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Original Article

High potassium concentration regulates the WNK3–SPAK–NKCC1 phosphorylation cascade via kelch-like protein 2 in mouse vascular smooth muscle cells

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Abstract

We have previously demonstrated that kelch-like protein 2 (KLHL2) forms a complex with Cullin3 that functions as an E3 ligase in the degradation of with-no-lysine kinases (WNKs). KLHL2 physiologically mediates the WNK3–SPAK–NKCC1 phosphorylation cascade in vascular smooth cells, thereby regulating vascular tone. A high-potassium diet can attenuate hypertension by reducing peripheral vascular resistance. In addition, we have reported that extracellular potassium can negatively regulate the WNK–OSR1/SPAK–NCC phosphorylation cascade in COS7 cells. However, the effect of high potassium concentrations on the role of WNKs in vascular tone regulation has not yet been clarified. In the present study, we investigated whether the WNK–SPAK–NKCC1 cascade in mouse vascular smooth muscle (MOVAS) cells could be regulated by high potassium concentrations. We confirmed that the WNK3–SPAK–NKCC1 phosphorylation cascade was significantly inhibited in high-potassium medium but that the protein level of WNK1 remained unchanged. Furthermore, KLHL2 levels increased when MOVAS cells were incubated in a high-potassium medium. KLHL2-knockdown experiments confirmed that the inhibition of the WNK3–SPAK–NKCC1 phosphorylation cascade induced by high potassium concentrations was attributable to KLHL2-mediated degradation of WNK3. Thus, the present study could explain one of the mechanisms underlying antihypertensive effects of high dietary potassium intake.

Keywords: KLHL2, hypertension, potassium, WNK3, vascular smooth muscle cells.

Introduction

Mutations in the with-no-lysine kinase 1 (WNK1) and WNK4 genes lead to pseudohypoaldosteronism type II (PHAII)[1], which is characterized by hereditary hypertension, hyperkalemia, metabolic acidosis, and thiazide sensitivity[2]. We have previously demonstrated that WNKs increase the phosphorylation of oxidative stress-responsive kinase 1 (OSR1) and STE20/SPS1-related proline/alanine-rich kinase (SPAK) in vivo, which are known as substrates of WNKs that in turn increase the phosphorylation of Na-K-Cl cotransporter (NKCC) and Na-Cl cotransporter (NCC)[3–6]. We have also reported that the over activation of the WNK–OSR1/SPAK–NCC phosphorylation cascade by mutated WNK4 is a major cause underlying PHAII[3].

Recently, kelch-like protein 3 (KLHL3) and Cullin3 (CUL3) have also been implicated in PHAII development[7]. KLHL3 forms a complex with CUL3 that mimics E3 ubiquitin ligase, which serves as a substrate adaptor for ubiquitination[8]. We have also reported that WNK4 is a major ubiquitination target of the KLHL3-CUL3 E3 ligase complex and that mutations in WNK4, KLHL3, or CUL3 impair the KLHL3-mediated ubiquitination of WNK4, which is responsible for PHAII development[9, 10]. Because KLHL3 binds to a highly conserved acidic domain belonging to WNKs, all WNKs can be substrates of the KLHL3-CUL3 E3 ligase complex[11]. KLHL2, the

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closest homolog of KLHL3, possesses a kelch domain (WNK-binding domain) that is highly similar to that of KLHL3; therefore, KLHL2 can also form a KLHL2-CUL3 E3 ubiquitin ligase and bind to all WNKs for degradation[12].

NKCC1 regulates vascular smooth muscle cell contractions via intracellular chloride accumulation and membrane depolarization, leading to the activation of voltage-gated calcium channels and, consequently, the elevation of peripheral resistance[13]. The WNK–OSR1/SPAK–NKCC1 phosphorylation cascade is also important for maintaining vascular tone in vascular smooth muscle cells. Bergaya et al.[14] and we[6, 15] have previously reported that WNK1 and WNK3–SPAK–NKCC1 signaling in arteries are critical in maintaining vascular tone in $WNK1^{+/-}$ and WNK3-knockout mice. Moreover, we have recently found that KLHL2 is expressed, but not KLHL3, in the mouse aorta and mouse vascular smooth muscle (MOVAS) cells, therefore, we focused on KLHL2 in WNK signaling cascade and excluded the influence of KLHL3. Using KLHL2 knockdown and overexpression, we have also confirmed that KLHL2 regulates WNK3 levels in MOVAS cells[16].

