Phenotypes of pseudohypoaldosteronism type II caused by the WNK4 D561A missense mutation are dependent on the WNK-OSR1/SPAK kinase cascade

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Summary
We recently reported increased phosphorylation of the NaCl cotransporter (NCC) in Wnk44D561A/+ knock-in mice, an ideal model of the human hereditary hypertensive disease pseudohypoaldosteronism type II (PHAII). Although previous in vitro studies had suggested the existence of a phosphorylation cascade involving the WNK, OSR1 and SPAK kinases, whether the WNK-OSR1/SPAK cascade is in fact fully responsible for NCC phosphorylation in vivo and whether the activation of this cascade is the sole mediator of PHAII remained to be determined. To clarify these issues, we mated the Wnk44D561A/+ knock-in mice with Spak and Osr1 knock-in mice in which the T-loop threonine residues in SPAK and OSR1 (243 and 185, respectively) were mutated to alanine to prevent activation by WNK kinases. We found that NCC phosphorylation was almost completely abolished in Wnk44D561A/+; SpakT243A/T243A/Osr1T185A/+ triple knock-in mice, clearly demonstrating that NCC phosphorylation in vivo is dependent on the WNK-OSR1/SPAK cascade. In addition, the high blood pressure, hyperkalemia and metabolic acidosis observed in Wnk44D561A/+ mice were corrected in the triple knock-in mice. These results clearly establish that PHAII caused by the WNK4 D561A mutation is dependent on the activation of the WNK-OSR1/SPAK-NCC cascade and that the contribution of other mechanisms to PHAII (independent of the WNK-OSR1/SPAK cascade) could be minimal.

Key words: Blood pressure, NaCl cotransporter, WNK, OSR1, SPAK

Introduction
Pseudohypoaldosteronism type II (PHAII) is an autosomal dominant hereditary hypertensive disease characterized by hyperkalemia and metabolic acidosis (Gordon, 1986). Since the discovery that the genes encoding WNK kinases are responsible for PHAII (Wilson et al., 2001), numerous studies concerning the role of WNK kinases with regard to renal transporters and channels have been performed to elucidate the pathogenic mechanisms of PHAII (Kahle et al., 2008; McCormick et al., 2008; Richardson and Alessi, 2008; Uchida, 2010). We previously generated Wnk44D561A/+ knock-in mice carrying a heterozygous D561A missense mutation in the Wnk4 gene, which corresponds to the D564A mutation found in PHAII patients (Yang, S. S. et al., 2007). We found increased phosphorylation of the NaCl cotransporter (NCC), and OSR1 and SPAK kinases. Previously, we had clarified through in vitro experiments that WNK1 and WNK4 phosphorylated and activated OSR1 and SPAK kinases, and that OSR1 and SPAK could phosphorylate threonine and serine residues in the N-terminal domain of NCC (Moriguchi et al., 2005; Vitari et al., 2005; Vitari et al., 2006). Phosphorylation of these residues (threonine 53 and 58, serine 71 in mouse NCC) was previously shown to be necessary for NCC function (Pacheco-Alvarez et al., 2006; Richardson et al., 2008) and possibly important for apical membrane localization of NCC in cells of the distal convoluted tubules (Yang, S. S. et al., 2007). We also showed that the phosphorylation of NCC at these three residues was similarly regulated in in vivo mouse kidney cells (Chiga et al., 2008). On the basis of these data, we speculated that a WNK-OSR1/SPAK-NCC phosphorylation cascade exists in vivo in kidney cells and postulated that the activation of this cascade by the mutant WNK4 is the major cause of PHAII. The increased numbers of phosphorylated NCC in the apical plasma membrane of cells of the distal convoluted tubules of kidneys might lead to increased Na reabsorption and salt-sensitive hypertension. Recently, we generated Spak and Osr1 knock-in mice in which the T-loop threonine residues (243 and 185, respectively) were mutated to alanine to prevent activation by WNK isoforms (Rafiqi et al., 2010). In fact, SpakT243A/T243A mice showed reduced NCC phosphorylation and decreased blood pressure, confirming the existence of a WNK-SPAK-NCC cascade in vivo in kidney cells (Rafiqi et al., 2010). Very recently, we also generated Spak knockout mice, further confirming the importance of SPAK in NCC phosphorylation and also the involvement of SPAK in the regulation of vascular resistance in the aorta (Yang et al., 2010).

