The CUL3/KLHL3-WNK-SPAK/OSR1 pathway as a target for antihypertensive therapy

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Ferdaus MZ, McCormick JA. The CUL3/KLHL3-WNK-SPAK/OSR1 pathway as a target for antihypertensive therapy. Am J Physiol Renal Physiol 310: F1389–F1396, 2016. First published April 13, 2016; doi:10.1152/ajprenal.00132.2016.—Chronic high blood pressure (hypertension) is the most common disease in the United States. While several classes of drugs exist to treat it, many patients (up to 10 million Americans) respond poorly to therapy, even when multiple classes are used. Recent evidence suggests that a significant portion of patients will always remain hypertensive despite maximum therapy with the drugs currently available. Therefore, there is a pressing need to develop novel antihypertensive agents. One limitation has been the identification of new targets, a limitation that has been overcome by recent insights into the mechanisms underlying monogenic forms of hypertension. The disease familial hyperkalemic hypertension is caused by mutations in with-no-lysine (WNK) kinases 1 and 4 and in cullin-3 and kelch-like 3, components of an E3 ubiquitin ligase complex that promotes WNK kinase degradation. The study of the mechanisms by which this pathway regulates blood pressure has identified several candidates for the development of new antihypertensive agents. This pathway is particularly attractive since its inhibition may not only reduce renal sodium reabsorption along multiple segments but may also reduce vascular tone. Here, we will describe the mechanisms by which this pathway regulate blood pressure and discuss the potential of targeting it to develop new antihypertensive drugs.
Familial Hyperkalemic Hypertension

Monogenic disorders that present with either high or low blood pressure have provided many insights into the mechanisms by which the kidney regulates Na+ reabsorption and, ultimately, extracellular fluid volume (1), and their study may help identify novel antihypertensive targets for use in non-Mendelian forms of hypertension (66). One such disease is familial hyperkalemic hypertension (FHHt; also called pseudohypoaldosteronism type II), characterized by hyperkalemia and hypertension. While modulation of the activities of other ion channels or transporters may contribute to the development of FHHt, the disease appears to result primarily from hyperactivation of NCC, reflected by increased NCC phosphorylation and abundance (26), and the ability of thiazide-like diuretics to completely normalize its blood pressure and electrolyte abnormalities (38). The Lifton group was the first to identify FHHt-causing mutations, in the atypical WNK kinases WNK1 and WNK4 (73), which led to a surge in interest in the regulation of NCC. Rather than directly phosphorylating NCC, WNK1 and WNK4 gene products activate two other kinases, SPAK and OSR1, which activate NCC by phosphorylating multiple residues (55). More recently, mutations in the cullin RING ligase family members kelch-like 3 (KLHL3) and cullin-3 (CUL3) were identified as causing the majority of cases of FHHt (4, 34). The severity of the disease can be ranked (with most severe causative mutations first) as CUL3 > recessive KLHL3 > dominant KLHL3 > WNK4 > WNK1 (4, 50). Cases of FHHt arising from mutations in KLHL3 or CUL3 involve small kindreds or are de novo, which precluded analysis by traditional positional cloning approaches to identify the causative mutations. The advent of whole exome sequencing allowed this limitation to be overcome.

