Antagonistic regulation of ROMK by long and kidney-specific WNK1 isoforms

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WNK kinases are serine-threonine kinases with an atypical placement of the catalytic lysine. Intronic deletions with increased expression of a ubiquitous long *WNK1* transcript cause pseudohypoaldosteronism type 2 (PHA II), characterized by hypertension and hyperkalemia. Here, we report that long *WNK1* inhibited ROMK1 by stimulating its endocytosis. Inhibition of ROMK by long *WNK1* was synergistic with, but not dependent on, *WNK4*. A smaller transcript of *WNK1* lacking the N-terminal 1–437 amino acids is expressed highly in the kidney. Whether expression of the KS-*WNK1* (kidney-specific, KS) is altered in PHA II is not known. We found that KS-*WNK1* did not inhibit ROMK1 but reversed the inhibition of ROMK1 caused by long *WNK1*. Consistent with the lack of inhibition by KS-*WNK1*, we found that amino acids 1–491 of the long *WNK1* were sufficient for inhibiting ROMK. Dietary K⁺ restriction decreases ROMK abundance in the renal cortical-collecting ducts by stimulating endocytosis, an adaptive response important for conservation of K⁺ during K⁺ deficiency. We found that K⁺ restriction in rats increased whole-kidney transcript of long *WNK1* while decreasing that of KS-*WNK1*. Thus, KS-*WNK1* is a physiological antagonist of long *WNK1*. Hyperkalemia in PHA II patients with PHA II mutations may be caused, at least partially, by increased expression of long *WNK1* with or without decreased expression of KS-*WNK1*.

dietary potassium intake | endocytosis | clathrin-coated vesicle | dynamin

WNK (with no lysine [K]) kinases are a new family of large serine-threonine protein kinases conserved in multicellular organisms with an atypical placement of the catalytic lysine. There are four mammalian WNK family members (1). *WNK1*, the first member identified, is >2,100 amino acids long (2). It contains an ≈270-aa kinase domain located near the amino terminus (e.g., amino acids 218–491 of the rat *WNK1*). *WNK2*, 3, and 4 are products of different genes and ≈1,200 to 1,600 amino acids in length (1, 2). The kinase domain of the four WNKs that share 85–90% sequence identity are unique in having the catalytic lysine located in the subdomain I instead of the conserved subdomain II of most protein kinases (1–3). Other conserved domains of WNK kinases include an autoinhibitory domain, 1–2 coiled-coil domains, and multiple PXXP proline-rich motifs for potential protein–protein interaction (1–4). Beyond the aforementioned conserved domains/motifs, sequence identity among the four WNKs is much lower and few homologous regions exist.

Pseudohypoaldosteronism type II (PHA II) is an autosomal-dominant disease characterized by hypertension and hyperkalemia (5). Recently, Wilson *et al.* (6) reported that mutations of *WNK1* and *WNK4* cause PHA II. Mutations in the *WNK1* gene are large deletions of the first intron leading to increased expression. Mutations in the *WNK4* gene are missense mutations in the coding sequence outside the protein kinase domain. Several recent studies have examined the mechanisms for hypertension and hyperkalemia in PHA II patients. *WNK4* inhibits the activity of the sodium chloride cotransporter. *WNK4* mutants that cause disease fail to inhibit the sodium chloride cotransporter, suggesting that an increase in sodium chloride cotransporter activity in the distal convoluted tubule is a cause

of hypertension (7, 8). Others have reported that *WNK4* phosphorylates claudins 1–4, the tight-junction proteins involved in the regulation of paracellular ion permeability (9, 10). The paracellular chloride permeability is greater in cells expressing *WNK4* mutants than in cells expressing wild-type proteins. Thus, hypertension in patients with *WNK4* mutations may be caused by an increase in NaCl reabsorption through the Na-Cl cotransporter and the paracellular pathway. Wild-type *WNK4* inhibits the ROMK1 channel and *WNK4* mutants that cause disease exhibit increased inhibition of ROMK (11), suggesting that *WNK4* mutations cause hyperkalemia by inhibiting ROMK.

