Original image was cropped to eliminate empty lanes and show the L-WNK1 and WNK3 lanes only. (D) Representative Western blot of phospho-WNKs (WNK3, WNK4, or mutant WNK4-L322F), total WNKs, phospho-NCC, NCC, and actin in control conditions and after LCHS, as stated. (E) Densitometric analysis of compiled results from six experiments for WNK4: three for WNK3, three for WNK4-L322F, and two for L-WNK1. White bars depict phosphorylation status in control conditions arbitrarily taken as 100%, and gray bars depict the phosphorylation status in LCHS, as normalized for its corresponding white bar. *$P<0.05$ versus control (Wilcoxon signed-rank test). (F) NCC activity in oocytes injected with mutant WNK4-LL-FF, WNK4-L322F, WNK4-LL-FF-S335A, or WNK4-L322F-S335A, as stated. Uptake in oocytes injected with NCC alone was arbitrarily set to 100%, and the corresponding groups were normalized accordingly. These series of experiments were performed in control conditions only. *n*=4. *$P<0.001$ versus corresponding group without S335A substitution.

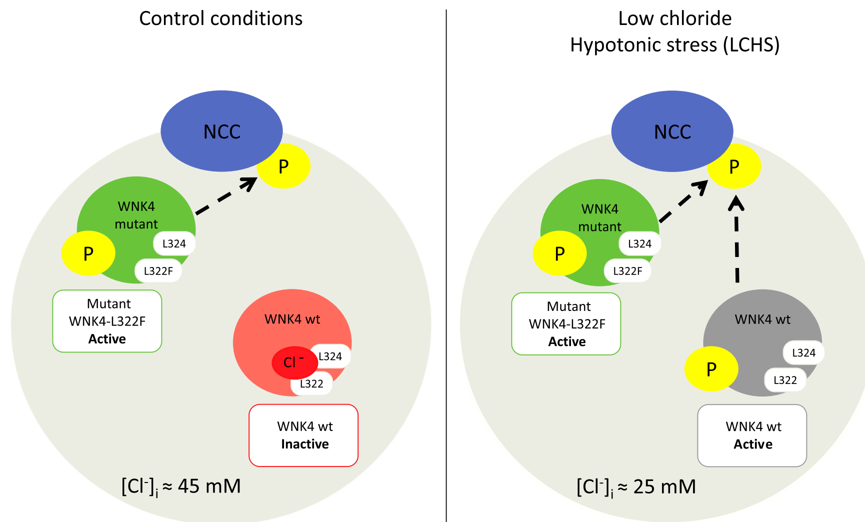

Figure 4. A cell scheme of a *X. laevis* oocyte illustrating the effect of intracellular chloride concentration on wild-type WNK4 and mutant WNK4-L322F effect on NCC. In control conditions, were intracellular chloride concentration fluctuates around 45 mM, wild-type (wt) WNK4 is inactive, resulting in a negative or neutral effect on NCC activity. Disruption of the chloride binding site in mutant WNK4-L322F turns it into a constituitively autophosphorylated actuate kinase that has a positive effect on NCC activity even without chloride depletion. Reduction of intracellular chloride to around 25 mM (after LCHS) is sufficient to promote wt WNK4 autophosphorylation, turning it into an activator of NCC.

Further experiments are required to define the IC_{50} for each WNK. In this regard, the regulation of WNK4 activity by angiotensin II and $[Cl^-]_i$ may be linked because angiotensin II promotes the opening of chloride channels,[25] which would result in a decreased $[Cl^-]_i$. Moreover, a recent study suggests the reduced activity of NCC in EAST/SeSAME syndrome results from Kcnj10 mutations that inhibit basolateral K^+ conductance, reducing the chloride exit from the cell.[26]

$[Cl^-]_i$ of distal convoluted tubule cells ranges from 14 to 20 mM.[27–29] Therefore, small changes in $[Cl^-]_i$, could have an important effect on NCC regulation by WNK4. Further experiments and mathematical modeling are required to explore this hypothesis and the role of $[Cl^-]_i$ in the regulation of NCC in physiologic conditions.

CONCISE METHODS

Mutagenesis and Constructs

All mutant WNK4 constructs were generated by site-directed mutagenesis, and their sequence was checked by automatic DNA sequencing.

Functional Expression of NCC

Our institutional committee on animal research approved the use of *X. laevis* frogs. The functional expression of NCC was assessed in *X. laevis* oocytes microinjected with NCC cRNA alone or together with wild-type WNK4, SLC26A9, or mutant WNK4. Two days later, the thiazide-sensitive $^{22}Na^+$ uptake was assessed in oocytes exposed to control conditions or to LCHS, following our previously published protocol.[11] In brief, the night before the uptake assay, groups of oocytes were incubated in two different osmolar conditions: isotonic (ND96, 210 mosm/kg H_2O) or low Cl^- hypotonic stress (Cl^--free 170 mosm/kg H_2O). Then, tracer $^{22}Na^+$ uptake was assessed in oocytes exposed to isotonicity using our usual isotonic uptake solution or NaCl containing hypotonic uptake medium (see Supplemental Material for details).

Measurement of Intracellular Chloride Concentration

Intracellular chloride concentration was assessed using ion selective microelectrodes. All measurements were performed in a Faraday cage to avoid electromagnetic interference. Each ion selective microelectrode was calibrated with 10^{-5} to 10^{-1} M KCl standard solution showing a nernstian behavior.