Other than the WNK-OSR1/SPAK-NCC cascade in kidney cells, several other regulatory mechanisms involving WNK kinases for renal transporters and channels have been postulated that are not related to the WNK-OSR1/SPAK signal cascade (Kahle et al., 2005; McCormick et al., 2008). For example, negative regulation of the renal potassium channel (ROMK) by mutant WNK4 has been postulated as one of the pathogenic mechanisms to explain hyperkalemia in PHAII (Kahle et al., 2003). This putative
mechanism is independent of WNK4 kinase activity and involves increased clathrin-dependent endocytosis of ROMK by WNK4 (Kahle et al., 2003). In the case of NCC and the epithelial Na channel (ENaC), wild-type WNK4 inhibits both NCC and ENaC expressed in Xenopus oocytes, but PHAII-causing mutant WNK4 alleviates the inhibitory effect (Ring et al., 2007; Yang et al., 2003; Yang, C. L. et al., 2007; Yang et al., 2005). Because these effects are also reported to be kinase activity independent, the WNK-OSR1/SPAK cascade must have nothing to do with these mechanisms of regulation.

The purpose of this study was to investigate whether the WNK-OSR1/SPAK cascade is in fact fully responsible for NCC phosphorylation in vivo and whether the activation of this cascade is the sole mediator of PHAII. For this purpose, we mated Wnk4<sup>D561A/+</sup> mice with Osr1<sup>T185A/+</sup> and Spak<sup>T243A/+</sup> knock-in mice to generate triple knock-in mice. Analyzing the effect of specifically interrupting the signal from WNK to OSR1/SPAK kinases in Wnk4<sup>D561A/+</sup> mice should test the relative contribution of the WNK-OSR1/SPAK signal cascade and other kinase-independent WNK4 mechanisms to the expression of PHAII phenotypes in vivo.

Results and Discussion

We bred the triple knock-in mice as shown in Fig. 1. Because homozygous Osr1 knock-in mice were lethal, we obtained mice with six different genotypes under Wnk4<sup>+/+</sup> and Wnk4<sup>D561A/+</sup> backgrounds. As previously reported by us, Wnk4<sup>T243A/+</sup> and Spak<sup>T243A/+</sup> knock-in mice show reduced NCC phosphorylation (Fig. 2A, upper panels), confirming the contribution of the WNK-SPAK kinase cascade to NCC phosphorylation under normal conditions. Here, we further confirmed the decrease in NCC phosphorylation in Wnk4<sup>D561A/+Osr1<sup>T185A</sup>/</sup> knock-in mice (Fig. 2A, upper panels). Interestingly, total OSR1 abundance was increased in Spak<sup>T243A/T243A</sup> knock-in mice, which could be a compensatory mechanism of SPAK inactivation. These results suggest that OSR1 and SPAK are both involved in NCC phosphorylation in vivo.

