Serine-threonine kinase with-no-lysine 4 (WNK4) controls blood pressure via transient receptor potential canonical 3 (TRPC3) in the vasculature

Hyun Woo Park[a,1], Joo Young Kim[a,1], Soo-Kyoung Choi[b], Young-Ho Lee[b], Weizhong Zeng[c], Kyung Hwan Kim[a], Shmuel Muallem[d], and Min Goo Lee[a,2]

[a]Department of Pharmacology, Brain Korea 21 Project for Medical Sciences, Severance Biomedical Science Institute, and [b]Department of Physiology, Yonsei University College of Medicine, Seoul 120-752, Korea; [c]Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX 75235; and [d]Epithelial Signaling and Transport Section, Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892

Edited* by Melanie H. Cobb, University of Texas Southwestern Medical Center, Dallas, TX, and approved May 23, 2011 (received for review March 18, 2011)

Mutations in the serine-threonine kinase with-no-lysine 4 (WNK4) cause pseudohypoaldosteronism type 2 (PHAII), a Mendelian form of human hypertension. WNK4 regulates diverse ion transporters in the kidney, and dysregulation of renal transporters is considered the main cause of the WNK4 mutation-associated hypertension. Another determinant of hypertension is vascular tone that is regulated by Ca^{2+}-dependent blood vessel constriction. However, the role of WNK4 in vasoconstriction as part of its function to regulate blood pressure is not known. Here, we report that WNK4 is a unique modulator of blood pressure by restricting Ca^{2+} influx via the transient receptor potential canonical 3 (TRPC3) channel in the vasculature. Loss of WNK4 markedly augmented TRPC3-mediated Ca^{2+} influx in vascular smooth muscle cells (VSMCs) in response to α-adrenoreceptor stimulation, which is the pathological hallmark of hypertension in resistance arteries. Notably, WNK4 depletion induced hypertrophic cell growth in VSMCs and increased vasoconstriction in small mesenteric arteries via TRPC3-mediated Ca^{2+} influx. In addition, WNK4 mutants harboring the Q562E PHAII-causing or the D318A kinase-inactive mutation failed to mediate TRPC3 inhibition. These results define a previously undescribed function of WNK4 and reveal a unique therapeutic target to control blood pressure in WNK4-related hypertension.

Hypertension, or elevated arterial blood pressure, is one of the most common diseases in industrialized countries, increasing the risk of a wide spectrum of cardiovascular illnesses including stroke, congestive heart failure, and myocardial infarction (1). More than 90% of hypertensive patients are classified as essential hypertension because of the lack of knowledge regarding the gene identity involved in blood pressure regulation (2, 3). In the last few years, most attention has been focused on the with-no-lysine (WNK) kinases, including WNK4, that were found mutated in patients with pseudohypoaldosteronism type 2 (PHAII; Online Mendelian Inheritance in Man no. 145260), which is a rare autosomal dominant disorder featuring hypertension associated with hyperkalemia, hyperchloremia, and metabolic acidosis (4).

Mice harboring the PHAII-causing WNK4 mutations Q562E (5) and D561A (6) reconstituted the phenotypes observed in PHAII patients. Deletion of the Na^+Cl^- cotransporter (NCC) reversed most of the phenotypes seen in the transgenic mice harboring the PHAII-causing WNK4 mutants (5), which indicated that aberrant regulation of NCC by the mutant WNK4 is critically involved in the pathogenesis of PHAII. This finding led to the suggestion that increased Na^+ in systemic fluids by the altered NCC activity is associated with elevated blood pressure (7–10). Another important aspect of hypertension is vascular tone. However, despite the diverse extrarenal tissue distribution of WNK4 (11), the expression and function of WNK4 in the resistance artery and the role in vasoconstriction as part of its function to regulate blood pressure has not been considered before.

Recent studies have suggested that the transient receptor potential canonical (TRPC) channels, such as TRPC1, TRPC3, and TRPC6, play an important role in the pathogenesis of several cardiovascular diseases and hypertension (12–14). TRPC channels activated by G-protein-coupled receptors (GPCRs) mediate $[Ca^{2+}]_i$ increase in vascular smooth muscle cells (VSMCs), which determine luminal diameter and contractility and thus peripheral resistance that markedly influences blood flow and pressure (12–14). In particular, accumulating evidence suggests that TRPC3 is up-regulated in hypertensive animal models (15), as well as in patients with hypertension (16–18), establishing a correlation between elevated TRPC3 activity and high blood pressure. Here, we report a molecular mechanism by which WNK4 regulates TRPC3 activity to determine vascular tone and its ablation by WNK4 mutations associated with hypertension. These results not only shed light on the previously undescribed signal pathway of WNK4-related hypertension, but also provide important therapeutic strategies to correct aberrant blood pressure.