Based on clinical studies, increased dietary potassium intake is recommended for preventing and treating hypertension[17, 18], regardless of the paradoxical tendency of raising blood pressure caused by the increase in aldosterone level induced by hyperkalemia[19]. Furthermore, high dietary potassium intake is inversely related to the occurrence of cardiovascular diseases and mortality[17]. Because of the health benefits of high dietary potassium, it is particularly important to elucidate the biochemical mechanism underlying the attenuation of hypertension by potassium. Among the many possible mechanisms underlying the antihypertensive effect of potassium, NCC, which is expressed in the distal convoluted tubules in the kidneys, is thought to play a key role[20]. In addition, potassium has been recognized as a vasodilator owing to its effect on vascular smooth muscle and direct reduction of peripheral vascular resistance[19], but the evidence to date is far from conclusive. Because extracellular potassium negatively regulates the WNK1–OSR1/SPAK–NCC phosphorylation cascade in COS7 cells[21], it is possible that high potassium concentrations suppress WNK1 and WNK3 activities, thereby inhibiting the SPAK–NKCC1 phosphorylation cascade in vascular smooth muscle cells.

Our study aimed to clarify whether the WNK–SPAK–NKCC1 phosphorylation cascade is suppressed by high potassium concentrations in vascular smooth muscle and whether KLHL2 is involved in this mechanism. We demonstrated that high potassium concentration suppressed the WNK3–SPAK–NKCC1 phosphorylation cascade via KLHL2 in MOVAS cells. This molecular mechanism could be responsible for antihypertensive effects of high dietary potassium intake.

Material and methods

Cell culture

We used the MOVAS cell line derived from mouse aorta (CRL-2797; ATCC) as the in vitro model. MOVAS cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 4.5 g/L d-glucose, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. The cells were grown in a humidified incubator at 37°C with 5% CO_2. We designated the osmolality and potassium concentration of DMEM as the standard condition (330 mOsm/kg H_2O, 5 mM K^+). The normal-potassium (5 mM K^+) and high-potassium (8.4 mM K^+) media comprised K^+ free isotonic solution (120 mM NaCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 44 mM $NaHCO_3$, 10 mM Hepes, and 10% FBS) and were supplemented with additional high concentration potassium gluconate solution. The NaCl concentration and osmolality of each type of K^+ solution were nearly equal to those of DMEM. The osmolality of the solutions was measured using Fiske One-Ten Osmometer (Fiske Associates). MOVAS cells were incubated in both high- and normal-potassium media for 60 min before performing analysis using Western blotting and RT-PCR.

Western blotting

MOVAS cells cultured in six-well collagen-coated culture plates were lysed using 150 μL of lysis buffer [150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 50 mM Tris/HCl (pH 7.5) 1 mM Na_3VO_4, 1 mM DTT, 1% Triton X-100, 0.27 M sucrose, and complete protease inhibitor cocktail (Roche): one tablet per 50 mL]. After 30 min of incubation on ice, the supernatants (100 μL) were collected by centrifugation at 12,000 ×*g* for 10 min at 4°C and were denatured for 20 min at 60°C using SDS sample buffer (Cosmo Bio). The processed samples were then subjected to SDS-PAGE, and blots were probed using the following primary antibodies: sheep anti-WNK3 antibody (S346C)[22], rabbit anti-WNK1 antibody (A301-516A; BETHYL), rabbit anti-phosphorylated SPAK antibody[23], rabbit anti-phosphorylated NKCC1 (T206) antibody[24, 25], rabbit anti-KLHL2/KLHL3 antibody (Santa Cruz Biotechnology), rabbit anti-phosphorylated OSR1 antibody (S325)[5] and rabbit anti-actin antibody (Cell Signaling Technology). Alkaline-phosphatase-conjugated anti-IgG antibodies (Promega) were used