Next, we investigated the effect of interrupting WNK-OSR1/SPAK signaling on NCC phosphorylation under a Wnk4<sup>D561A/+</sup> background (Fig. 2A, lower panels). As we previously reported, NCC phosphorylation is significantly increased in Wnk4<sup>D561A/+</sup> mice. We had been presenting NCC phosphorylation data as the ratio of phosphorylated NCC to total NCC. However, we learned that the increase in NCC phosphorylation is always accompanied by an increase in total NCC abundance (Chiga et al., 2008; Rafiqi et al., 2010). Phosphorylation of NCC might somehow have implications for the stability of the protein. Therefore, the magnitude of the change in the ratio is always smaller than the change in NCC phosphorylation itself (see Fig. 2B). Here, we show both phosphorylated NCC data corrected by actin abundance and the ratio of phosphorylated NCC to total NCC, as before. However, statistical analyses based on either parameter gave almost the same results as shown in Fig. 2B. The increase in NCC phosphorylation by the WNK4 D561A mutation decreased as the number of inactivated alleles in Osr1 and Spak increased. Moreover, the total amount of SPAK and OSR1 was inversely correlated with NCC phosphorylation (Fig. 2A). Most significantly, NCC phosphorylation...
was almost abolished in \textit{Wnk4}^{D561A/+}\textit{Osr1}^{T185A/+}\textit{Spak}^{T243A/T243A} mice. Because \textit{Osr1} homozygous knock-in mice were not viable, two alleles of \textit{Spak} inactivation plus one allele of \textit{Osr1} inactivation was the maximum possible inactivation of total \textit{OSR1}/\textit{SPAK} activity in live mice. Previously, \textit{Spak}^{T243A/T243A} mice and \textit{Spak} knockout mice showed decreased NCC phosphorylation in kidney cells (Rafiqi et al., 2010; Yang et al., 2010). However, the decrease in NCC phosphorylation seemed to be more evident in \textit{Spak}^{T243A/T243A}\textit{Osr1}^{T185A/+} mice even under the \textit{Wnk4}^{D561A/+} background. This indicates that \textit{OSR1} is also involved in NCC phosphorylation in vivo in kidney cells. This interpretation might be further supported by the fact that the total \textit{OSR1} protein level increased as the number of inactivated alleles in \textit{SPAK} increased (Fig. 2A). We can conclude that the contribution of kinases other than \textit{OSR1}/\textit{SPAK} to NCC phosphorylation at specific residues is highly unlikely in vivo in kidney cells.

As shown in Fig. 2A,B, NCC phosphorylation in \textit{Wnk4}^{D561A/+}\textit{Osr1}^{T185A/+}\textit{Spak}^{T243A/+} knock-in mice was almost the same as that in wild-type mice. In those mice and in \textit{Wnk4}^{D561A/+}\textit{Osr1}^{T185A/+}\textit{Spak}^{T243A/T243A} mice, the increased blood pressure (Fig. 3A), hyperkalemia (Fig. 3B) and metabolic acidosis (Fig. 3C) observed in \textit{Wnk4}^{D561A/+} PHAII model mice were reversed to the levels of wild-type mice. These results clearly indicate that the PHAII phenotypes observed in \textit{Wnk4}^{D561A/+} knock-in mice were totally dependent on the WNK-\textit{OSR1}/\textit{SPAK} signal cascade. Previously, we showed that PHAII phenotypes in \textit{Wnk4}^{D561A/+} knock-in mice are completely reversed to normal levels with thiazide treatment (Yang, S. S. et al., 2007), which is one of the important characteristics of PHAII. Similarly, the PHAII phenotypes in mutant WNK4 transgenic mice were also reversed by crossing the mice with NCC-null mice (Lalioti et al., 2006). Such in vivo evidence had already suggested that activation of NCC function is the major mechanism underlying the pathogenesis of PHAII. However, heterologous overexpression studies have reported \textit{OSR1}/\textit{SPAK}-independent regulation of channels and transporters, including NCC (Yang et al., 2003; Yang, C. L. et al., 2007; Yang et al., 2005), in wild-type and mutant WNK4 mice, and have suggested that these mechanisms might be involved in the pathogenesis of PHAII. The ROMK channel was shown to be inhibited by wild-type WNK4. Because PHAII-causing mutant WNK4 inhibits ROMK more than wild-type WNK4 when expressed in \textit{Xenopus} oocytes (Kahle et al., 2003), this mechanism was speculated to be involved in the pathogenesis of hyperkalemia in PHAII. The inhibitory mechanisms have been shown to be independent of the kinase activity of WNK4 and rather involve a mechanism that regulates endocytosis independent of \textit{OSR1} and \textit{SPAK} kinases (Kahle et al., 2003). Accordingly, the reversal of PHAII phenotypes by the interruption of WNK-\textit{OSR1}/\textit{SPAK} signaling clearly indicates that ROMK inhibition by the mutant WNK4 in vitro might have only a minor role in the development of hyperkalemia in PHAII in vivo.