Western Blotting of *X. laevis* Oocyte Proteins

Flag-tagged NCC protein, human Flag-tagged WNK4, Myc-tagged WNK3, and Myc-tagged L-WNK1 protein extracts were analyzed by Western blot. The following commercial HRP-conjugated antibodies were used: anti-Flag 1:5000 (Sigma-Aldrich), anti-Myc 1:1000 (Sigma-Aldrich), and anti-beta-actin 1:2500 (Santa Cruz Biotechnology). The following sheep antibodies were used at a concentration of 1–2 μg/ml: anti-NCC (recognizing residues 906–925 of human NCC, CHTKRFEDMIAPFRLNDGFKD); anti-phosphorylated NCC at threonine 45, threonine 50, and threonine 55 (T44, 48, and 53 in rats); anti-WNK4 (recognizing residues 1221–1243 of human WNK4, SSTGSQEQRASKGVTFAGDVGRM); and anti-phosphorylated L-WNK1 at S382. The blots done with the sheep antibodies were incubated for 1.5 hour at room temperature with secondary anti-sheep HRP-conjugated antibody 1:5000 (Santa Cruz Biotechnology) in blocking solution. The signal was detected by chemiluminescence using the Luminata Forte Western HRP substrate (EMD Millipore).

Statistical Analysis

The significance of the difference between the two groups was tested with *t* test and for three or more groups by one-way ANOVA with multiple comparisons with Bonferroni correction, using GraphPad Prism version 6.00 for Mac (GraphPad Software, San Diego, CA).

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DISCLOSURES

None

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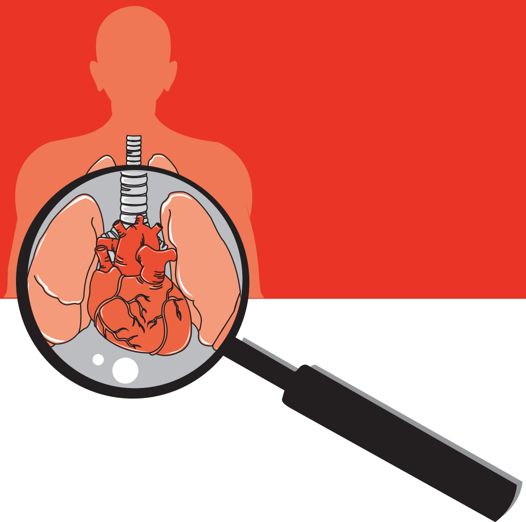

Manejo renal del calcio, fósforo y magnesio

Lorena Rojas Vega, Gerardo Gamba

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CALCIO

Distribución corporal de calcio

El calcio (Ca^{2+}) es un catión con gran variedad de funciones intracelulares y extracelulares, incluidas la preservación de la integridad de membranas celulares, actividad neuromuscular, regulación de actividades secretorias endocrinas y exocrinas, coagulación sanguínea, activación del complemento y metabolismo óseo, así como la mediación de gran cantidad de procesos celulares, como segundos mensajeros. Por lo general, el Ca^{2+} libre intracelular se encuentra a baja concentración (100 nM), pero puede alcanzar grandes y rápidos incrementos (hasta 100 veces su concentración basal). El Ca^{2+} extracelular se mantiene con escasa variación (casi siempre, 8.5 a 10.5 mg/dL o 2.2 a 2.6 mM).

Alrededor de 40% del Ca^{2+} total está unido a proteínas, por lo que variaciones en las concentraciones séricas de proteínas alteran las concentraciones de Ca^{2+} total y unido a proteínas. En la hipoalbuminemia (disminución de la concentración de albúmina sérica), una reducción de 1 g/dL de albúmina resulta en un descenso de 0.8 mg/dL de Ca^{2+} unido a proteínas. El pH también afecta el Ca^{2+} unido a proteínas: el incremento o disminución de 0.1 en el pH aumenta o reduce, respectivamente, 0.12 mg/dL de Ca^{2+} unido a proteínas.

El Ca^{2+} sérico se divide en ionizado y no ionizado, el primero biológicamente activo. La concentración sérica de Ca^{2+} ionizado en sujetos normales es de 4.0 a 4.9 mg/dL (1.2 mM), o 50% del Ca^{2+} sérico total. La porción de Ca^{2+} difusible no ionizado (~10% del total) forma complejos con algunos aniones, en particular con bicarbonato, fosfato y acetato.

El Ca^{2+} corporal varía de 1.0 a 1.5 kg, 95% del total de Ca^{2+} se almacena en el esqueleto, sólo 1% se halla en el líquido extracelular y el resto en el intracelular. La absorción gastrointestinal de Ca^{2+} es incompleta y son las variaciones de la absorción intestinal, así como de la liberación de Ca^{2+} del hueso y su excreción urinaria, las que lo regulan.

Excreción urinaria de Ca^{2+}

La excreción urinaria de Ca^{2+} varía en grado considerable en individuos normales. La excreción máxima diaria es < 300 mg en varones y < 250 mg en mujeres o 4 mg/kg de peso corporal. Alrededor de 97 a 99% del Ca^{2+} filtrado se resorbe. El 20% del Ca^{2+} que aparece en la orina es ionizado y el resto corresponde a complejos cálcicos,

como citrato de calcio, sulfato de calcio, fosfato de calcio y gluconato de calcio.

Se calcula que 50 a 70% del Ca^{2+} filtrado se resorbe en el túbulo proximal (TP), 30 a 40% en el asa ascendente de Henle (AAH) y un 10% en la nefrona distal. Por lo tanto, la mayor fracción del Ca^{2+} filtrado se resorbe en TP y en la parte gruesa de la rama de la AAH. El epitelio en estos sitios hace posible un flujo selectivo y regulado del Ca^{2+} desde la superficie luminal hasta la superficie basolateral, en especial mediante flujo paracelular pasivo entre las células, dirigido por el gradiente electroquímico, pero también por paso transcelular a través de ellas. En el segmento grueso de la rama ascendente del asa de Henle, la reabsorción de Na^+, Ca^{2+} y Mg^{2+} sigue la misma dirección. El voltaje positivo en la luz, generado por el cotransportador Na^+-K^+-$2Cl^-$ (NKCC2) y el conducto apical de potasio ROMK, es la fuerza directriz de la reabsorción del Ca^{2+}; las uniones estrechas constituyen la vía para el paso de cationes divalentes en este segmento de la nefrona (figura 29-1).