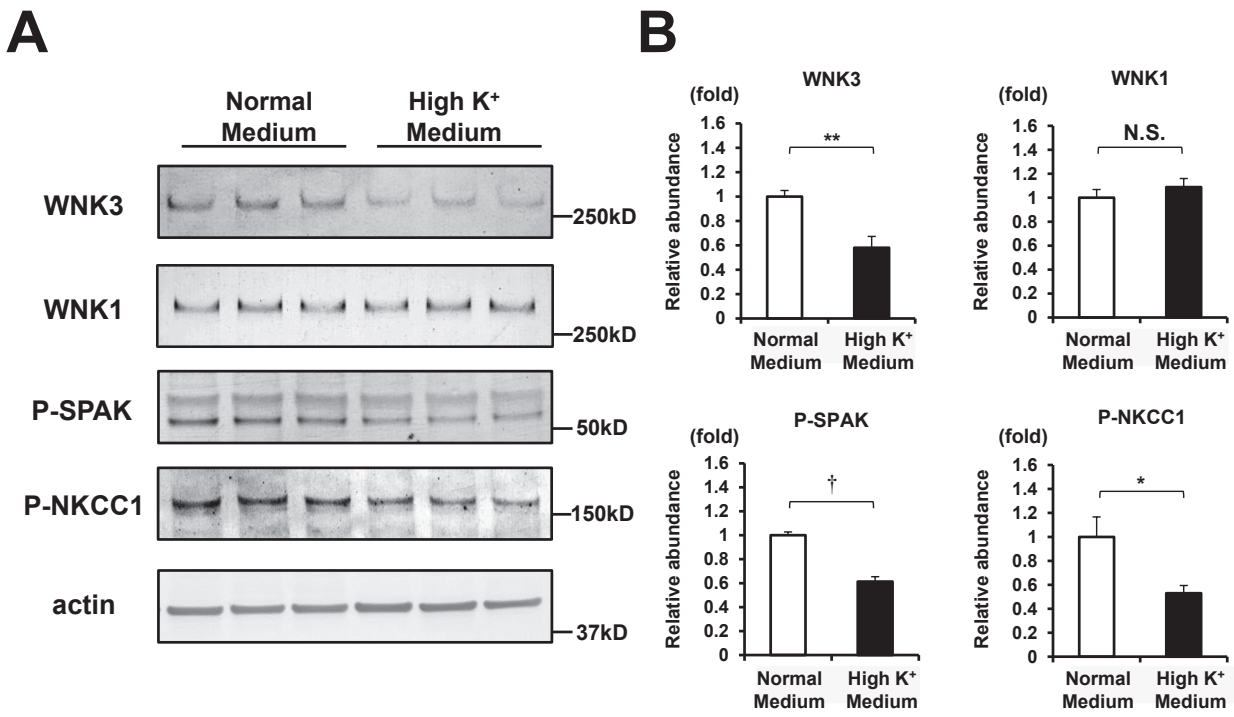

Figure 1. Expression of WNK3, WNK1, and SPAK-NKCC1 phosphorylation cascade in MOVAS cells treated with normal-potassium and high-potassium media.

(A) Representative immunoblots and (B) densitometry analyses of WNK3, WNK1, P-SPAK, and P-NKCC1 after 60 min of incubation in normal- and high-potassium media. The protein expression level in normal-potassium groups and high-potassium groups were evaluated by relative abundance. *p < 0.05; **p < 0.01; †p < 0.001; N.S., not statistically significant. White bars: normal-potassium groups; black bars: high-potassium groups. See Supplementary Table 1 for values used for Fig 1B.

as the secondary antibodies. Western Blue (Promega) was used to detect the signals. The relative intensity levels of the immunoblot bands were measured using densitometry analysis with ImageJ.

RT-PCR

TRIzol reagent (Invitrogen) was used for extracting total RNA from MOVAS cells. Total RNA was reverse transcribed using Omniscript RT (Qiagen). For quantitative RT-PCR (qRT-PCR), the target cDNAs were amplified using PCR with SYBR Premix Ex Taq II (TAKARA BIO). The sequences of WNK3 primers for qRT-PCR were constructed on the basis of the designs used in our previous studies[15, 16]. The commercially available GAPDH primers (TAKARA BIO) were used for loading control. A sample size of 5 subjects per group was set for this study.

KLHL2 knockdown experiments

For KLHL2 knockdown in MOVAS cells, triple duplexes of mouse KLHL2 small interfering RNAs (siRNAs) purchased from Origene were used; detailed protocols have been described in our previous reports[16]. Briefly, mouse KLHL2 siRNA was transfected into MOVAS cells using Lipofectamine 2000 reagent (Invitrogen). All cells were harvested after 48 h of siRNA transfection. A sample size of 5 subjects per group was set for this study.

Statistical analysis

Comparison of statistical significance between the two groups was performed using unpaired Student's *t*-test. ANOVA with Tukey's *post hoc* test was used to evaluate statistical significance in the comparison among multiple groups. All data were expressed as mean ± standard error of means. Differences with p values of <0.05 were considered statistically significant and calculated with IBM SPSS statistics software.

Results

High-potassium medium inhibits the WNK3-SPAK-NKCC1 phosphorylation cascade in MOVAS cells

To investigate whether high potassium concentration

affects WNKs signaling in vascular smooth muscle cells, we examined WNK–SPAK–NKCC1 phosphorylation cascade in MOVAS cells after incubating with normal- or high-potassium medium (Figure 1). As shown in Figure 1B and Supplementary Table 1, after incubating with the high-potassium medium, WNK3 levels were found to have decreased ($n = 6$; $p < 0.01$). However, the differences in WNK1 levels were not statistically significant ($n = 6$). We also observed a reduction in phosphorylated SPAK and NKCC1 levels in MOVAS cells treated with the high-potassium medium ($n = 6$; P-SPAK, $p < 0.001$; P-NKCC1, $p < 0.05$). Furthermore, we measured protein levels of phosphorylated OSR1 ($n = 6$), which showed no significant differences following normal and high-potassium treatments (Supplementary Figure 1 and Supplementary Table 4).