In the case of ENaC, wild-type WNK4 inhibits ENaC expressed in \textit{Xenopus} oocytes and the mutant WNK4 alleviates the inhibitory effect (Ring et al., 2007). This inhibitory effect was also shown to be a kinase-independent function of WNK4. Accordingly, if this mechanism significantly contributes to the pathogenesis of hypertension in PHAII, hypertension must remain to some extent after interrupting the signal from WNK to \textit{OSR1} and \textit{SPAK} in \textit{Wnk4}^{D561A/+} mice. In fact, we previously showed activation of ENaC in \textit{Wnk4}^{D561A/+} mice (Yang, S. S. et al., 2007). However, this observation was not primarily an effect of the WNK4 mutation, but a secondary effect of activated NCC, because the activation was lost after treating \textit{Wnk4}^{D561A/+} mice with thiazide. In this respect, this study also suggests that involvement of ENaC activation in the pathogenesis in PHAII is unlikely, at least as the primary cause of hypertension in PHAII by the D564A WNK4 mutation.

Fig. 3. PHAII phenotypes in triple knock-in mice of each genotype. Values are means ± s.e.m. The number in parenthesis is the number of mice examined.

(A) Systolic blood pressure of the triple knock-in mice. \$P<0.01, \#P<0.05 compared with wild-type mice; \$\#P<0.01, \#\#P<0.05 compared with \textit{Wnk4}^{D561A/+}\textit{Osr1}^{T185A/+}\textit{Spak}^{T243A} mice. (B) Serum potassium levels of the triple knock-in mice. \$\#P<0.01, \#\#P<0.05 compared with wild-type mice; \$\#\#P<0.01, \#\#\#P<0.05 compared with \textit{Wnk4}^{D561A/+}\textit{Osr1}^{T185A/+}\textit{Spak}^{T243A} mice. (C) Serum bicarbonate levels of the triple knock-in mice. \$\#P<0.01, \#\#P<0.05 compared with wild-type mice. \$\#\#P<0.01, \#\#\#P<0.05 compared with \textit{Wnk4}^{D561A/+}\textit{Osr1}^{T185A/+}\textit{Spak}^{T243A} mice.
Similarly, wild-type WNK4 has been shown to inhibit NCC, whereas PHAII-causing mutant WNK4 showed less inhibition. This effect was also shown to be a kinase-independent function of WNK4 (Yang et al., 2003; Yang, C. L. et al., 2007; Yang et al., 2010). If this kinase-independent inhibitory effect of wild-type WNK4 on NCC is highly active in vivo, as consistently observed in Xenopus oocytes, we should have observed PHAII phenotypes after specifically interrupting the kinase signal to OSR1 and SPAK in the Wnk4D561A/+ knock-in mice. However, hypertension as well as hyperkalemia and acidosis were at normal levels in SpakT243A/+;Osr1T185A/+ and SpakT243A/T243A;Osr1T185A/+ mice with the Wnk4D561A/+ background. This implies that the inhibitory power of wild-type WNK4 on NCC might not be as strong in vivo kidney cells as in the Xenopus oocyte expression system.

In conclusion, NCC phosphorylation in vivo in the kidney is fully dependent on the WNK-OSR1/SPAK signal cascade. The development of PHAII phenotypes induced by mutant WNK4 is also fully dependent on this signal cascade. Our findings provide further in vivo validation of the concept that inhibitors of OSR1/SPAK could be useful in the treatment of hypertension.