Expression of *WNK1* abolishes inhibition of the sodium chloride cotransporter caused by *WNK4* in *Xenopus* oocytes (7), suggesting that *WNK1* mutations cause hypertension by releasing *WNK4*-mediated inhibition of the cotransporter in the distal convoluted tubule. However, PHA II patients with *WNK1* mutations are not particularly sensitive to thiazide diuretics (12). Moreover, patients with *WNK1* mutations do not have hypercalciuria, whereas patients with *WNK4* mutations have hypercalciuria that is ≈6-fold more sensitive to thiazide treatment than normal individuals (13, 14). A recent study by Xu *et al.* (15) shows that *WNK1* activates SGK leading to activation of ENaC. Thus, hypertension in PHA II patients with *WNK1* mutations may be caused by increased activity of Na-Cl cotransporter and ENaC. The mechanism for hyperkalemia in patients with *WNK1* mutations is unknown.

Although *WNK4* is expressed predominantly in kidney and several extrarenal epithelial tissues, *WNK1* is widely expressed in multiple spliced forms (2, 16). A long *WNK1* transcript (produced from 28 exons) encoding a polypeptide of >2,100 amino acids in length is expressed in all cell lines and tissues examined (2, 17–19). A shorter *WNK1* transcript encoding a polypeptide (≈1,700 amino acids in length) lacking the amino terminal 1–437 amino acids of the long *WNK1* is expressed highly in the kidney but not in other tissues (18, 19). The KS-*WNK1* (KS, kidney-specific) is produced by replacing the first 4 exons with an alternative 5' exon (exon 4A). The remaining exons 5–28 are the same as the long transcript. Quantitative analysis of *WNK1* transcripts reveals that KS-*WNK1* is expressed in kidney more abundantly than long *WNK1* (≈85% vs. ≈15%) (18, 19). A large deletion of intron 1 causes increased expression of the long *WNK1* isoform (6). Whether the expression of KS-*WNK1* is altered in PHA II and the physiological role of KS-*WNK1* are currently unknown.

K⁺ secretion by kidney is critical for controlling serum K⁺ levels and overall K⁺ homeostasis (20, 21). ROMK K⁺ channels present on the apical membrane of the distal renal tubules are important for baseline renal K⁺ secretion (20–23). Another type

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Abbreviations: CCV, clathrin-coated vesicle; HEK, human embryonic kidney; KS, kidney-specific; PHA II, pseudohypoaldosteronism type II; siRNA, small interference RNA.

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Fig. 1. Effect of WNK1 on ROMK1 expressed in HEK cells. (A) Whole-cell recording, voltage-clamp protocol, and current-voltage (I-V) relationships of currents. (B) Dose-dependent inhibition of ROMK1 by WNK1. Cells were transfected with ROMK1 plus WNK1 (0–2.5 μ g of plasmid DNA). In each experiment, the total amount of DNA for transfection was balanced by using empty vector. Ba²⁺-sensitive (after subtraction of residual currents in the presence of 10 mM Ba²⁺) inward current density is shown. *, $P < 0.05$ vs. ROMK1 alone. (C) Effect of wild-type (WT-DII) or dominant-negative (K44A) dynamin II (DN-DII) on WNK1 inhibition of ROMK1. (D) Effect of WNK1 on wild-type ROMK1 (WT-RK) vs. N375I ROMK1 mutant. Experiments above were repeated 3–5 times with similar results. NS, not significant.

of K⁺ channels, maxi-K, are also present in the distal renal tubules and important for K⁺ secretion in response to increase in tubular fluid flow (23, 24). To maintain K⁺ homeostasis, the abundance of ROMK in the distal nephron decreases or increases during low or high dietary K⁺ intake, respectively (25, 26). Alteration of abundance of ROMK during changes of dietary K⁺ intake involves endocytosis and subsequent degradation of the channel protein (27, 28).

In the present study, we show that long WNK1 inhibits ROMK1, whereas KS-WNK1 reverses inhibition of ROMK1 caused by long WNK1. Dietary K⁺ restriction in rats increases the abundance of transcript for long WNK1 and decreases the abundance for KS-WNK1 in the kidney. These results suggest that the physiological role of KS-WNK1 as an antagonist of long WNK1. Furthermore, hyperkalemia in PHA II patients with *WNK1* mutations may be caused, at least partially, by the inhibition of ROMK channels resulting from increased expression of long WNK1 with or without decreased expression of KS-WNK1.