Over the past 15 yr, using FHHt-causing gene mutations as a starting point, the model of phosphorylation-dependent NCC activation shown in Fig. 1 has been developed. In this model, CUL3 and KLHL3 are components of an E3 ubiquitin ligase complex that regulates protein turnover, with KLHL3 serving as a substrate-binding adaptor and CUL3 acting as a scaffold protein (35). Ubiquitination of WNK kinases by an E3 ubiquitin ligase complex consisting of a scaffold protein (CUL3), a substrate adaptor (KLHL3), and a ubiquitin ligase (RING) tags them for proteasomal degradation, decreasing their abundance. According to this scheme, high WNK kinase abundance decreases NCC activation, reducing NaCl retention. Only WNK4 is shown since current data suggest it is the major WNK regulating NCC, although there is likely to be cross-compensation after disruption of each kinase (Refs. 41 and 69 and our unpublished observations). SPAK and OSR1 are expressed in the renal epithelia, since constitutive global disruption is embryonic lethal (12). Overall, the data suggest that, in vivo, OSR1 mainly activates NKCC2, whereas SPAK mainly activates NCC, although there is likely to be cross-compensation after disruption of each kinase (Refs. 41 and 69 and our unpublished observations). SPAK and OSR1 are expressed in the renin-angiotensin system, which has been intensely investigated for almost 15 yr, as a potential source of novel antihypertensive agents.
vasculature, and SPAK knockouts and OSR1 heterozygotes display reduced phosphorylation of NKCC1, which in SPAK knockout mice was correlated with impaired responses to the selective α1-adrenergic agonist phenylephrine. Thus, in addition to stimulating renal Na\(^+\) reabsorption, SPAK and OSR1 may play roles in hypertension by increasing vascular tone. It is also worth noting that interactions of SPAK and OSR1 with the scaffolding protein mouse protein-25 (MO25; also known as CAB39) dramatically enhances their ability to phosphorylate NCC, NKCC2, and NKCC1 (17), and MO25 may also permit WNK4 to activate cation cotransporters independently of SPAK and OSR1 (53). Thus, while the physiological roles of MO25 are currently unknown, it may represent a novel target for antihypertensive therapy.

A recent screen of >20,000 small-molecule compounds and 840 existing drugs identified 2 lead compounds with similar structures that showed promise as potential SPAK inhibitors (29). The first, IS-14279, rapidly reduced NCC phosphorylation in mice but did not affect SPAK/OSR1-dependent NKCC2 phosphorylation. Unfortunately, IS-14279 displayed toxic effects, so it was not further studied. The other compound, closantel, is widely used as an antiparasitic agent in livestock. Both acute and chronic administration to mice resulted in reduced phosphorylation of NCC and NKCC2 in the kidney and of NKCC1 in the aorta, suggesting that closantel may inhibit both SPAK and OSR1. However, blood pressure was only transiently reduced by closantel after acute administration, most likely due to vasodilation. It is worth noting that these studies were performed in normotensive animals, so the effect of closantel in hypertensive models warrants further investigation. Furthermore, neither compound competed for ATP binding to SPAK, which makes them attractive targets for investigation. Furthermore, neither compound competed for ATP binding to SPAK, which makes them attractive targets for investigation. Furthermore, neither compound competed for ATP binding to SPAK, which makes them attractive targets for investigation. Furthermore, neither compound competed for ATP binding to SPAK, which makes them attractive targets for investigation. Furthermore, neither compound competed for ATP binding to SPAK, which makes them attractive targets for investigation.
increase their abundance in vivo as a result of reduced ubiquitination (Fig. 2). Similar mutations have been reported recently for WNK1, but these appear to cause the electrolyte defects observed in FHHt without causing hypertension (51). In addition to a causative role in FHHt, polymorphisms in both WNK1 (45, 46, 54, 65) and WNK4 (23) have been associated with increased risk of hypertension and have been identified in non-FHHt patients, indicating a broader rationale for targeting these kinases.