**Materials and Methods**

**Animals**

Wnk4D561A/+, Osr1T185A/+, and SpakT243A/+ knock-in mice were generated and characterized as described previously (Rafiqi et al., 2010; Yang, S. S. et al., 2007). We crossed these three knock-in mice and first obtained triple heterozygous knock-in mice. Using these triple heterozygous knock-in mice, we obtained mice of each genotype as shown in Fig. 1. Even in the presence of the D561A allele in the Wnk4 gene, homozygous Osr1 knock-in mice were not born. Mice were maintained under specific pathogen-free conditions, and all procedures and experiments were approved by the Animal Care and Use Committee of Tokyo Medical and Dental University. All experiments were performed at the age of 12±1 weeks.

**Blood analysis and blood pressure measurement**

Blood for electrolyte analyses was obtained from the retro-orbital sinus under light ether anesthesia. Electrolyte levels were determined with an i-STAT analyzer (Fuso, Osaka, Japan). The blood pressure of restrained conscious mice at steady state was measured with a programmable tail-cuff sphygmomanometer (MK-2000A; Murornachi, Tokyo, Japan).

**Immunoblotting**

Semiquantitative immunoblotting was performed as described previously using whole kidney homogenate without nuclear fraction (600 g) of the crude membrane fraction (17,000 g). The intensity of the bands was analyzed using ImageGauge software (Fujiﬁlm). To detect total OSR1, SPAK and NCC, OSR1-specific (M09; Abnova, Taipei, Taiwan), SPAK-specific (Cell Signaling Technology, Danvers, MA) and anti-total NCC (Chemicon, Billerica, MA) antibodies were used. Anti-phosphorylated-NCC antibody (pSer71) was previously generated and characterized (Yang, S. S. et al., 2007). Alkaline-phosphatase-conjugated anti-IgG antibodies (Promega, Madison, WI) were used as secondary antibodies for immunoblotting.

**Statistical analysis**

Data are presented as means ± s.e.m., with n representing the number of analyzed mice. Mean values were compared by ANOVA with Fischer’s post-hoc test. P<0.05 was considered statistically significant.

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**Fig. 4. Regulation of NCC by WNK kinases in kidney cells.** In wild-type mice (left panel), WNK kinases send a positive signal to OSR1 and SPAK kinases by phosphorylation, and activated OSR1 and SPAK phosphorylate and activate NCC. Red arrows indicate positive regulation of NCC. NCC has been shown to be negatively regulated by wild-type WNK4 when assayed in Xenopus oocytes, which is independent of the WNK-OSR1/SPAK cascade. Blue arrows indicate negative regulation of NCC. In Wnk4D561A/+ mice (middle panel), the PHAII-causing D561A mutation in a single WNK4 allele increases WNK kinase activity towards OSR1 and SPAK. Whether the increased WNK kinase activity is due to an increase in kinase activity of mutant WNK4 itself, or other mechanisms involving other WNK kinases, remains to be determined. The activated OSR1 and SPAK then activate NCC by phosphorylation. The disease-causing WNK4 mutants reportedly lack the inhibitory effect of wild-type WNK4 on NCC, as indicated by the dotted light blue arrow. Both mechanisms might work in parallel to activate NCC in PHAII. However, PHAII phenotypes can be restored only by the partial interruption of the signal from the WNK kinases to OSR1 and SPAK in Wnk4D561A/+;SpakT243A/Osr1T185A/+ triple knock-in mice (right panel). Because the level of NCC phosphorylation in wild-type mice and Wnk4D561A/+;SpakT243A/Osr1T185A/+ triple knock-in mice is almost equal (Fig. 2), the triple knock-in mice should still show some PHAII-like phenotypes if the inhibitory power of wild-type WNK4 (dark blue arrow) is substantial. However, these mice showed normal phenotypes (Fig. 3), clearly suggesting that the inhibitory effect of wild-type WNK4 might not be as strong in vivo kidney cells as in the Xenopus oocyte expression system.

In conclusion, NCC phosphorylation in vivo in the kidney is fully dependent on the WNK-OSR1/SPAK signal cascade. The development of PHAII phenotypes induced by mutant WNK4 is also fully dependent on this signal cascade. Our findings provide further in vivo validation of the concept that inhibitors of OSR1/SPAK could be useful in the treatment of hypertension.
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