Results

Long WNK1 Inhibits ROMK1 by Stimulating Endocytosis of the Channel.

We examined regulation of ROMK1 by long WNK1 coexpressed in the human embryonic kidney (HEK) cells by using whole-cell patch-clamp recording. Cells transfected with ROMK1 exhibited the characteristic weak inward-rectifying Ba²⁺-sensitive K⁺ currents (Fig. 1A). K⁺ currents were not detected in mock-transfected cells (Fig. 1A). Coexpression with long WNK1 inhibited K⁺ currents through ROMK1 (Fig. 1A). Fig. 1B shows dose-dependent inhibition of ROMK1 current by long WNK1. The mean inward ROMK current density (at -100 mV) were 135 ± 14 pA/pF and 37 ± 13 pA/pF for without WNK1 and coexpression with 2.5- μ g long WNK1 DNA, respectively (Fig. 1B). No further inhibition of ROMK1 was observed by coexpression with >2.5- μ g WNK1 DNA (not shown). It has been reported that WNK4 regulates membrane trafficking of ion channels and transporters (7, 8, 11).

Fig. 2. Effect of WNK1 or WNK4 siRNA on ROMK1 expression in HEK cells. (A) Cells were cotransfected with ROMK1 (0.5 μ g) plus WNK1 siRNA (200 nM in transfection mixture) or control oligonucleotide. (B) Cells were cotransfected with ROMK1 (0.5 μ g) plus WNK4 siRNA (200 nM) or control oligonucleotide. (B Inset) mRNA expression of endogenous WNK4 analyzed by reverse-transcription PCR. Cells were mock-transfected (Mock) or transfected with control oligos (Control) or siRNA for WNK4 (WNK4-si). Experiments above were repeated 2–3 times with similar results.

ROMK1 undergoes clathrin-coated vesicle (CCV)-mediated endocytosis, a process believed to be an important mechanism for regulating K⁺ secretion in physiological and/or pathophysiological states (27, 28). To determine whether WNK1 inhibits ROMK1 by stimulating CCV-mediated endocytosis of the channel, we examined the effect of WNK1 on ROMK1 by coexpression with wild-type or a dominant-negative dynamin II. As reported previously, coexpression with dominant-negative dynamin II increased basal ROMK1 current density (Fig. 1C; 124 ± 10 pA/pF vs. 364 ± 20 pA/pF for coexpression with wild-type vs. dominant-negative dynamin II, respectively; $P < 0.01$), indicating that endocytosis of ROMK1 occurs in the basal state. Coexpression with dominant-negative dynamin II (lysine-44 to alanine; K44A) prevented the inhibition of ROMK1 by long WNK1. For comparison, coexpression with wild-type dynamin II had no effect on long WNK1-induced inhibition. Dynamin II may also be involved in internalization via caveolae (29). ROMK1 contains a canonical NPXY motif for internalization via CCV (30), and mutation of the critical asparagine-375 in the motif abolished endocytosis (28). We found that WNK1 failed to inhibit N375I mutant while inhibiting wild-type ROMK1 (Fig. 1D). Thus, WNK1 inhibits ROMK1 by stimulating endocytosis of the channel via CCV. The incomplete inhibition of ROMK by WNK1 (Fig. 1B) is probably due to limitation of endogenous endocytic machinery.

Endogenous WNK1 and WNK4 Are Important for Regulating ROMK1 Channels.

WNK1 and WNK4 are widely expressed. In the next series of experiments, we examined whether endogenous WNK1 or WNK4 in HEK cells contributes to the basal endocytosis of ROMK1. We found that cells transfected by small interference RNA (siRNA) for WNK1 expressed ≈ 2.2 fold higher ROMK1 current density than cells transduced by control RNA oligonucleotide (current density: 380 ± 24 pA/pF vs. 170 ± 13 pA/pF for WNK1 siRNA vs. control RNA, respectively, $P < 0.01$; Fig. 2A). Knockdown of endogenous WNK1 by siRNA was confirmed by Western blot analysis of endogenous WNK1 in ref. 31. Similarly, cells transfected with WNK4 siRNA expressed higher ROMK1 currents (current density: 341 ± 36 pA/pF vs. 192 ± 15 pA/pF for WNK4 siRNA vs. control RNA, respectively, $P < 0.01$; Fig. 2B). Knockdown of endogenous WNK4 messenger RNA by siRNA was confirmed by reverse-transcription PCR analysis (Fig. 2B Inset). We could not consistently detect endogenous WNK4 protein by using the antibodies against WNK4 available to us.

antagonist of long WNK1. Consistent with the finding that WNK1 (1–491) inhibits ROMK1 equally to the full-length WNK1, KS-WNK1 also reversed inhibition of ROMK1 caused by WNK1 (1–491) (Fig. 5B).