Results

WNK4 Depletion Increases TRPC3 Activity and Promotes Hypertrophic Cell Growth in VSMCs. To determine the role of WNK4 in TRPC3-mediated Ca^{2+} influx in primary cultured rat aortic VSMCs, endogenous WNK4 and TRPC3 proteins were knocked down by using specific siRNAs for each protein (Fig. 1*A*). Ba^{2+} influx was measured to isolate Ca^{2+} influx by TRPC3 in Fura-2 loaded cells, because TRPCs are highly permeable to Ba^{2+}. TRPC3 was stimulated with its direct activator, 1-oleoyl-2-acetyl-sn-glycerol (OAG; ref. 19). Influx through voltage-gated L-type Ca^{2+} channels was excluded by including nifedipine in the bath solution. Treatment with TRPC3-specific siRNA had minimal effect on Ca^{2+} influx in resting cells, suggesting that TRPC3 primarily mediates Ca^{2+} influx in receptor-stimulated cells. Notably, depletion of WNK4 in VSMCs significantly increased Ba^{2+} influx that was largely abolished by knockdown of native TRPC3 (Fig. 1 *B* and *C*). siRNA against TRPC3 had no effect on the expression of TRPC1 and TRPC6 (Fig. S1), which are also known to induce GPCR-mediated $[Ca^{2+}]_i$ increase in VSMCs (12–14). These results indicate that WNK4 exerts an inhibitory effect on Ca^{2+} entry via TRPC3.

Author contributions: J.Y.K. and M.G.L. designed research; H.W.P., J.Y.K., S.-K.C., Y.-H.L., and W.Z. performed research; H.W.P., J.Y.K., K.H.K., S.M., and M.G.L. analyzed data; and H.W.P. and M.G.L. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]H.W.P. and J.Y.K. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: mlee@yuhs.ac.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1104271108/-/DCSupplemental.

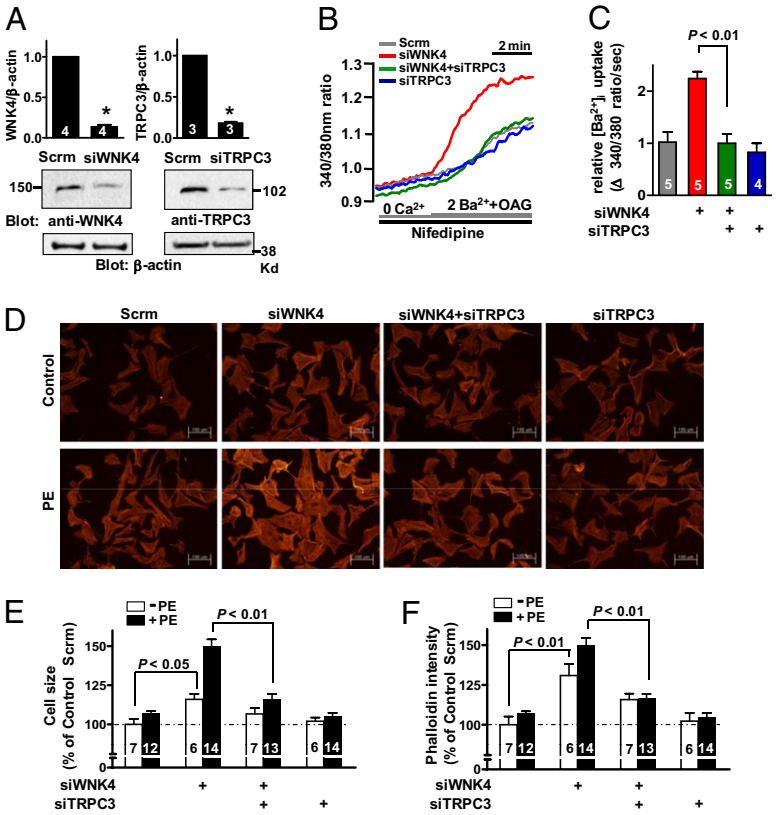

Fig. 1. Effects of WNK4 depletion on TRPC3-mediated Ba^{2+} influx and hypertrophic growth of VSMCs. (*A*) Immunoblot analysis of endogenous WNK4 and TRPC3 expression in cultured VSMCs treated with scrambled (Scrm) or indicated siRNA (80 nmol/L). The lower blots of β-actin were used as controls. siRNAs specific for WNK4 and TRPC3 reduced their protein expressions by 87% and 82%, respectively. $*P < 0.05$, from control scrambled siRNA. (*B* and *C*) WNK4 depletion augments TRPC3-mediated Ba^{2+} influx by OAG (100 μmol/L) stimulation. Nifedipine (10 μmol/L) was applied to block voltage-gated L-type Ca^{2+} channel. (*D*) Fluorescence images of rhodamine-phalloidin-stained VSMCs that were treated with (*Lower*) or without (*Upper*) 1 μmol/L PE for 48 h in the absence of serum, after treatment with the indicated siRNA. (Scale bars, 100 μm.) (*E* and *F*) Analysis of hypertrophic cell growth. Measurements of relative cell size (*E*) and stress fiber formation represented by rhodamine-phalloidin fluorescence intensity (*F*) of VSMC images in *D*. In each experiment, 300–500 cells were analyzed. Values are the means ± SEM of indicated numbers.

Ca^{2+} has two well established effects in the cardiovascular system. Ca^{2+} mediates the acute response of vasoconstrictors (20, 21) and the long-term GPCR-mediated cell hypertrophy (22). A prominent Ca^{2+} channel associated with these vascular activities is TRPC3 (23). To assess the role of WNK4 on the TRPC3-mediated vascular hypertrophy, VSMCs were stimulated with the α-1-adrenoreceptor agonist phenylephrine (PE) for 48 h in the absence of serum, and cell hypertrophy was analyzed by measuring cell size and stress fiber formation (Fig. 1*D*). To better observe the WNK4 effect, the VSMCs were stimulated with a low dose (1 μmol/L) of PE, which caused a marginal cell size increase in control cells. Significantly, depletion of WNK4 alone was sufficient to cause 16.0 ± 3.4% and 30.8 ± 7.3% increase in cell size and stress fiber (F-actin) formation, respectively. Treatment with PE further increased the hypertrophic cell growth indices (Fig. 1 *E* and *F*). Most notably, the WNK4 depletion-induced hypertrophic changes were abolished by knockdown of TRPC3. Collectively, these results indicate that WNK4 negatively regulates TRPC3 activity and that the loss of WNK4 function induces a TRPC3-mediated hypertrophic cell growth in VSMCs.