Global WNK1 knockout mice die from cardiovascular defects in utero, but heterozygotes display a mild reduction in blood pressure (79); mice lacking WNK1 protein expression specifically along the renal epithelia have not yet been reported. Two WNK4 knockout mouse lines and one WNK4 hypomorph line have been reported (7, 47, 63). In all three models, abundances of total and phosphorylated NCC were reduced on a normal diet. However, systolic blood pressure (47) and plasma K+ concentration (7) were slightly reduced in one of the models, but not in the other two. When challenged with dietary Na+ restriction or infusion of ANG II, the effects of WNK4 disruption are clearer. Both stimuli were reported to activate NCC in wild type mice but not in WNK4 knockout mice (7), with Na+ restriction also causing hypotension (63). Targeting WNK1 and WNK4 may be complicated by the fact that both have been reported to regulate ion transport pathways in addition to cation cotransporters, including the epithelial Na+ channel (ENaC) and renal outer medullary K+ channel (for a review, see Ref. 24) and large-conductance K+ channels (32, 72), which are expressed distally along the connecting tubule and collecting duct (although ENaC is coexpressed with NCC along the late DCT). In WNK4 knockout mice, the major defect appears to be impaired activation of NCC (63), which may limit the therapeutic usefulness of specific WNK4 inhibition since chronic adaptation [as seen with thiazide-like diuretics (30)] is a possibility.

Two strategies have been taken to begin to target the actions of WNK kinases pharmacologically. The first strategy used a commercially available library of 86 kinases and showed that inhibitors of the Src family of tyrosine kinases and the EGF receptor inhibited WNK1 kinase activity in the micromolar range (76). The effects of these compounds on WNK4 or on blood pressure were not determined. A weakness of this approach is that the inhibitors identified are not WNK1 specific. A potentially more specific approach was used by Uchida and colleagues and leveraged knowledge of how WNK kinases interact with SPAK and OSR1. A region termed the conserved carboxy-terminal (CCT) has been shown to be essential for interactions with both WNKs and cation cotransporters (including NCC and NKCC2) (68). Knockin mice homozygous for a mutation in the CCT of SPAK (SPAKL502A/L502A) displayed reduced total abundance and phosphorylation of both NKCC2 and NCC, suggesting a dominant negative effect of mutant SPAK on wild-type OSR1 as well as lower blood pressure (83). Uchida and colleagues screened 17,000 compounds and identified 2 compounds that disrupted binding of WNK1 or WNK4 to SPAK, thereby preventing WNK-mediated activation of SPAK (43). In cultured cells, these compounds reduced the abundance of phosphorylated NCC and NKCC1. It was not determined whether these compounds also inhibit interactions between WNK kinases and OSR1 or between SPAK/OSR1 and cation cotransporters, both of which might enhance their efficacy. The effects of these compounds on blood pressure have not been examined, but they represent a potentially specific and potent approach to inhibit both the renal and vascular effects of WNK kinases.

**KLHL3**

KLHL3 acts as a substrate adaptor in an E3 ubiquitin ligase consisting of the scaffold protein CUL3 and a RING ubiquitin ligase (Fig. 1). FHHt-causing mutations in KLHL3 mainly include missense mutations in the BTB domain, which interacts with CUL3, or in the kelch propeller blades, which directly interact with substrates of E3 ligase (Fig. 2) (4, 34). In a few cases, there are premature termination mutations, frameshifts, or splice site mutations. Interestingly, while most FHHt-causing mutations are dominant, some KLHL3 mutations follow a recessive pattern of inheritance. The mechanism by which mutations in KLHL3 lead to FHHt is a functional loss of the CUL3-KLHL3 ubiquitin ligase complex, which results in the accumulation of WNK kinases and ultimately increased NCC activation. KLHL3 interacts with WNK kinases but not with SPAK or NCC (48), and mutations in the kelch propeller blades of KLHL3 impair the binding, ubiquitination, and degradation of WNK kinases (48, 60). Interactions between KLHL3 and WNK kinases appear to be physiologically regulated, as demonstrated by the observation that ANG II promotes PKC-dependent phosphorylation of a serine in the kelch propeller blade (Ser433), which prevents its interaction with WNK4 (59). Mutations in the BTB domain disrupt binding to CUL3, while one mutation (S410L) affects both KLHL3 stability and WNK kinase binding (44). While a mouse model recapitulating KLHL3-mediated FHHt has been generated (62), the phenotype of KLHL3 knockout mice has not been reported. However, since FHHt-causing mutations in KLHL3 cause loss of function of KLHL3, it is likely that such mice would also display a FHHt phenotype. Therefore, rather than inhibiting KLHL3, stabilization of KLHL3 (or its interactions with CUL3, see below) might provide an approach to develop an antihypertensive agent. The effects of disrupting KLHL3 function appear to be extremely specific to the DCT (i.e., they cause FHHt), which may reflect the observation that KLHL3 is highly enriched in this segment of the nephron (34, 42, 52). Therefore, targeting KLHL3 may also lead to chronic adaptation, as seen with thiazide-like and loop diuretics. Other members of the kelch-like family may also provide potential targets. KLHL2, another CUL3 adaptor, has been reported to play a role in WNK kinase degradation (64, 81) and has been implicated in mediating the effects of ANG II on vascular tone (80).