It has been reported that the region of amino acids 480–660 of long WNK1 interacts with the region of amino acids 1–491 (32). Amino acids 1–253 of KS-WNK1 contain the region of amino acids 480–660 of long WNK1 (Fig. 5A). We found that KS-WNK1 (1–253), like full-length KS-WNK1, was capable of reversing long WNK1-mediated inhibition of ROMK1 (Fig. 5B). KS-WNK1 (1–253) by itself had no effect on ROMK. We next examined the interaction between WNK1 (1–491) and KS-WNK1 (1–253) by coexpressing the respective Myc- and Flag-tagged proteins. As shown in Fig. 5C, anti-Myc antibody immunoprecipitated Myc-WNK1 (1–491) (indicated by a 60-kDa protein band), which coimmunoprecipitated Flag-KS-WNK1 (1–253) (indicated by a 32-kDa protein band). Pod1 is an unrelated transcription factor protein (33). As a control, Myc-

Fig. 6. Effect of dietary K^+ intake on long and KS-WNK1 expression in kidney. (A) Relative abundance of transcript (normalized to the control K^+ diet) for long WNK1 in rat kidney fed low (LK), control (CK) or high K^+ diet (HK). (B) Relative abundance for KS-WNK1. (C) Ratio of abundance of transcript for L-WNK1 vs. for KS-WNK1. Ratio relative to the control K^+ diet is shown. *, $P < 0.05$ vs. CK.

WNK1 (1–491) did not coimmunoprecipitate Flag-pod1 [see lack of protein in 22 kDa position (predicted molecular mass of pod1); Fig. 5C]. Conversely, anti-Flag antibody immunoprecipitated Flag-KS-WNK1 (1–253), which coimmunoprecipitated Myc-WNK1 (1–491). Also, Flag-pod1 did not coimmunoprecipitate Myc-WNK1 (1–491) (Fig. 5C). These results suggest that KS-WNK1 antagonizes inhibition of ROMK1 by long WNK1 by binding to amino acids 1–491 of long WNK1.

Effects of Changes of Dietary K⁺ Intake on Expression of Long and KS-WNK1. ROMK channel in the apical membrane of the distal nephron is an exit pathway for baseline renal K⁺ secretion (20–22). To maintain K⁺ homeostasis, the abundance of ROMK in the apical membranes of distal nephron increases and decreases with high and low dietary K⁺ intake, respectively (25, 26). Dietary K⁺ restriction decreases the abundance of ROMK of cortical collecting ducts by stimulating endocytosis (27). To see whether the opposite regulation of ROMK1 by long and KS-WNK1 is physiologically important, we examined the expression of long and KS-WNK1 in rats fed K⁺-deficient (LK), control (CK) or high K⁺ (HK) diet. The abundance of transcript for K⁺-deficient and high K⁺ diet relative to the control K⁺ diet was determined by using quantitative real-time PCR. As shown in Fig. 6, the transcript for long WNK1 was apparently increased by feeding a K⁺-restriction diet (Fig. 6A; $115 \pm 7\%$ of the control, $P = 0.05$; mean $\pm$ SEM, $n = 11$ for each) but not significantly altered by a high K⁺ diet ($88 \pm 11\%$ of the control, not significant). As reported in refs. 18 and 19, we found that KS-WNK1 is much more abundant than long WNK1 in rat kidney ($91 \pm 8\%$ vs. $9 \pm 4\%$). In contrast to the apparent increase for the long WNK1, the abundance of transcript for KS-WNK1 was decreased by K⁺-deficient diet (Fig. 6B, $50 \pm 6\%$ of the control). Conversely, the transcript for KS-WNK1 was increased by high K⁺ diet ($142 \pm 10\%$ of control). Besides the opposite direction of changes, the magnitude of changes of KS-WNK1 by variations of dietary K⁺ intake was much greater than that in long WNK1. The ratio (relative to control) of abundance of transcript for long over KS-WNK1 for K⁺-deficient and high K⁺ diet were calculated at 230% of control and 62% of control, respectively (Fig. 6C).