WNK4 Depletion Augments TRPC3-Mediated Vasoconstriction. Next, the direct role of WNK4 and TRPC3 in vasoconstriction was examined by measuring the PE-induced vasoconstriction in isolated small mesenteric arteries. Small arteries greatly contribute to blood pressure because they are highly innervated by sympathetic nerves and react dynamically to vasoactive compounds (20). siRNAs were successfully introduced into intact arterial smooth muscle cells by a reversible permeabilization procedure (ref. 24; Fig. 2*A*). Immunoblot and RT-PCR analyses demonstrated the markedly reduced expression of endogenous proteins within 2 d after siRNA treatment (Fig. 2*B*). Arteries were mounted in an arteriograph chamber attached to a pressure myograph, and intraluminal pressure was maintained at 40 mmHg. Inner diameter of mesenteric artery was measured by using a video-recording system. The mesenteric arteries showed dose-dependent constriction in response to PE. The 500 nmol/L PE, which is within the logarithmic phase of the dose–response curve (Fig. 2*C*), was applied in subsequent experiments. A summary of multiple experiments is depicted in Fig. 2*D*, and individual examples of video images and inner diameter recordings are presented in Fig. 2 *E*–*H* and *I*–*L*, respectively. Compared with controls treated with scrambled siRNA, depletion of TRPC3 alone in resistance arteries induced a 47% reduction in the PE-induced vasoconstriction (Fig. 2 *D*, *H*, and *L*), indicating that TRPC3 plays an important role in the agonist-induced blood pressure control. Of note, depletion of WNK4 dramatically augmented the PE-induced vasoconstriction (Fig. 2 *F* and *J*). However, knockdown of TRPC3 nearly abolished the effect of depleting WNK4 (Fig. 2 *G* and *K*), indicating that the increased TRPC3 activity is responsible for most of the WNK4 depletion-induced up-regulation of vascular contraction. Hence, in addition to its effect on VSMC hypertrophy, WNK4 directly regulates the acute α-1-adrenoreceptor-induced constriction of small