En las uniones intercelulares estrechas (*tight junctions*) se expresa una proteína llamada paracelina 1 o claudina 16 que, si bien parece ser más importante en la reabsorción de Mg^{2+}, también favorece la reabsorción paracelular de Ca^{2+}.

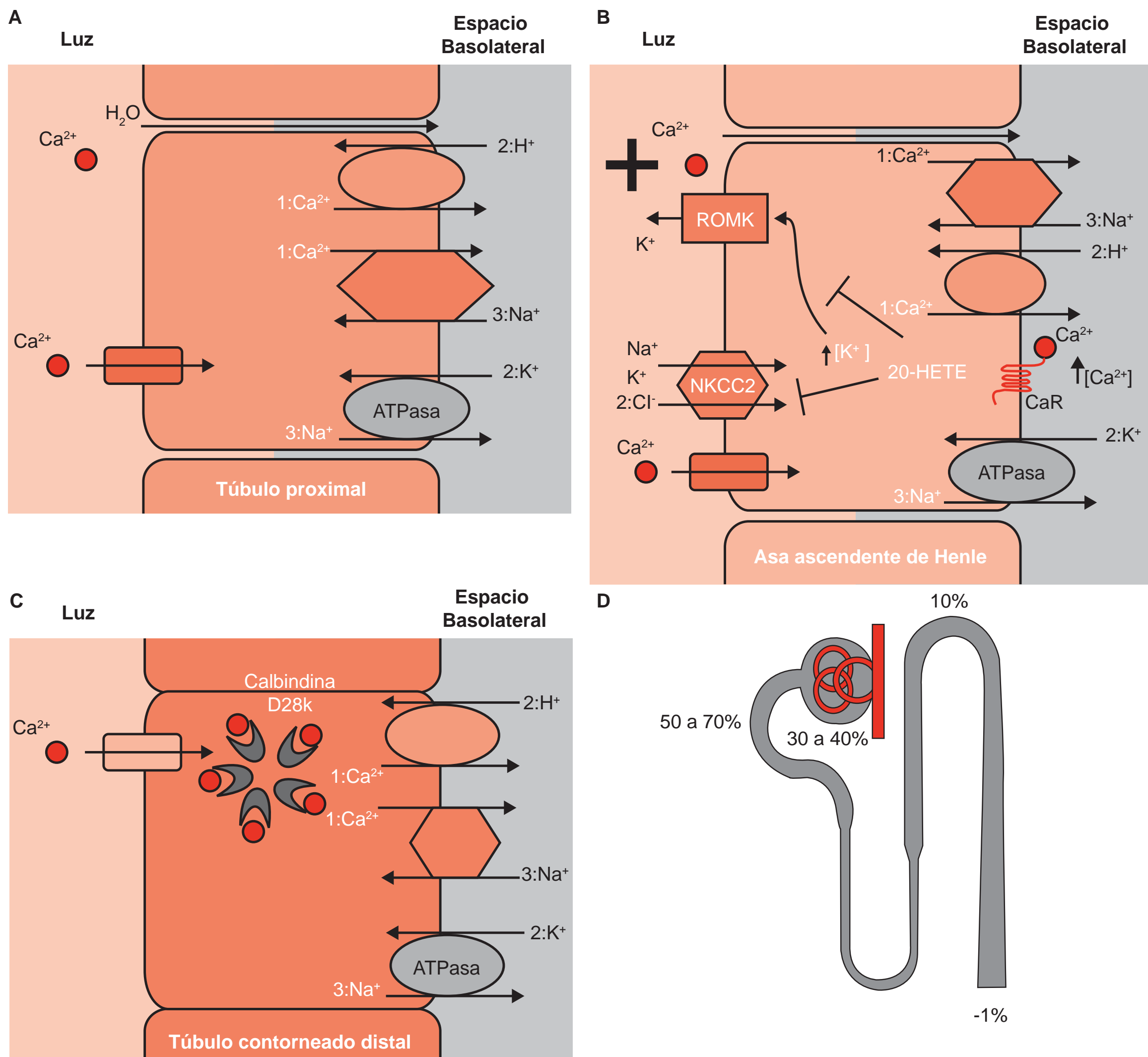

Figura 29-1. Manejo renal del calcio. A. La reabsorción en el TP es sobre todo paracelular, junto con otros iones y el agua; se cree que existen conductos (aún no identificados) de calcio expresados en la membrana apical. A lo largo de toda la nefrona se expresan la bomba plasmática de calcio ($1Ca^{2+}$:$2H^+$) y el intercambiador Na^+/Ca^{2+} ($3Na^+$:$1Ca^{2+}$), encargados de la salida de la célula al espacio basolateral de Ca^{2+}. **B.** La reabsorción de Ca^{2+} en el AAH tiene lugar a través de la creación de un voltaje luminal positivo por la función de NKCC2 y ROMK. NKCC2 resorbe de la luz a la célula Na^+, K^+ y Cl^-; ROMK recicla el K^+ a la luz y crea un gradiente de reabsorción de cationes paracelular. **C.** La reabsorción en el TCD ocurre a través de un conducto apical llamado TRPV5; el Ca^{2+} se une a la calbinida D28K, que lo transporta a la membrana basolateral. **D.** El Ca^{2+} se resorbe en especial en el TP (50 a 70%), en la AAH en 30 a 40% y en el TCD en 10%.

