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

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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 Ca2+-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 Ca2+ influx via the transient receptor potential canonical 3 (TRPC3) channel in the vasculature. Loss of WNK4 markedly augmented TRPC3-mediated Ca2+ 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 Ca2+ 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 [Ca2+]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 Ca2+ influx in primary cultured rat aortic VSMCs, endogenous WNK4 and TRPC3 proteins were knocked down by using specific siRNAs for each protein (Fig. 1A). Ba2+ influx was measured to isolate Ca2+ influx through voltage-gated L-type Ca2+ channels was excluded by including nifedipine in the bath solution. Treatment with TRPC3-specific siRNA had minimal effect on Ca2+ influx in resting cells, suggesting that TRPC3 primarily mediates Ca2+ influx in receptor-stimulated cells. Notably, depletions of WNK4 in VSMCs significantly increased Ba2+ influx that was largely abolished by knockdown of native TRPC3 (Fig. 1B and C). siRNA against WNK4 had no effect on the expression of TRPC1 and TRPC6 (Fig. S1), which are also known to induce GPCR-mediated [Ca2+]i increase in VSMCs (12–14). These results indicate that WNK4 exerts an inhibitory effect on Ca2+ entry via TRPC3.

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The authors declare no conflict of interest.

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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 \(\alpha\)-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. 1D). To better observe the WNK4 effect, the VSMCs were stimulated with a low dose (1 \(\mu\)mol/L) of PE, which caused a marginal cell size increase in control cells. Significantly, depletion of WNK4 alone was sufficient to cause 16.0 \(\pm\) 3.4\% and 30.8 \(\pm\) 7.3\% increase in cell size and stress fiber (F-actin) formation, respectively. Treatment with PE further increased the hypertrophic cell growth indices (Fig. 1E 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. 2A). Immunoblot and RT-PCR analyses demonstrated the markedly reduced expression of endogenous proteins within 2 d after siRNA treatment (Fig. 2B). 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. 2C), was applied in subsequent experiments. A summary of multiple experiments is depicted in Fig. 2D, and individual examples of video images and inner diameter recordings are presented in Fig. 2E–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. 2D, 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. 2F and J). However, knockdown of TRPC3 nearly abolished the effect of depleting WNK4 (Fig. 2G 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 \(\alpha\)-1-adrenoreceptor-induced constriction of small arteries.**
arteries (Fig. 2D). 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.** PHAI-II-causing mutations in WNK4 have been linked to hypertension. Therefore, it was of interest to determine the effects of PHAI-II-causing mutation WNK4^{D318E} 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_{3}R), TRPC3, and the indicated WNK4 constructs and were then stimulated with the M_{3}R agonist carbachol (Fig. 3A). WT-WNK4 strongly inhibited TRPC3-mediated Ba^{2+} influx, whereas WNK4^{D318E} was much less effective (Fig. 3A 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 PHAI-II 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 (WK; 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_{3}R, 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(WK); 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. 5A, WNK4 depletion

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**Fig. 2.** Effects of WNK4 depletion on the TRPC3-mediated vasconstriction 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 knockout 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.
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 PHAI1-causing WNK4<sup>Q562E</sup> and kinase-inactive WNK4<sup>Q562E D318A</sup> mutants failed to decrease TRPC3 activity. (Figs. 1A and B) PHAI1 mutations 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. Commmunoprecipitation experiments in HEK 293T cells and rat aorta (Fig. 6A 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. 6C) and performed communoprecipitation assays with the truncated proteins to identify the WNK4 and TRPC3 interacting domains. WNK4 interacts with constructs bearing the ankyrin repeats at the N terminus of TRPC3 (Fig. 6D), which are known to be involved in the targeting of TRPC3 to the plasma membrane (25). TRPC3 interacts with WNK4<sup>441–799</sup> and WNK4<sup>441–1032</sup>, which share the first coiled-coil domain and the acidic motif where the PHAI1-causing mutations are clustered (Fig. 6E; constructs 2 and 4 of Myc-WNK4). However, the interaction of WNK4<sup>441–799</sup> with TRPC3 was insufficient to retain the inhibition of TRPC3 activity by WNK4 (Fig. 6F), which accords with the importance of the kinase activity of WNK4 for this inhibition (Fig. 4).

**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<sup>+</sup> 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<sup>Q562E</sup> 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-

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**Fig. 3.** Inhibition of TRPC3 activity by WNK4. Ba<sup>2+</sup> influx in Fura-2 loaded HEK 293T cells was measured after transfection with indicated constructs. WT WNK4 abolished TRPC3-mediated Ba<sup>2+</sup> influx. In contrast, PHAI1-causing WNK4<sup>Q562E</sup> mutant only partially reduced TRPC3 activity. *P < 0.05 from WT, *P < 0.05 from mock-transfected control.

**Fig. 4.** WNK4-induced inhibition of TRPC3 is dependent on WNK4 kinase activity. (A and B) Measurement of Ba<sup>2+</sup> influx in Fura-2 loaded HEK 293T cells. TRPC3 was directly activated with 100 μmol/L OAG. *P < 0.05 from mock-transfected control. (C) Whole cell current measurements of TRPC3 were performed in HEK 293T cells. However, the PHAI1-causing WNK4<sup>Q562E</sup> and kinase-inactive WNK4<sup>Q562E D318A</sup> mutants failed to decrease TRPC3 activity. (D) Whole cell current measurements of TRPC3 were performed in 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<sup>i</sup>) WNK4<sup>D318A</sup> mutant failed to inhibit TRPC3 activity. *P < 0.05 from TRPC3 + WNK4(WT).

**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 (Scr). (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<sup>Q562E</sup> and the kinase inactive WNK4(K) mutants showed no significant reduction in surface TRPC3. *P < 0.05 from TRPC3 + WNK4(WT).
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 TRPC3/6 subsequently induces membrane depolarization, which further increases \([\text{Ca}^{2+}]_i\) by activation of the \(\alpha_{1,2}\) L-type \(\text{Ca}^{2+}\) channel. Neither surface expression of TRPC6 nor that of \(\text{Ca}^{2+}\) influx. The interaction of WNK4 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.

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 1-198 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 PHAI-III-causing mutations are clustered. (F) The effect of WNK4\(_{441-799}\) on TRPC3-mediated \(\text{Ba}^{2+}\) 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.

Materials and Methods

Plasmids, Cell Culture, and siRNAs. The mammalian expressible plasmids for mWNK4 (7), hTRPC3 (34) pR5K-HA-TRPC6 (34), and rCa\(_{1.2}\) (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 (a) 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 = (β − α)a × 100. Full methods are described in SI Materials and Methods.

Measurements of Ba2+ Influx and TRPC3 Current. Ba2+ 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,R-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.

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