This suggested that WNK3 plays a critical role in high potassium concentration-induced decreased phosphorylation of SPAK–NKCC1 signaling, which leads to decreased vascular tonus.

High potassium concentration increases KLHL2 protein levels in MOVAS cells

We further investigated how potassium regulates WNK3 levels. Our previous reports have clarified that KLHL2 is the closest homolog of KLHL3 and that the kelch domain (WNK-binding domain) of KLHL2 is highly similar to that of KLHL3. Furthermore, all WNKs, including WNK3, can be ubiquitinated by KLHL2 and KLHL3, thereby leading to WNK degradation sequence by KLHL2/3–Cul3 E3 ligase[12, 16]. In particular, WNK3 binds to KLHL2 and KLHL3 with a stronger affinity than other WNKs[12]. Therefore, we hypothesized that WNK3 is degraded by KLHL2 in MOVAS cells in the presence of high potassium concentration. To test this hypothesis, we analyzed WNK3 mRNA and KLHL2 protein levels in MOVAS cells. qRT-PCR showed no significant difference in WNK3 mRNA levels between treatments with normal and high potassium concentrations ($n = 5$; Figure 2A and Supplementary Table 2). Western blotting revealed that KLHL2 levels were significantly 1.5 times higher in MOVAS cells incubated in the high-potassium medium than in those incubated in normal-potassium medium ($n = 6$; $p < 0.001$; Figure 2C and Supplementary Table 2).

These results indicated that high potassium concentrations increased KLHL2 levels in MOVAS cells, leading to a proportionate increase in WNK3 degradation without influencing WNK3 transcription.

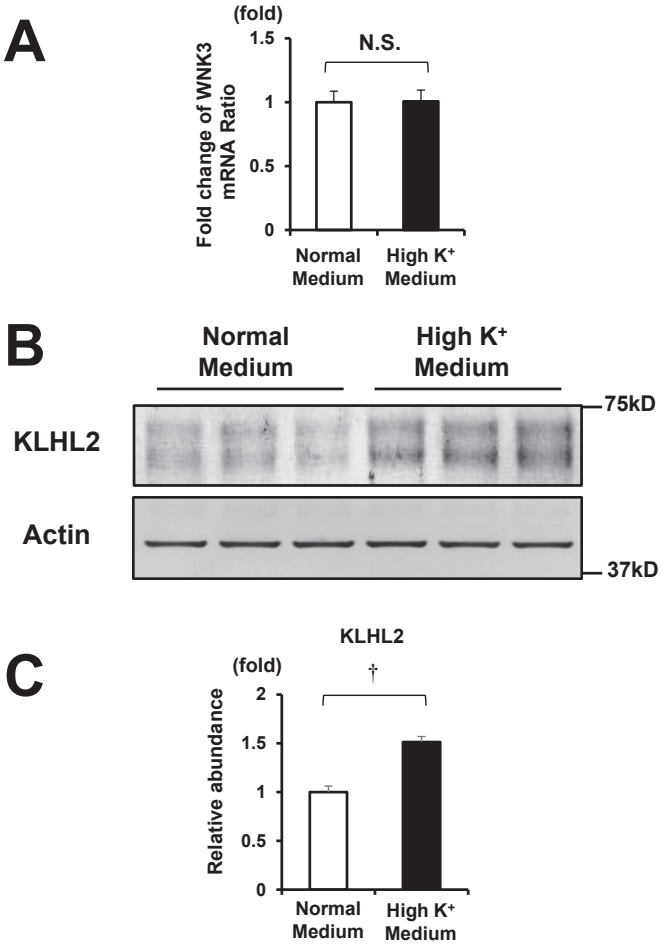

Figure 2. WNK3 mRNA levels and KLHL2 protein levels in MOVAS cells in the presence of high potassium concentration.

(A) WNK3 mRNA levels in MOVAS cells analyzed by quantitative RT-PCR after incubation in normal- and high-potassium media for 60 min. (B) Representative immunoblots and (C) densitometry analyses of KLHL2 levels in MOVAS cells after incubation in normal- and high-potassium media for 60 min. The protein expression level in normal-potassium groups and high-potassium groups were evaluated by relative abundance. †$p < 0.001$; N.S., not statistically significant. White bars: normal-potassium groups; black bars: high-potassium groups. See Supplementary Table 2 for values used for Fig 2A and 2C.