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Por otro lado, el sensor receptor de Ca^{2+} (CaR), un receptor acoplado a proteína G, funciona como un "calciostato" con la suficiente sensibilidad para detectar elevaciones de las cifras de Ca^{2+} (figura 29-1). El CaR puede activarse por acción de varios ligandos, entre ellos el propio Ca^{2+} y Mg^{2+}. El CaR tiene su sitio de expresión más abundante en la membrana basolateral de las células de la AAH, en donde al reconocer elevaciones de las concentraciones de Ca^{2+} plasmáticos se activa e inicia una cascada de señalización a través de la activación de la fosfolipasa A_2 (PLA_2), hacia la producción de metabolitos de P-450, como el ácido 20-hidroxieicosatetraenoico (20-HETE). El 20-HETE es un potente inhibidor de ROMK, NKCC2 y Na^+/K^+-ATPasa, por lo que no sólo impide la reabsorción de sodio y cloro, sino que evita la formación del potencial eléctrico luminal (figura 29-1) que favorece la reabsorción paracelular de Ca^{2+}, Mg^{2+} y Na^+. Es decir, el CaR "percibe" las concentraciones normales o elevadas de Ca^{2+} y activa la cascada de señales de transducción, lo que resulta en disminución de la reabsorción de Ca^{2+}. Por el contrario, concentraciones bajas de Ca^{2+} (hipocalcemia) dan lugar a una falla para activar la vía de señal de transducción, lo que a su vez induce la reabsorción de Ca^{2+} y por tanto la reducción del Ca^{2+} en la orina (hipocalciuria). Por consiguiente, la intensa rebsorción de Ca^{2+} en la nefrona proximal se relaciona de manera directa con la reabsorción de sal y volumen.

El control fino de la reabsorción tubular de Ca^{2+} se regula por transporte activo transcelular en los segmentos distales de la nefrona (figura 29–1). En el riñón se expresa el conducto TRPV5, perteneciente a la familia TRPV (*transient receptor potential cation chanel*), también conocido como ECaC1, que se expresa en la membrana apical del túbulo contorneado distal (TCD) y el túbulo conector de la corteza renal. El TRPV5 constituye el mecanismo de entrada apical del transporte activo transcelular del calcio. Después de la entrada al citosol, el Ca^{2+} se une a una proteína transportadora (calbindina D28K), la cual acarrea al catión hacia los transportadores en la membrana basolateral (el intercambiador Na^+/Ca^{2+} y la Ca^{2+}-ATPasa), los cuales desplazan el Ca^{2+} hacia el espacio basolateral (figura 29-1).

En contraste con la furosemida, que incrementa la excreción de Na^+ y Ca^{2+} de manera paralela, los diuréticos tiazídicos inhiben la reabsorción de sodio e incrementan la reabsorción de Ca^{2+}. A pesar de que los mecanismos no son claros, se presupone que al inhibir el cotransporte apical de sodio y cloro en el túbulo distal, la disminución de la concentración de cloro intracelular hiperpolariza a la célula y esto a su vez activa a TRPV5 e incrementa la entrada apical de Ca^{2+} y la salida basolateral de Ca^{2+}, lo que se refleja en una disminución de la excreción de Ca^{2+}.

La disminución de Ca^{2+} ionizado plasmático (hipocalcemia) incrementa la excitabilidad nerviosa y muscular. El aumento de Ca^{2+} ionizado plasmático (hipercalcemia) atenúa la excitabilidad neuronal y produce arritmias cardiacas, letargia, desorientación e incluso la muerte.

FÓSFORO

Distribución corporal de fósforo

El plasma contiene ~12 mg/dL de fósforo, del cual ~8 mg es orgánico y está contenido en fosfolípidos; una traza es anión de ácido pirofosfórico y el resto es fosfato inorgánico. La fracción inorgánica es la principal forma circulante de fósforo y es la que se cuantifica para fines clínicos. Alrededor de 90% del fósforo inorgánico es ultrafiltrable, de cual, alrededor del 53% es fosfato monohidrógeno (HPO_4^{2-}) que es divalente, y fosfato dihidrógeno que es monovalente ($H_2PO_4^-$). A pH normal la proporción relativa de estas dos formas es 4:1. El resto es fosfato en forma de sales de Na^+, Ca^{2+} y Mg^{2+}.

El fósforo corporal es de 0.5 a 0.8 kg, 85% almacenado en el esqueleto, 1% se halla en el líquido extracelular y el resto en el intracelular. Mientras que la absorción gastrointestinal de fosfato es incompleta, los verdaderos reguladores de las concentraciones plasmáticas de fosfato son las variaciones en la absorción intestinal, la liberación desde hueso y su excreción urinaria.

Excreción urinaria de fósforo

Alrededor de 85% del fósforo inorgánico es filtrado, del que se resorbe 80 a 90%. El TP resorbe casi 70% y, como la AAH, el TCD y los colectores resorben cantidades muy pequeñas de fosfato; aproximadamente 20 a 30% del fosfato filtrado se excreta en la orina. La reabsorción tubular de fosfato es un proceso saturable y muestra capacidad de reabsorción tubular máxima, es decir, un fenómeno Tmax. Este último varía de manera considerable con los cambios de fosfato en la dieta y el mejor método para determinarlo consiste en medir la reabsorción máxima de fosfato por unidad de volumen de la tasa de filtración glomerular (TmaxP/TFG) durante infusiones agudas de fosfato.