Tissue Culture, Immunoprecipitation, and Western Blots. HEK293 cells were maintained in Dulbecco's modified Eagle's medium with 10% FBS/2 mM L-glutamine/100 units/ml penicillin/streptomycin and transfected as described in ref. 15. Transfected cells were harvested in isotonic lysis buffer containing 1% Triton X-100 and phosphatase and protease inhibitors as described in ref. 15. For coimmunoprecipitation experiments, cells were lysed with a Dounce homogenizer in buffer lacking Triton X-100. Proteins were immunoprecipitated from cell lysates by using monoclonal anti-Myc or anti-Flag (2, 15, 31, 32) antibodies at 1:100 dilution. For Western blots, total lysates or immunoprecipitates were resolved by SDS/PAGE and proteins were transferred onto nitrocellulose membranes. The membranes were incubated with the indicated antibodies and developed by using enhanced chemiluminescence.

Whole-Cell Patch-Clamp Recording of ROMK Channels. HEK cells were transfected with cDNAs (0.5 μ g each) for GFP-ROMK. As indicated, cells were cotransfected with long WNK1, WNK4, KS-WNK1, wild-type, and/or dominant-negative rat dynamin II (gifts of Joseph Albanesi, University of Texas Southwestern Medical Center). In each experiment, the total amount of DNA for transfection was balanced by using empty vectors. Approximately 36–48 h after transfection, whole-cell currents were recorded by using an Axopatch 200B amplifier as described in refs. 15 and 39. Transfected cells were identified by using epifluorescent microscopy. The bath and pipette solution contained 145 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes (pH 7.4) and 145 mM KCl, 2 mM EDTA, 10 mM Hepes (pH 7.4), respectively. Capacitance and access resistance were monitored and 75% compensated. The voltage protocol consists of 0 mV holding potential and 400 ms steps from –100 to 100 mV in 20-mV increments. Statistical comparison was made by using the unpaired Student *t* test.

Experimental Animals, Diets, and Real-Time PCR. Male or female Sprague–Dawley rats (150–200 g) were used for the study. To study

the effects of low dietary K⁺ intake, rats were pair-fed either a K⁺-deficient diet (no added K⁺; TD95006, Harlan TEKLAD, Madison, WI), a control K⁺ diet (K⁺ content 6.7 g/kg in KCl; TD88238) or a high K⁺ diet (K⁺ content 49.5 g/kg; TD94121) for 48–72 h as described in ref. 27. All animals were allowed to drink distilled water freely. Food intake and body weight were measured daily. Kidneys from control and K⁺-deficient rats were dissected immediately after killing. After homogenization, RNA was extracted from kidney by using RNeasy (Invitrogen) according to the manufacturer's instructions. Two hundred nanograms was used for RT with the TaqMan RT kit (Applied Biosystems). Real-time quantitative PCR was performed on ABI 7000 as described in Applied Biosystems User Bulletin no. 2 by using the TaqMan assay. Forward and reverse primers and TaqMan probe from the 5' sequence of exon1 (WNK1 P1 forward, 5'-GGC ACT CCT GGC TTC CTT TC-3'; P1 reverse, 5'-ATC GGA GCT TGA GCC ATT CTT-3'; P1 TaqMan probe, 5'-CCT CCG GCT CCA GTC-3') were used to amplify the full-length WNK1 isoform under P1 promoter control (18, 19). Primer from exon 4a (RP forward, 5'-GCT GCT GTT CTC AAA AGG ATT GTA T-3') and from exon 5 (RP reverse, 5'-CAG GAA TTG CTA CTT TGT CAA AAC TG-3') and TaqMan probe (5'-TGA GGG AGT GAA GCC A-3') were used to amplify the kidney-specific isoform (18, 19). Relative WNK1 mRNA levels were calculated with 18s rRNA as the internal control.

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