KLHL2 regulates the WNK3–SPAK phosphorylation cascade in the presence of high potassium concentration

To confirm whether reduced WNK3 levels in the presence of high potassium concentration are regulated by KLHL2, we performed KLHL2-knockdown experiments using MOVAS cells. As shown in Figure 3 and Supplementary Table 3, KLHL2 knockdown decreased KLHL2 levels and increased WNK3 levels in the presence of normal potassium concentrations, with a

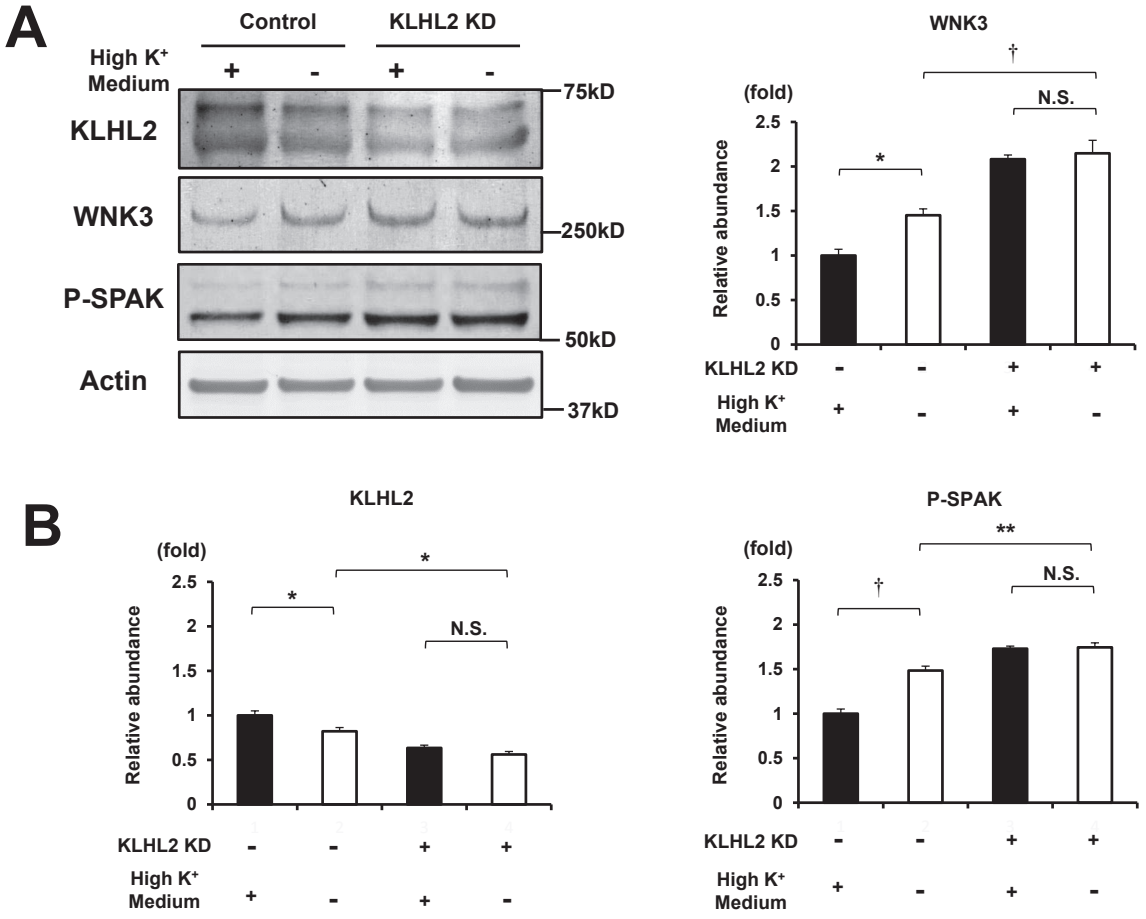

Figure 3. KLHL2 is involved in the mechanisms of high-potassium induced WNK3 degradation.
(A) Representative immunoblots and (B) densitometry analyses of KLHL2, WNK3, and P-SPAK levels in control knockdown (KD) MOVAS cells and KLHL2-KD MOVAS cells, treated with normal- and high-potassium media for 60 min. The protein expression level in normal-potassium groups and high-potassium groups were evaluated by relative abundance. *$p < 0.05$; **$p < 0.01$; †$p < 0.001$; N.S., not statistically significant. White bars: normal-potassium groups; black bars: high-potassium groups. Minus marks: control groups; plus marks: experimental groups. See Supplementary Table 3 for values used for Fig 3B.

concomitant increase in SPAK phosphorylation. This confirmed that the process of knockdown was effective and KLHL2-mediated WNK3 degradation in MOVAS cells normally occurred in the presence of normal potassium concentrations (n = 5; KLHL2 KD (-) and high potassium (-) groups versus KLHL2 KD (+) and high potassium (-) groups; KLHL2, $p < 0.05$; WNK3, $p < 0.001$; P-SPAK, $p < 0.01$). In particular, we observed that in the control knockdown group, KLHL2 levels were increased and the WNK3-SPAK phosphorylation cascade was decreased due to high potassium concentrations (n = 5; KLHL2 KD (-) and high potassium (-) groups versus KLHL2 KD (-) and high potassium (+) groups; KLHL2, $p < 0.05$; WNK3, $p < 0.05$; P-SPAK, $p < 0.001$), as expected, but these phenomena did not occur in case of KLHL2 knockdown. The decrease in WNK3-SPAK phosphorylation cascade due to the high-potassium media was canceled in KLHL2-knockdown MOVAS cells (n = 5; KLHL2 KD (+) and high potassium (-) groups versus KLHL2 KD (+) and high potassium (+) groups; KLHL2, WNK3, P-SPAK, not statistically significant).