El fósforo penetra la membrana en borde de cepillo del TP mediante cotransporte de Na^+-fosfato en contra de un gradiente electroquímico (figura 29-2). La energía de este último la proporciona el gradiente de Na^+ generado por la bomba Na^+-K^+-ATPasa basolateral. El cotransporte Na^+-fosfato es afectado por el pH debido a alteraciones en la proporción de las formas de fosfato divalente y monovalente. El HPO_4^{2-} es la forma reabsorbida a pH fisiológico, mientras que el $H_2PO_4^-$ se absorbe a pH bajo. Cambios en la concentración de H^+ alteran la afinidad del cotransportador por el Na^+ y en consecuencia por el fosfato. La acidificación luminal atenúa la afinidad del transportador por el fosfato y en consecuencia su reabsorción. Por otro lado, la acidificación intracelular incrementa la liberación de fosfato por parte del transportador e incrementa su reabsorción.

Las cantidades intracelulares de fósforo se mantienen por un ajuste estrecho de la entrada luminal con su salida basolateral. El fósforo sale de la célula por la membrana basolateral por transporte dependiente de Na^+ (70%) y

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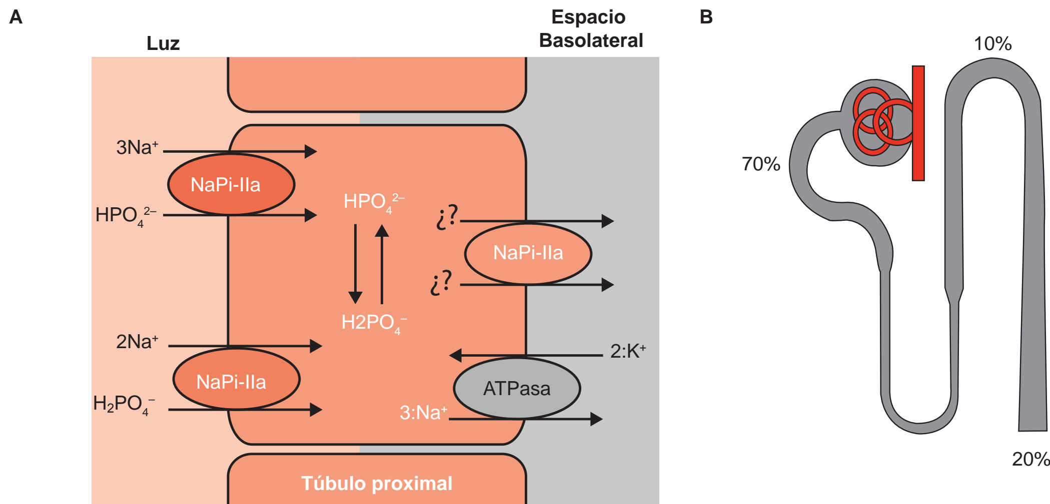

Figura 29-2. A. La célula del túbulo proximal reabsorbe fosfato a través de los cotransportadores NaPi-IIa ($3Na^+:HPO_4^-$) y NaPiIIc ($2Na^+:HPO_4^-$); los mecanismos de salida al espacio basolateral no se han dilucidado del todo. **B.** El fosfato se reabsorbe en un 70% en el túbulo proximal (TP), casi nada en el asa ascendente de Henle (AAH), un 10% en el túbulo contorneado distal (TCD) y los colectores. Un 20% se elimina en la orina.

parcialmente (30%) por un sistema de intercambio de aniones independiente de Na^+.

Tres familias de cotransportadores de Na^+-fosfato (NaPi) se han identificado en el plano molecular y se han llamado cotransportadores NaPi tipos I, II y III. Los cotransportadores I y II se expresan de manera predominante en el riñón y se localizan en la membrana de borde en cepillo de las células del TP. El tipo III se expresa de forma ubicua. Los NaPi IIa y NaPi IIc son los principales encargados de la reabsorción en el TP ($3Na^+$-HPO_4^{2-} o $2Na^+$-$H_2PO_4^-$, respectivamente). El transporte basolateral es sobre todo pasivo, aunque no es del todo claro el mecanismo; NaPi III se expresa en la membrana basolateral y puede transportar $3Na^+$-HPO_4^{2-}.

Las modificaciones del Ca^{2+} extracelular en el transporte de fosfato parecen mediadas por la PTH. Además, la ingestión por vía oral aumentada de Ca^{2+} se relaciona con disminución de la excreción urinaria de fósforo; dos factores pueden contribuir a este efecto: a) el Ca^{2+} reduce la absorción intestinal de fosfato por formación de complejos no absorbibles y b) grandes cantidades de Ca^{2+} por vía oral pueden suprimir la secreción de PTH y aminorar la excreción de fósforo.

La administración intravenosa de glucosa puede producir un efecto doble en el metabolismo de fósforo: a) la glucosa intravenosa tiende a disminuir el fósforo sérico, quizá por incorporación intracelular de fósforo durante la fosforilación de la glucosa y b) la glucosa parece tener un efecto renal directo al suprimir la reabsorción e incrementar la excreción de fosfato. Esta competencia puede ser más importante en estados de glucosuria masiva como la diabetes mellitus descontrolada.

Por otra parte, existe una serie de péptidos llamados colectivamente "fosfatoninas" que se han identificado como encargados de varias enfermedades vinculadas con hipofosfatemia, por lo que se han relacionado con funciones de la regulación de la homeostasis de fosfato; entre ellos figuran el factor de crecimiento fibroblástico 23 y el 7 (FGF 23 y FGF 7). El FGF23 se secreta en el hueso e inhibe la reabsorción de fosfato y la producción de calcitriol. El FGF23 se estimula por la hiperfosfatemia sostenida, la PTH y el calcitriol.