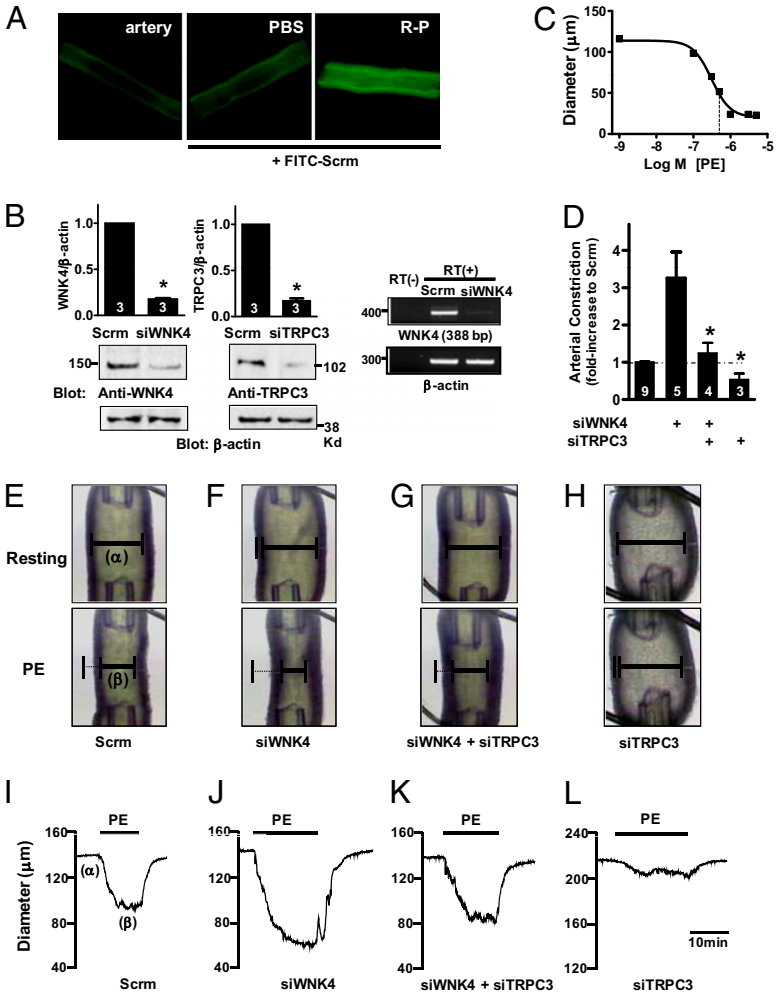

Fig. 2. Effects of WNK4 depletion on the TRPC3-mediated vasoconstriction in mesenteric arteries. (*A*) Incorporation of fluorescence (FITC)-labeled scrambled siRNA (Scrm) into intact rat mesenteric artery smooth muscle cells. Fluorescence Images indicate effective siRNA entry by reversible permeabilization (R-P) procedure (*Right*) compared with those exposed to PBS (*Center*). (*B Left*) Immunoblot of endogenous WNK4 and TRPC3 expression in reversibly permeabilized arteries treated with scrambled (Scrm) or the indicated siRNA (20 nmol/L) and cultured for 48 h. siRNAs specific for WNK4 and TRPC3 reduced their protein expressions by 82% and 83%, respectively. $*P < 0.05$ from control scrambled siRNA. (*Right*) The knockdown effect of WNK4 siRNA was additionally verified by RT-PCR. (*C*) Dose–response curve of cultured artery to PE stimulation. (*D*) Summarized results of arterial constriction measurement. Relative arterial constriction is represented in fold increase compared with scrambled siRNA-treated arteries. $*P < 0.05$ from siWNK4 alone. (*E–H*) Images of resting (*Upper*) and PE-stimulated (*Lower*) arteries mounted on an arteriograph chamber pressurized at 40 mmHg. The horizontal line indicates inner diameter at resting state, and vertical lines show the change in width after maximum constriction. (*I–L*) Measurements of inner diameter of arteries treated with the indicated siRNA and stimulated with 500 nmol/L PE. α, diameter at resting state; β, diameter after PE stimulation; RT, reverse-transcriptase.

arteries (Fig. 2*D*). Depletion or reduction of WNK4 activity is thus likely to elicit a steep increase in peripheral vascular resistance.

Human Mutations in WNK4 Alleviate TRPC3 Inhibition. PHAII-causing mutations in WNK4 have been linked to hypertension. Therefore, it was of interest to determine the effects of PHAII-causing mutation $WNK4^{Q562E}$ on the activation of TRPC3 by GPCRs and OAG. For these experiments, HEK 293T cells were cotransfected with the G_q-coupled M_3 muscarinic receptor (M_3R), TRPC3, and the indicated WNK4 constructs and were then stimulated with the M_3R agonist carbachol (Fig. 3*A*). WT-WNK4 strongly inhibited TRPC3-mediated Ba^{2+} influx, whereas $WNK4^{Q562E}$ was much less effective (Fig. 3 *A* and *B*). Similar results were obtained when TRPC3 was directly activated by OAG (ref. 19; Fig. 3 *C* and *D*). Therefore, it is conceivable that impaired inhibition of TRPC3 in the vasculature by mutant WNK4 contributes to the high blood pressure observed in PHAII patients and to the related vascular pathology.

Mechanisms Associated with WNK4-Induced Inhibition of TRPC3. To gain insight into the molecular mechanism by which WNK4 regulates TRPC3, first we analyzed the role of WNK4 kinase activity. Fig. 4 shows that the inhibitory effects of WNK4 on TRPC3 were dependent on the kinase activity of WNK4. Inactivation of kinase activity (K_i, $WNK4^{D318A}$) significantly released the inhibition of the TRPC3-mediated Ba^{2+} influx by WNK4 (Fig. 4 *A* and *B*). More direct evidence was obtained in the whole cell current measurements. HEK 293T cells were cotransfected with M_3R, and receptor-stimulated TRPC3-mediated current was measured. Similar to the fluorescence-based Ba^{2+} influx measurements, WNK4 reduced the TRPC3-mediated cation currents activated by receptor stimulation [WNK4(WT); Fig. 4 *C–E*]. Notably, inactivation of WNK4 kinase activity abolished this inhibition [WNK4(K_i); Fig. 4 *C–E*].

Analyses on TRPC3 surface expression reveal that WNK4-induced TRPC3 inhibition is mainly mediated by decreasing its cell surface expression. As shown in Fig. 5*A*, WNK4 depletion

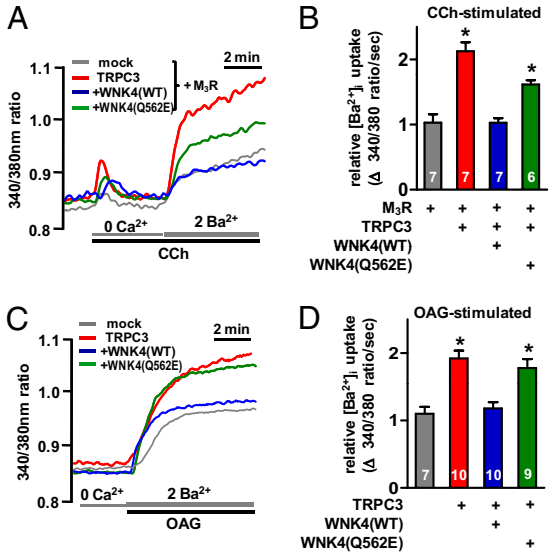

Fig. 3. Inhibition of TRPC3 activity by WNK4. Ba^{2+} influx in Fura-2 loaded HEK 293T cells was measured after transfection with indicated constructs. WT WNK4 abolished TRPC3-mediated Ba^{2+} influx. In contrast, PHAII-causing $WNK4^{Q562E}$ mutant only partially reduced TRPC3 activity. (*A* and *B*) The M_3 muscarinic receptor (M_3R) was cotransfected and stimulated with 100 μmol/L carbachol (CCh) to activate TRPC3 by a G-protein-dependent mechanism. *$P < 0.05$ from M_3R alone. (*C* and *D*) TRPC3 was directly activated with 100 μmol/L OAG. *$P < 0.05$ from mock-transfected control.