These results indicated that the KLHL2-mediated WNK3 ubiquitin degradation was necessary for the inhibition of the WNK3–SPAK–NKCC1 phosphorylation cascade in MOVAS cells caused by high potassium concentrations.

Discussion

In vascular smooth muscle cells, NKCC1 plays a critical role in regulating the vascular tone and blood pressure. Increased activation of NKCC1 leads to intracellular chloride accumulation, causing membrane depolarization and, consequently, opening of the voltage-dependent calcium channels; this results in high calcium ion influx and peripheral resistance of smooth muscles[13]. Therefore, the WNK–SPAK–NKCC1 phosphorylation cascade and NKCC1 activation are important in the regulation of vasoconstriction. We have previously shown that both dietary salt and angiotensin II (AngII) regulate the WNK3–SPAK–NKCC1 phosphorylation cascade in mouse aorta and MOVAS cells[15]. However, limited information is available on other regulatory factors that influence the WNK–SPAK–NKCC1 phosphorylation cascade.

The beneficial effects of high dietary potassium intake in treating and managing hypertension[17-19, 26], irrespective of the paradox of increased blood pressure due to hyperkalemia-induced elevated aldosterone levels, have been universally acknowledged for decades. One of the mechanisms underlying this phenomenon is renal natriuresis caused by potassium. Loffing's group showed that a high-potassium diet suppresses phosphorylation of NCC, which leads to natriuresis[28]. In addition, we have recently demonstrated that high-potassium load rapidly dephosphorylates NCC[27]. Because NCC plays a role in reabsorbing sodium in the distal convoluted tubules, inhibited NCC activation induced by high potassium concentration causes natriuresis and decreases blood pressure. Furthermore, we believe that the present study substantiates one of the mechanisms underlying the antihypertensive effect of potassium through KLHL2–WNK3–SPAK–NKCC1 signaling in vascular smooth muscle cells. By focusing on the effect of high extracellular potassium concentration on the WNK–SPAK–NKCC1 phosphorylation cascade in MOVAS cells, we found that high potassium concentration reduced WNK3 levels, which in turn suppressed the downstream SPAK and NKCC1 phosphorylation. We also observed that this regulating mechanism was KLHL2-dependent. This could be a clue for the elucidation of the mechanism of antihypertensive effects by potassium.

We have previously confirmed that potassium regulates the WNK1–OSR1/SPAK phosphorylation cascade in COS7 cells[21]. On the basis of these observations, we hypothesized that high potassium concentrations can also decrease WNK levels in other cells, including vascular smooth muscle cells. To our knowledge, only WNK1 and WNK3 are expressed in vascular smooth muscle cells[6, 14, 15], and both can phosphorylate their downstream SPAK and NKCC1[29-31]. However, the high-potassium medium used in the present study only decreased WNK3 levels and did not affect WNK1 levels, as seen in Figure 1; this is different from the result of our study on COS7 cells. These data suggested that WNK3 is the most predominant WNK regulated by high potassium concentrations. We cannot ignore the influence of KLHL2 on WNK1 in the presence of high potassium concentrations, but considering that WNK3 showed the highest binding affinity with KLHL2[12], it is likely that WNK3 is the major regulator of KLHL2 in MOVAS cells in the presence of high potassium concentrations. Indeed, our present results showed that the WNK3–SPAK–NKCC1 phosphorylation cascade was inhibited owing to high potassium concentrations in MOVAS cells. Such a potassium signaling pathway in vascular smooth muscle cells can provide new insights in terms of the antihypertensive effect of potassium although further in vivo experiments will be required to confirm this phenomenon.

We have previously demonstrated that phosphorylated NKCC1 levels decrease in aortic tissue in SPAK-knockout ($SPAK^{-/-}$) mice, despite the compensational increase in phosphorylated OSR1. Moreover, the aortic contractility of $SPAK^{-/-}$ mice showed approximately 50% of maximal contraction compared with that of wildtype mice after stimulation by phenylephrine, a selective 1-adrenergic agonist. In the presence of bumetanide (an NKCC1 inhibitor), aortic contraction in the wildtype mice significantly reduces to a level similar to that in the $SPAK^{-/-}$ mice without bumetanide treatment, and the aortic contractility of the $SPAK^{-/-}$ mice shows no response to bumetanide inhibition[24]. In the present study, there was no significant difference in phosphorylated OSR1 levels following normal and high-potassium treatment (Supplementary Figure 1). These results indicate that SPAK, rather than OSR1, is crucial for the regulation of NKCC1-mediated aortic contractility.