Efecto de la vitamina D en el manejo renal de calcio y fósforo

El colecalciferol o vitamina D se somete a la acción de la 25-hidroxilasa en el hígado para producir 25-hidroxivitamina D3 (25-hidroxicolecalciferol o 25(OH)D3) y después se hidroxila en las células del túbulo proximal del riñón por la 1α-hidroxilasa para dar origen a la 1,25-dihidroxivitamina D3 (1,25-dihidroxicolecalciferol, 1,25(OH)2D3 o calcitriol), la cual es el metabolito más activo conocido de la vitamina D. La formación de calcitriol se estimula de manera fundamental por la PTH, la hipocalcemia y la hipofosfatemia. El calcitriol se une a receptores intracelulares (receptor de vitamina D [VDR]) expresados en todas las células y regula la transcripción de genes. La activación de VDR promueve la diferenciación de enterocitos y potencia la disponibilidad de Ca^{2+} y fosfato para la formación de hueso y la prevención de hipocalcemia e hipofosfatemia (figura 29-3). Por otra parte, la hipercalcemia y la hiperfosfatemia reducen la producción de calcitriol.

El calcitriol potencia las acciones de la PTH sobre la reabsorción renal distal de Ca^{2+}. La vitamina D también induce la expresión/actividad de la proteína ligadora de Ca^{2+} (calbindina) en los segmentos distales de la nefrona, lo cual incrementa la reabsorción de Ca^{2+}. La vitamina D actúa de modo directo en el tejido paratiroideo al suprimir la secreción de PTH y formar un sistema de retroalimenta-

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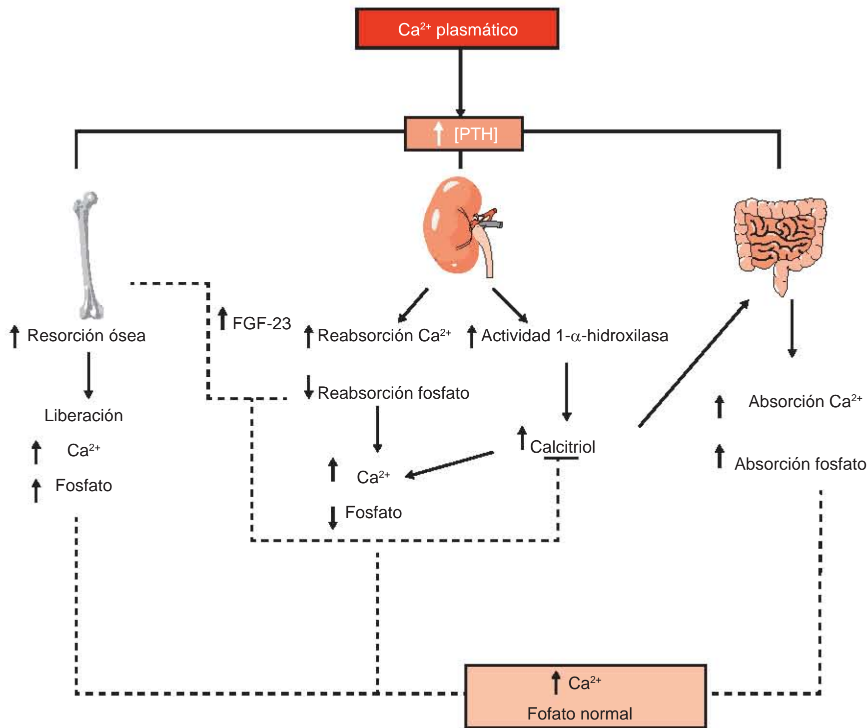

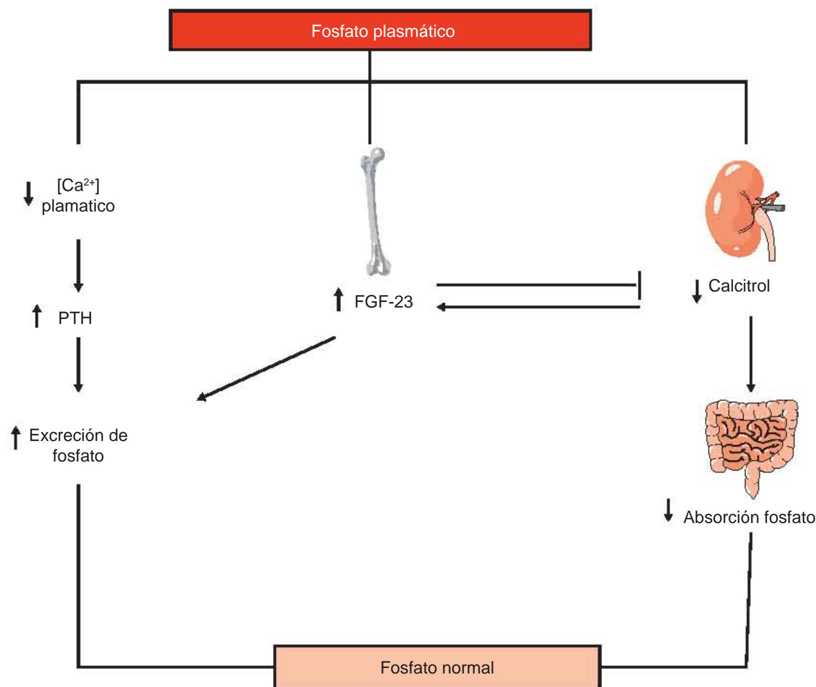

Figura 29-3. Sinopsis de la regulación de la concentración plasmática de calcio y fosfato. **A.** La hipocalcemia es el principal estímulo de secreción de PTH. La PTH estimula la resorción de hueso de calcio y fósforo, incrementa la reabsorción y la excreción urinaria de calcio y fosfato, respectivamente, y estimula la producción de calcitriol que activa la absorción de Ca^{2+} y fosfato en el intestino. Debido a que los cambios en el fosfato por el intestino, el riñón y el hueso tienden a equilibrarse entre sí, la PTH puede incrementar las concentraciones de calcio sin afectar las cantidades de fosfato. La PTH puede favorecer la secreción FGF-23 del hueso, el FGF-23 atenúa los efectos de la PTH sobre el fosfato y reduce la síntesis de calcitriol. B. Ante incrementos en el fosfato plasmático, el fosfato disminuye la producción de calcitriol, y por tanto la concentración plasmática de calcio (lo que a su vez estimula la liberación de PTH) y la absorción intestinal de fosfato, e incrementa la formación de FGF-23.