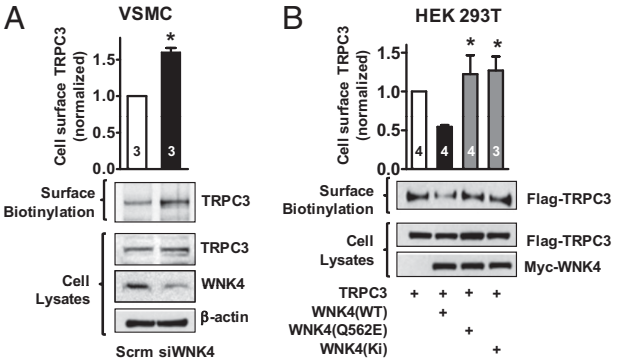

Fig. 5. WNK4 reduces surface expression of TRPC3. (*A*) Cell surface biotinylation of TRPC3 in VSMCs. WNK4 depletion (siWNK4) increased surface expression of TRPC3. *$P < 0.05$ from control scrambled siRNA (Scrm). (*B*) HEK 293T cells were transfected with indicated constructs, and cell surface expression of TRPC3 was quantified by using biotinylation assay. WNK4(WT) decreased surface expression of TRPC3, whereas $WNK4^{Q562E}$ and the kinase inactive WNK4(K_i) mutants showed no significant reduction in surface TRPC3. *$P < 0.05$ from TRPC3 + WNK4(WT).

induced a 60% increase in the surface expression of native TRPC3 in VSMCs. Furthermore, expression of WT-WNK4 caused a significant reduction in the surface biotinylated fractions of TRPC3 without affecting its total protein levels in HEK 293T cells. However, the PHAII-causing $WNK4^{Q562E}$ and kinase-inactive $WNK4^{D318A}$ mutants failed to decrease TRPC3 surface expression (Fig. 5*B*). These results imply that WNK4 may modulate TRPC3 activity by maintaining proper copy number at the cell surface via a WNK4 kinase-dependent mechanism. The TRPC6 channel, which is also activated by GPCRs, and the $Ca_v1.2$ L-type Ca^{2+} channel, which opens in response to TRPC3-mediated depolarization, are other candidates to elevate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in VSMCs (23). However, neither WT nor mutant WNK4 altered the surface expression of both TRPC6 and $Ca_v1.2$ (Fig. S2), in agreement with the findings that knockdown of TRPC3 is sufficient to alleviate most of the WNK4 depletion-induced pathologic responses in VSMCs (Figs. 1 and 2).

To further explore how WNK4 regulates TRPC3, we analyzed protein–protein interaction between the two proteins. Coimmunoprecipitation experiments in HEK 293T cells and rat aorta (Fig. 6 *A* and *B*) indicate that WNK4 and TRPC3 are present in a protein complex both in vitro and in vivo. Next, we designed truncated constructs of each protein (Fig. 6*C*) and performed coimmunoprecipitation assays with the truncated proteins to identify the WNK4 and TRPC3 interacting domains. WNK4 strongly interacts with constructs bearing the ankyrin repeats at the N terminus of TRPC3 (Fig. 6*D*), which are known to be involved in the targeting of TRPC3 to the plasma membrane (25). TRPC3 interacts with $WNK4_{441–799}$ and $WNK4_{441–1032}$, which share the first coiled-coil domain and the acidic motif where the PHAII-causing mutations are clustered (Fig. 6*E*; constructs 2 and 4 of Myc-WNK4). However, the interaction of $WNK4_{441–799}$ with TRPC3 was insufficient to retain the inhibition of TRPC3 activity by WNK4 (Fig. 6*F*), which accords with the importance of the kinase activity of WNK4 for this inhibition (Fig. 4).

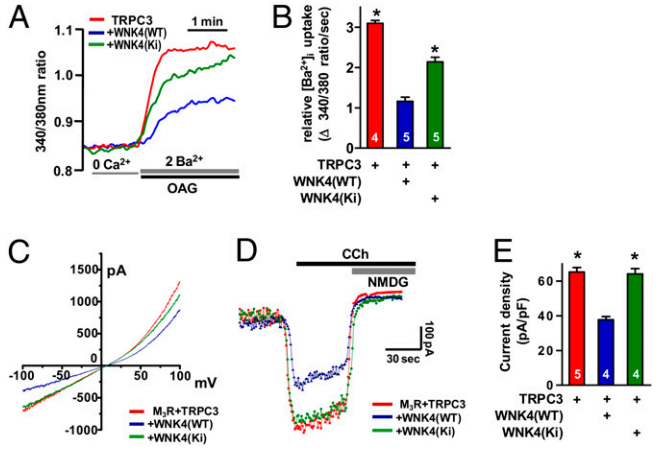

Fig. 4. WNK4-induced inhibition of TRPC3 is dependent on WNK4 kinase activity. (*A* and *B*) Measurement of Ba^{2+} influx in Fura-2 loaded HEK 293T cells. TRPC3 was directly activated with 100 mmol/L OAG. Inactivation of kinase activity (K_i, $WNK4^{D318A}$) significantly decreased the inhibition of the TRPC3-mediated Ba^{2+} influx by WT WNK4. *$P < 0.05$ from TRPC3 + WNK4 (WT). (*C–E*) Whole cell current measurements of TRPC3 were performed in M_3R cotransfected HEK 293T cells. The *I–V* relationship (*C*) and the cation current at −100 mV holding potential (*D*) were measured in response to 100 μmol/L carbachol (CCh) stimulation. Summarized results are presented in *E*. The kinase inactive (K_i) $WNK4^{D318A}$ mutant failed to inhibit TRPC3 activity. *$P < 0.05$ from TRPC3 + WNK4(WT).