We also assessed WNK levels as surrogate markers of the function of WNK kinases. In our previous reports[9, 32], we have shown that increased WNK kinase levels in WNK4 transgenic mice and $KLHL3^{R528H/+}$ mice significantly enhance the WNK-OSR1/SPAK-NCC cascade and cause severe PHAII phenotypes. Although we cannot exclude the possibility of an unknown mechanism regulating the function of WNK kinases as opposed to their protein levels, evaluating the exact function of WNK kinases remains a challenge.

In conclusion, we confirmed that high potassium concentrations suppress the WNK3–SPAK–NKCC1

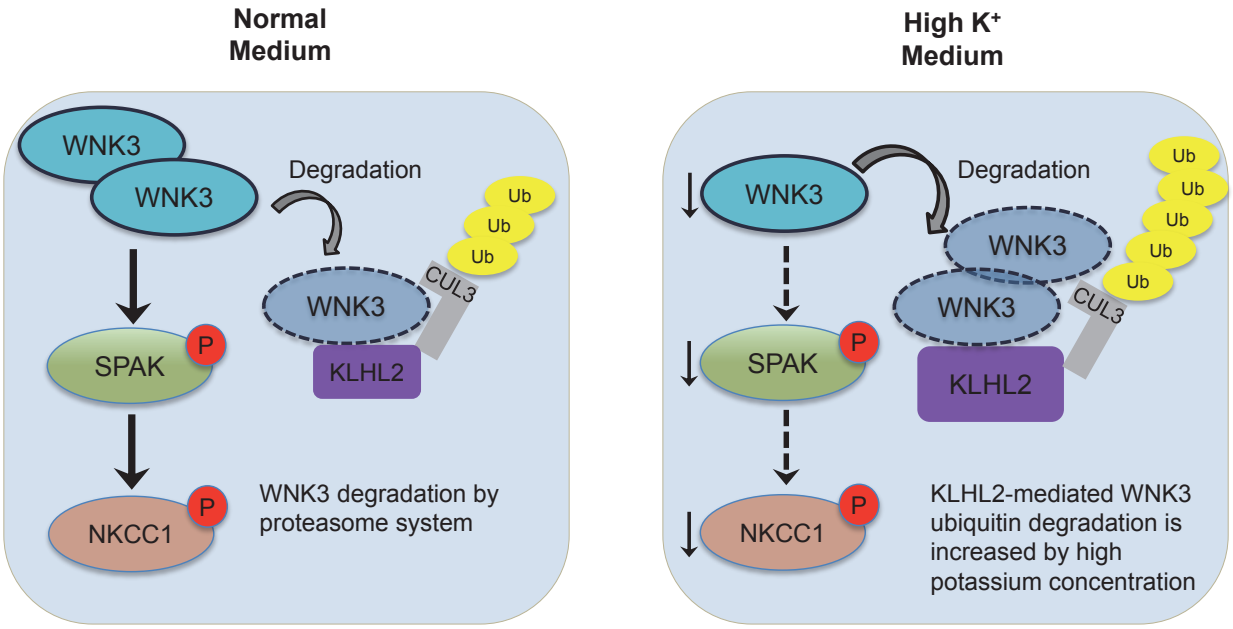

Figure 4. Mechanism of reduced activity of WNK3–SPAK–NKCC1 phosphorylation cascade caused by high potassium concentration via KLHL2.
In vascular smooth muscle cells, WNK3 is degraded by KLHL2–CUL3-mediated ubiquitination. With high potassium stimulation, KLHL2-mediated WNK3 ubiquitin degradation is enhanced. Ub, ubiquitin; P, phosphorylation.

phosphorylation cascade and increase KLHL2 levels in MOVAS cells (Figure 4). Increased KLHL2-mediated degradation of WNK3 plays a key role in SPAK–NKCC1 de-phosphorylation regulated by high potassium concentration and this mechanism could be responsible for the antihypertensive effects of high dietary potassium intake in clinical treatment.