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ción negativa para la PTH, al inhibir las concentraciones de ARNm y proteína. Grandes dosis por vía oral de vitamina D causan hipercalcemia. Por último, el FGF23 suprime la producción renal de calcitriol al inhibir a la 1α-hidroxilasa.

Efecto de la PTH en el manejo renal de calcio y fósforo

La PTH es una hormona polipeptídica de cadena única con 84 aminoácidos y actividad biológica en el extremo N-terminal 1-34. Su biosíntesis inicia como prepro-PTH (110 aminoácidos), que después de la escisión de 21 aminoácidos se convierte en pro-PTH. A continuación se forma PTH cuando cinco aminoácidos se remueven de la pro-PTH. El CaR controla las cifras de Ca^{2+} en las glándulas paratiroideas. Incrementos mínimos lo activan y esto reduce la liberación de PTH, mientras que la disminución de Ca^{2+} sérico permite la síntesis y liberación de PTH. Por lo regular se ha considerado que el fosfato no se regula mediante retroalimentación negativa y por tanto sus valores varían en límites relativamente más amplios que el Ca^{2+}. Sin embargo, algunos datos recientes señalan que las concentraciones séricas de fosfato pueden relacionarse de manera directa con la secreción de PTH, de tal modo que las cantidades séricas altas o bajas de fosfato incrementan o disminuyen, respectivamente, la síntesis y secreción de PTH, cualesquiera que sean las concentraciones de Ca^{2+} sérico y vitamina D.

Ante descensos de las cifras séricas de Ca^{2+} y fósforo, la secreción de PTH ejerce sus acciones a través de lo siguiente: a) en presencia de calcitriol, estimula la reabsorción ósea y libera Ca^{2+} y fosfato desde el hueso hasta el líquido extracelular; b) incrementa la reabsorción renal de Ca^{2+} y la excreción de fosfato; y c) aumenta la absorción intestinal de Ca^{2+} y fosfato al promover la formación de calcitriol.

Los efectos renales de la PTH tienen en parte la mediación de la activación del sistema de la adenilato ciclasa en el TP, la AAH y el TCD. El efecto principal de la PTH sobre la reabsorción renal del Ca^{2+} se realiza en la nefrona distal a través de la activación de TRPV5. La PTH puede incrementar también el número de intercambiadores Na^+/Ca^{2+} en la membrana basolateral (a través de los cuales se realiza hasta 70% de la expulsión del Ca^{2+} intracelular). La PTH atenúa la reabsorción proximal de fosfato al disminuir la cantidad/actividad de NaPiII.

MAGNESIO

Distribución corporal de magnesio

El Mg^{2+} juega un papel crítico en muchos procesos metabólicos y participa como cofactor en más de 300 reacciones enzimáticas, dentro de las cuales se incluyen la hidrólisis de ATP por ATPasas dependientes de Mg^{2+}, la transcripción de RNA y la sintesis de proteínas. En terminos extracelulares, el Mg^{2+} interviene de modo amplio en procesos básicos, como el control neuromuscular y el mantenimiento del tono vascular.

El cuerpo humano tiene un total de 21 a 28 g de Mg^{2+}. Menos del 2% se encuentra distribuido fuera de las células y el resto está almacenado en los huesos (dos tercios del Mg^{2+} corporal total); los músculos tienen cerca de 20%.

La tasa de obtención de Mg^{2+} a partir del hueso y músculo es muy lenta, lo cual da lugar a que el Mg^{2+} utilizado para los requerimientos diarios provenga sobre todo del equilibrio entre la absorción intestinal y la excreción renal. Sólo 30 a 40% del Mg^{2+} contenido en la dieta se absorbe en el tracto gastrointestinal, en particular en el intestino delgado. Las concentraciones plasmáticas de Mg^{2+} oscilan entre 0.8 y 1.9 mM (1.8 a 2.2 mg/dL). En el plasma, el 55% del Mg^{2+} se encuentra libre, 32% está unido a proteínas y alrededor de 15% forma complejos como fosfato de magnesio o citrato de magnesio.

Excreción urinaria de Mg^{2+}

En condiciones normales, menos del 5% del Mg^{2+} filtrado aparece en la orina. La concentración de Mg^{2+} en el TP depende del filtrado por el glomérulo (figura 29-4). El Mg^{2+} filtrado es el que se encuentra libre en plasma. El Mg^{2+} tiene un patrón de reabsorción diferente de otros solutos, ya que del 15 al 25% se resorbe por el TP; 60 a 70% del Mg^{2+} lo hace sobre todo en la AAH. La reabsorción es secundaria al transporte de Na^+ y Cl^- mediante transporte pasivo utilizando el mismo sistema de reabsorción de Ca^{2+}. En las uniones estrechas de la AAH, la claudina 16 es necesaria para la reabsorción paracelular de Mg^{2+} y mutaciones en ella producen una enfermedad autosómica recesiva caracterizada por la pérdida grave de Mg^{2+}.

El TCD es el sitio de regulación fina del Mg^{2+}, aunque sólo resorbe 5 a 10%; en el colector no existe una reabsorción significativa. En el TCD, el transporte es activo y transcelular por la expresión apical del conducto TRPM6 (*transient receptor potential melastatin 6*) cuyas mutaciones producen hipomagnesemia con hipocalcemia secundaria. El transporte basolateral no es del todo claro, pero puede deberse a la diferencia de voltaje entre el lado apical y el basolateral o el intercambio con el sodio.