Discussion

Despite its morbid consequences as a major risk factor for diverse cardiovascular diseases, only few genes have been identified to date that impart significant effects on blood pressure (26). Hence, regulatory pathways of renal ion transport that have been revealed by the discovery of WNK4 mutations in PHAII patients provide valuable insights into the pathogenesis of hypertension. To date, fluid retention due to the increased Na^+ and fluid reabsorption by the kidney through increased activity of NCC is considered as the sole mechanism for the increased blood pressure in PHAII patients harboring the $WNK4^{Q562E}$ mutation (5, 27–29). However, the expression of WNK4 in diverse extrarenal tissues (11) suggests that an additional pathway may contribute to the elevated blood pressure. In addition to blood volume ex-

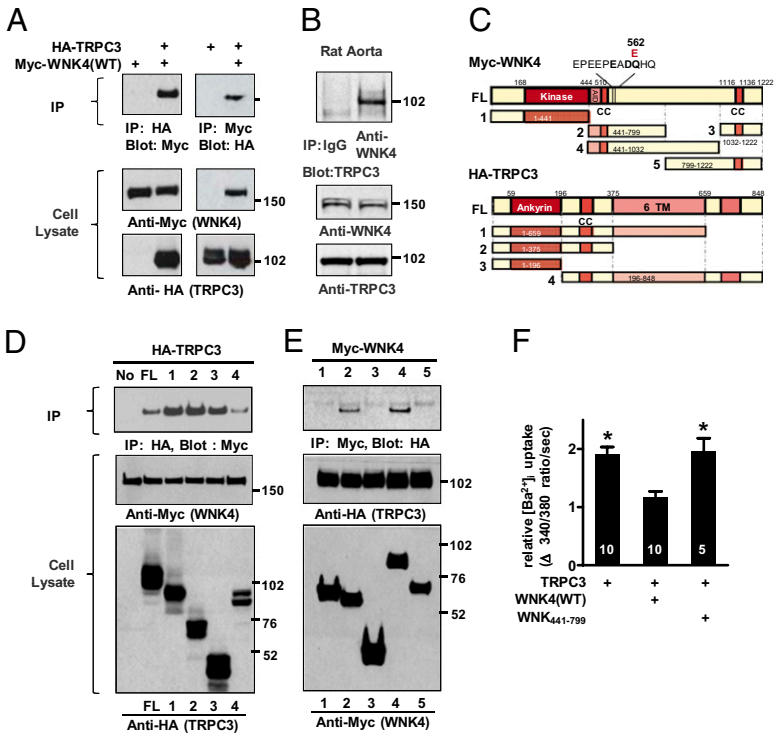

Fig. 6. WNK4 associates with TRPC3 in a protein complex. (*A*) Coimmunoprecipitation (IP) of Myc-WNK4 and HA-TRPC3 heterologously expressed in HEK 293T cells. (*B*) Coimmunoprecipitation of endogenous WNK4 and TRPC3 in rat aorta. (*C*) Diagrams for full-length and truncated constructs of Myc-WNK4 and HA-TRPC3 used in this study. (*D*) $TRPC3_{1–196}$ containing the N-terminal ankyrin repeats of TRPC3 is sufficient to coimmunoprecipitate WNK4. (*E*) Association of TRPC3 with $WNK4_{441–799}$ and $WNK4_{441–1032}$. The $WNK4_{441–799}$ region contains the first coiled-coil domain and the acidic motif where the PHAII-causing mutations are clustered. (*F*) The effect of $WNK4_{441–799}$ on TRPC3-mediated Ba^{2+} influx. The interaction of $WNK4_{441–799}$ with TRPC3 is not sufficient to exert the inhibitory effect of full-length WNK4. $^*P < 0.05$ from TRPC3 + WNK4(WT). AID, auto-inhibitory domain; CC, coiled-coil domain; FL, full-length; TM, transmembrane domain.

pansion, increased vascular resistance is a major determinant of hypertension. Accordingly, the present study provides an important reference on the expression and function of WNK4 in the vasculature and its direct role in the control of vascular resistance.

Our findings indicate that regulation of blood pressure by WNK4 is more complex than previously assumed. Notably, WNK4 is directly involved in the control of vascular tone by reducing TRPC3 activity (Fig. 2). TRPC3 and TRPC6 mediate cation influx evoked by stimulation of GPCRs that controls vasoconstriction in the resistance artery (14, 15, 23). The cation influx by TRPC3/TRPC6 subsequently induces membrane depolarization, which further increases $[Ca^{2+}]_i$ by activation of the $Ca_v1.2$ L-type Ca^{2+} channel. Neither surface expression of TRPC6 nor that of $Ca_v1.2$ was altered by WNK4 (Fig. S2), which suggests that the primary effect of WNK4 on the vasculature is mediated by regulation of TRPC3 function.