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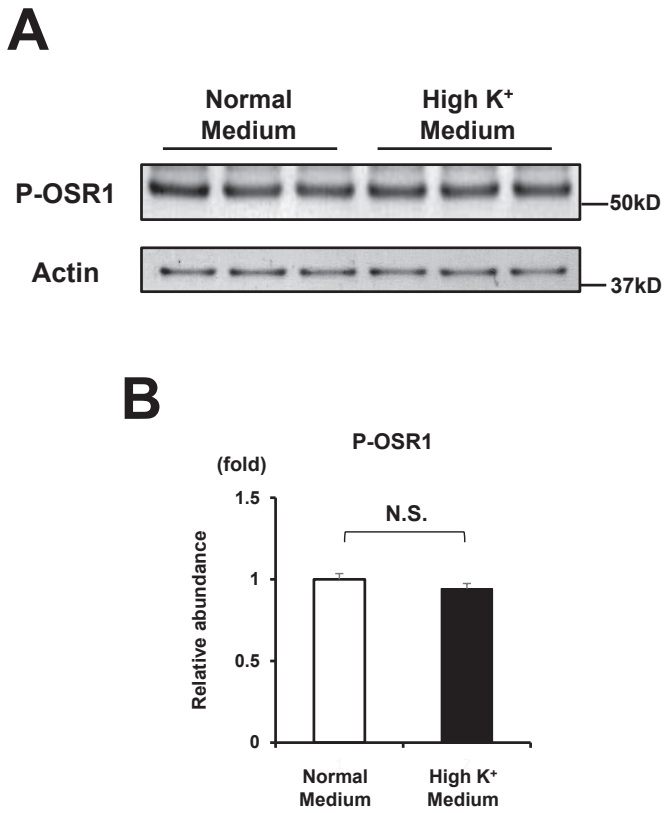

Supplementary Figure 1. Expression of phosphorylated OSR1 in MOVAS cells treated with normal-potassium and high-potassium media.

(A) Representative immunoblots and (B) densitometry analyses of P-OSR1, after 60 min of incubation in normal- and high-potassium media. The protein expression level in normal-potassium groups and high-potassium groups were evaluated by relative abundance. N.S., not statistically significant. White bars: normal-potassium groups; black bars: high-potassium groups. See Supplementary Table 4 for values used for Supplementary Fig 1B.

Supplementary Table 1. Densitometric values of Western blots from Figure 1A.

WNK3	Normal K^+	High K^+
Mean	1	0.58
SEM	0.050	0.093
P value		p = 0.004

WNK1	Normal K^+	High K^+
Mean	1	1.09
SEM	0.069	0.071
P value		p = 0.393

P-SPAK	Normal K^+	High K^+
Mean	1	0.61
SEM	0.027	0.041
P value		p < 0.001

P-NKCC1	Normal K^+	High K^+
Mean	1	0.53
SEM	0.166	0.063
P value		p = 0.036

Supplementary Table 2. Values of relative increase in mRNA from Figure 1A and densitometric values of Western blots from Figure 2B.

mRNA of WNK3	Normal K^+	High K^+
Mean	1	1.01
SEM	0.034	0.047
P value		p = 0.923

KLHL2	Normal K^+	High K^+
Mean	1	1.51
SEM	0.061	0.056
P value		p < 0.001

Supplementary Table 3. Densitometric values of Western blots from Figure 3A.

KLHL2				
KLHL2 KD	–	–	+	+
High K^+	+	–	+	–
Mean	1	0.82	0.63	0.56
SEM	0.051	0.042	0.032	0.033
P value	KLHL2 KD (–), High K^+ (–) vs. KLHL2 KD (–), High K^+ (+) KLHL2 KD (–), High K^+ (–) vs. KLHL2 KD (+), High K^+ (–) KLHL2 KD (+), High K^+ (–) vs. KLHL2 KD (+), High K^+ (+)			p = 0.029 p = 0.048 p = 0.974
WNK3				
KLHL2 KD	–	–	+	+
High K^+	+	–	+	–
Mean	1	1.45	2.08	2.15
SEM	0.070	0.072	0.047	0.145
P value	KLHL2 KD (–), High K^+ (–) vs. KLHL2 KD (–), High K^+ (+) KLHL2 KD (–), High K^+ (–) vs. KLHL2 KD (+), High K^+ (–) KLHL2 KD (+), High K^+ (–) vs. KLHL2 KD (+), High K^+ (+)			p = 0.018 p < 0.001 p = 0.956
P-SPAK				
KLHL2 KD	–	–	+	+
High K^+	+	–	+	–
Mean	1	1.48	1.73	1.74
SEM	0.052	0.049	0.029	0.051
P value	KLHL2 KD (–), High K^+ (–) vs. KLHL2 KD (–), High K^+ (+) KLHL2 KD (–), High K^+ (–) vs. KLHL2 KD (+), High K^+ (–) KLHL2 KD (+), High K^+ (–) vs. KLHL2 KD (+), High K^+ (+)			p < 0.001 p = 0.006 p = 0.996

Supplementary Table 4. Densitometric values of Western blots from Supplementary Figure 1A.

P-OSR1	normal K^+	high K^+
Mean	1	0.94
SEM	0.035	0.031
P value		p = 0.253