Debido a que la reabsorción de Mg^{2+} depende de la activación del NKCC2 sensible a diuréticos de asa, el empleo de estos fármacos (furosemida) reduce la reabsorción de Mg^{2+}. La PTH, calcitonina, glucagon y hormona antidiurética aumentan la reabsorción de Mg^{2+} al incrementarse el voltaje transepitelial, así como la permeabilidad paracelular del Mg^{2+}, mientras que los aumentos de las concentraciones de Mg^{2+} pueden inhibir la liberación de PTH. El CaR también es capaz de detectar las modificaciones en la concentración del Mg^{2+} plasmático. El factor de crecimiento epidérmico (EFG) y el 17-β-estradiol son hormonas magnesiotrópicas debido a que favorecen la actividad o abundancia de TRPM6.

Las alteraciones más frecuentes del metabolismo del Mg^{2+} se deben a pérdidas anormales del catión por una mala absorción intestinal, como en cuadros de diarrea, enfermedad renal, y como consecuencia de la administra-

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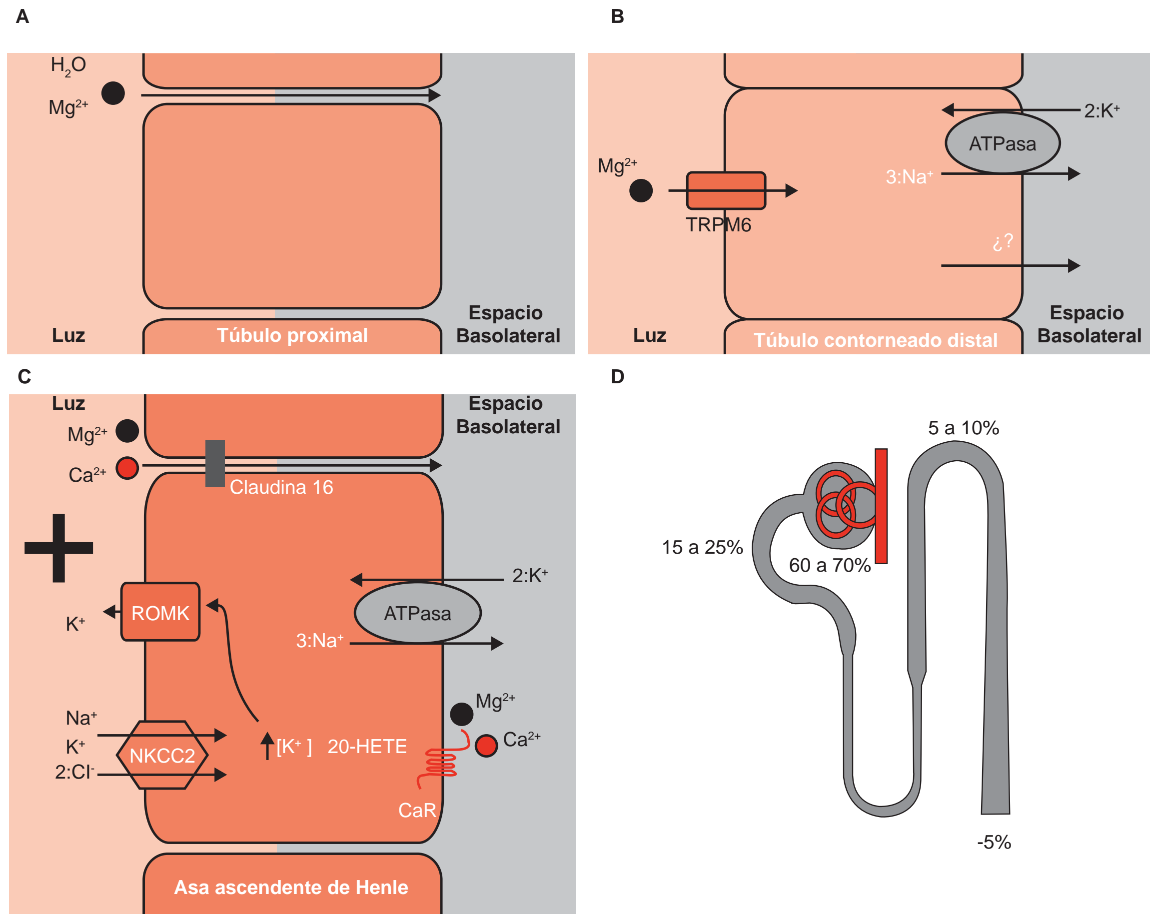

Figura 29-4. Manejo renal del magnesio. A. En el TP, el Mg^{2+} se reabsorbe de manera paracelular. **B.** En la AAH, la reabsorción del Mg^{2+} comparte los mismos mecanismos que el Ca^{2+}; con excepción de la claudina 16, es más afín por Mg^{2+} que por Ca^{2+}. **C.** En el TCD, la reabsorción ocurre a través del conducto recién descubierto TRPM6; los mecanismos de salida al espacio basolateral no se conocen. **D.** El Mg^{2+}, a diferencia de otros iones en el TP, sólo se reabsorbe en 15 a 25%, 60 a 70% en la AAH y 5 a 10% en el TCD.

ción de diuréticos. La hipomagnesemia produce una mayor excitabilidad neuronal y predispone el desarrollo de arritmias cardiacas.

Por otro lado, mayores consumos de Mg^{2+} o la insuficiencia renal provocan hipermagnesemia. El exceso de Mg^{2+} tiende a desplazar al Ca^{2+} y generar datos clínicos similares a los de la hipocalcemia.

Sin embargo, algunos estudios muestran evidencia de que incrementos moderados de la ingestión de Mg^{2+} pueden reducir la presión sanguínea y la incidencia de hipertensión, en tanto que la hipomagnesemia es un factor de riesgo para la calcificación vascular.

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