Previous reports suggested that TRPC3 activity is essential for cardiovascular hypertrophy induced by angiotensin II (23). Accordingly, TRPC3 depletion abolished α-adrenoreceptor–induced hypertrophic cell growth in VSMCs (Fig. 1 *D–F*), which is likely to increase the wall thickness/lumen diameter (W/L) ratio and raise peripheral resistance. Activations of angiotensin II receptor and α-adrenoreceptor are critical signaling cascades in the progression of human hypertension and related cardiovascular diseases. Importantly, WNK4 negatively regulated TRPC3 and greatly reduced TRPC3-mediated vascular hypertrophy (Fig. 1). Of note, histological analysis of mice harboring the Q562E (5) and D561A (6) mutations showed significant increase in the luminal surface area and size of the distal convoluted tubules (DCTs) by an unknown mechanism. In addition, increased expression of TRPV6 was recently observed in the DCTs of the $WNK4^{D561A/+}$ mouse (30). Similar to TRPC3 in VSMCs, the aberrant regulation of Ca^{2+}-permeable channels in the kidney (31) might be responsible for the hypertrophic phenotype observed in the DCTs of transgenic mice. Together, these results raise the possibility that WNK4 may be a potential therapeutic target both to acutely control blood pressure and to chronically modulate vascular remodeling.

Similar to WNK4 regulation on NCC (29, 32, 33), WT-WNK4 inhibits TRPC3 activity by regulating its surface abundance, which was dependent on the kinase activity of WNK4. Similarly, parallel to the effect on NCC, the aberrant regulation by $WNK4^{Q562E}$ increased the surface expression of TRPC3 and thus raised $[Ca^{2+}]_i$. The combined effect of WNK4 on NCC and TRPC3 would likely function to set both adequate blood volume and peripheral resistance in normal subjects. Mutations in WNK4, such as $WNK4^{Q562E}$, or altered WNK4 activity due to mutations in WNK1 (32, 33), would increase blood volume and induce vasoconstriction to raise blood pressure in PHAII patients. The present findings have direct clinical implications, in which a combination of vasodilators in addition to the NCC inhibitor thiazide diuretics should be considered in the treatment of WNK4-related hypertension, especially in those who exhibit limited response to thiazide. Further characterization of WNK4 and its signaling pathways in the vasculature are needed to provide insight for possible therapeutic strategies to control aberrant blood pressure.

Materials and Methods

Plasmids, Cell Culture, and siRNAs. The mammalian expressible plasmids for mWNK4 (7), hTRPC3 (34) pRK5-HA-TRPC6 (34), and $rCa_v1.2$ (35) were described. HEK 293T cells were cultured in Dulbecco's modified Eagle medium. Full methods are described in *SI Materials and Methods*.

Isolation and Culture of VSMCs and Mesenteric Artery. The rat aortic VSMCs were isolated as reported (36). To isolate mesenteric artery, the third and fourth branch of mesenteric arteries (120–220 μm, inner diameter) were cut into 2- to 3-mm segments for subsequent analysis. Full methods are available in *SI Materials and Methods*.

Immunoblotting, Immunoprecipitation, Cell Surface Biotinylation, RT-PCR, and Real-Time PCR Analysis. Conventional protocols were used in these experiments. Full methods are described in *SI Materials and Methods*.

Cell Hypertrophy Analysis. Fluorescent images of 300–500 cells were taken from 10–15 randomly chosen fields, and relative cell size and rhodamine-phalloidin fluorescence intensity of VSMCs were analyzed by using MetaMorph software (Molecular Devices). Full methods are described in *SI Materials and Methods*.

Reversible Permeabilization and Arterial Constriction Analysis in Mesenteric Artery. siRNAs were introduced into intact mesenteric arteries through reversible permeabilization procedure as reported (24). The difference in diameter at resting state (α) and maximum constricted state after PE stimulation (β) was measured as percent constriction, and each group was compared by the fold increase relative to the scrambled siRNA transfected group. % constriction = $(\alpha - \beta)/\alpha \times 100$. Full methods are described in *SI Materials and Methods*.

Measurements of Ba^{2+} Influx and TRPC3 Current. Ba^{2+} influx was measured in cultured VSMCs and HEK 293T cells by using Fura-2 (Invitrogen) as described (34). For current recording of TRPC3, whole cell current measurement was accomplished in M_3R-cotransfected HEK 293T cells. Full methods are described in *SI Materials and Methods*.

Statistical Analysis. The results of multiple experiments are presented as the means ± SEM. Statistical analysis was performed with Student's *t* tests or with ANOVA followed by Tukey's multiple comparison test, as appropriate. $P < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS. We thank Dr. D. G. Welsh for technical advice on the reversible permeabilization procedure and the Yonsei-Carl Zeiss Advanced Imaging Center for technical assistance. This work was supported by National Research Foundation of Korea (NRF) Grants 2010-0001670 and 2010-0017752 (to M.G.L.) and 2007-0054658 (to J.Y.K.), which were funded by the Korean Ministry of Education, Science, and Technology. M.G.L. was supported by the faculty research program of Yonsei University College of Medicine.

1. Burt VL, et al. (1995) Prevalence of hypertension in the US adult population. Results from the Third National Health and Nutrition Examination Survey, 1988-1991. *Hypertension* 25:305–313.
2. Carretero OA, Oparil S (2000) Essential hypertension. Part I: Definition and etiology. *Circulation* 101:329–335.
3. Lifton RP (1996) Molecular genetics of human blood pressure variation. *Science* 272: 676–680.
4. Wilson FH, et al. (2001) Human hypertension caused by mutations in WNK kinases. *Science* 293:1107–1112.
5. Lalioti MD, et al. (2006) Wnk4 controls blood pressure and potassium homeostasis via regulation of mass and activity of the distal convoluted tubule. *Nat Genet* 38: 1124–1132.
6. Yang SS, et al. (2007) Molecular pathogenesis of pseudohypoaldosteronism type II: generation and analysis of a Wnk4(D561A/+) knockin mouse model. *Cell Metab* 5: 331–344.
7. He G, Wang HR, Huang SK, Huang CL (2007) Intersectin links WNK kinases to endocytosis of ROMK1. *J Clin Invest* 117:1078–1087.
8. Kahle KT, Ring AM, Lifton RP (2008) Molecular physiology of the WNK kinases. *Annu Rev Physiol* 70:329–355.
9. Kahle KT, et al. (2003) WNK4 regulates the balance between renal NaCl reabsorption and K+ secretion. *Nat Genet* 35:372–376.
10. Rossier BC (2003) Negative regulators of sodium transport in the kidney: key factors in understanding salt-sensitive hypertension? *J Clin Invest* 111:947–950.
11. Kahle KT, et al. (2004) WNK4 regulates apical and basolateral Cl- flux in extrarenal epithelia. *Proc Natl Acad Sci USA* 101:2064–2069.
12. Inoue R, et al. (2006) Transient receptor potential channels in cardiovascular function and disease. *Circ Res* 99:119–131.
13. Watanabe H, Murakami M, Ohba T, Takahashi Y, Ito H (2008) TRP channel and cardiovascular disease. *Pharmacol Ther* 118:337–351.
14. Reading SA, Earley S, Waldron BJ, Welsh DG, Brayden JE (2005) TRPC3 mediates pyrimidine receptor-induced depolarization of cerebral arteries. *Am J Physiol Heart Circ Physiol* 288:H2055–H2061.
15. Liu D, et al. (2009) Increased transient receptor potential canonical type 3 channels in vasculature from hypertensive rats. *Hypertension* 53:70–76.
16. Thilo F, et al. (2009) Transient receptor potential canonical type 3 channels and blood pressure in humans. *J Hypertens* 27:1217–1223.
17. Thilo F, Loddenkemper C, Berg E, Zidek W, Tepel M (2009) Increased TRPC3 expression in vascular endothelium of patients with malignant hypertension. *Mod Pathol* 22: 426–430.
18. Yu Y, et al. (2004) Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. *Proc Natl Acad Sci USA* 101:13861–13866.
19. Hofmann T, et al. (1999) Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature* 397:259–263.
20. Mulvany MJ, Aalkjaer C (1990) Structure and function of small arteries. *Physiol Rev* 70: 921–961.
21. Papageorgiou P, Morgan KG (1991) Increased Ca2+ signaling after alpha-adrenoceptor activation in vascular hypertrophy. *Circ Res* 68:1080–1084.
22. Nishida M, Kurose H (2008) Roles of TRP channels in the development of cardiac hypertrophy. *Naunyn Schmiedebergs Arch Pharmacol* 378:395–406.
23. Onohara N, et al. (2006) TRPC3 and TRPC6 are essential for angiotensin II-induced cardiac hypertrophy. *EMBO J* 25:5305–5316.
24. Lesh RE, Somlyo AP, Owens GK, Somlyo AV (1995) Reversible permeabilization. A novel technique for the intracellular introduction of antisense oligodeoxynucleotides into intact smooth muscle. *Circ Res* 77:220–230.
25. Wedel BJ, Vazquez G, McKay RR, St J Bird G, Putney JW, Jr. (2003) A calmodulin/inositol 1,4,5-trisphosphate (IP3) receptor-binding region targets TRPC3 to the plasma membrane in a calmodulin/IP3 receptor-independent process. *J Biol Chem* 278: 25758–25765.
26. Lifton RP, Gharavi AG, Geller DS (2001) Molecular mechanisms of human hypertension. *Cell* 104:545–556.
27. Cope G, Golbang A, O'Shaughnessy KM (2005) WNK kinases and the control of blood pressure. *Pharmacol Ther* 106:221–231.
28. Richardson C, Alessi DR (2008) The regulation of salt transport and blood pressure by the WNK-SPAK/OSR1 signalling pathway. *J Cell Sci* 121:3293–3304.
29. Yang CL, Zhu X, Ellison DH (2007) The thiazide-sensitive Na-Cl cotransporter is regulated by a WNK kinase signaling complex. *J Clin Invest* 117:3403–3411.
30. Yang SS, et al. (2010) Mechanisms for hypercalciuria in pseudohypoaldosteronism type II-causing WNK4 knock-in mice. *Endocrinology* 151:1829–1836.
31. Woudenberg-Vrenken TE, Bindels RJ, Hoenderop JG (2009) The role of transient receptor potential channels in kidney disease. *Nat Rev Nephrol* 5:441–449.
32. Yang CL, Angell J, Mitchell R, Ellison DH (2003) WNK kinases regulate thiazide-sensitive Na-Cl cotransport. *J Clin Invest* 111:1039–1045.
33. Yang CL, Zhu X, Wang Z, Subramanya AR, Ellison DH (2005) Mechanisms of WNK1 and WNK4 interaction in the regulation of thiazide-sensitive NaCl cotransport. *J Clin Invest* 115:1379–1387.
34. Huang GN, et al. (2006) STIM1 carboxyl-terminus activates native SOC, I(crac) and TRPC1 channels. *Nat Cell Biol* 8:1003–1010.
35. Zhang H, et al. (2006) Ca1.2 and CaV1.3 neuronal L-type calcium channels: differential targeting and signaling to pCREB. *Eur J Neurosci* 23:2297–2310.
36. Chamley-Campbell J, Campbell GR, Ross R (1979) The smooth muscle cell in culture. *Physiol Rev* 59:1–61.