**CUL3**

Mutations in CUL3 cause the most severe form of FHHt, with 94% of patients presenting with hypertension by the age of 18 yr; <20% of patients are hypertensive at this age for each of the other forms of FHHt (4). The mutant form of CUL3 generated may cause severe hypertension not only due to effects on the kidney but also by effects on the vasculature, where wild-type CUL3 is normally expressed at significant levels (49, 58). The fact that CUL3 interacts with multiple adaptor proteins, including KLHL2, may also lead to enhanced Na+ transport in renal segments other than the DCT when it is mutated in FHHt. The CUL3 mutations that cause FHHt cluster
at sites involved in splicing of exon 9 and lead to its loss (4). The result is the expression of a modified form of CUL3 with a 57-amino acid deletion (CUL3 Δ403–459). While CUL3 Δ403–459 expression leads to reduced degradation of WNK4 (48), the precise mechanism by which this occurs is controversial. CUL3 is modified by a process termed neddylation, during which an 81-amino acid protein, Nedd8, is covalently attached to CUL3 at a lysine near the carboxy-termi

nus (K712), resulting in CUL3 activation. We have reported that CUL3 Δ403–459 is a gain-of-function mutant, as reflected by enhanced neddylation and an increased ability to ubiquitinate substrates (42), a finding confirmed by other groups (50, 58). We found that CUL3 Δ403–459 displays modified activity and ubiquitinates KLHL3, thus promoting its degradation. Therefore, we proposed that the effect of CUL3 mutations is to disrupt the E3 ligase complex by reducing KLHL3 levels, leading to the accumulation of WNK kinases. The ability of CUL3 to target its own substrate adaptor has been previously reported with respect to oxidative stress responses (82). Sigmund and colleagues (25) concluded that CUL3 Δ403–459 may act dominantly by forming unstable dimers with wild-type CUL3, reducing the availability of active CUL3 dimers to efficiently ubiquitinate substrates. Kurz and colleagues (58) recently reported that CUL3 Δ403–459 autoubiquitinates and promotes its own degradation, possibly as a result of altered structural flexibility. They also generated knockin mice with the CUL3 Δ403–459 mutation, and, consistent with their finding of autoubiquitination, they found that CUL3 Δ403–459 was expressed at very low levels but KLHL3 abundance was unchanged. In addition to the expected increased activation of NCC via an increase in WNK abundance and altered renal electrolyte handling, these mice also displayed a vascular defect, characterized by increased arterial stiffness and enhanced agonist-mediated contraction in vivo. The overall conclusion of Kurz and colleagues was that FHHt due to CUL3 mutations is the result of reduced abundance of wild-type CUL3, and, consistent with this, nonspecific inhibition of cullin activity with a neddylation inhibitor also enhanced agonist-mediated contraction in aortic rings from normal mice and increased arterial pressure in vivo (49). However, an attempt by Uchida and colleagues to generate a similar mouse model inadvertently resulted in the generation of mice with a null CUL3 allele (3), and mice heterozygous for CUL3 did not display any features of FHHt. We generated inducible kidney-specific (KS-)CUL3 knockout mice (i.e., CUL3 loss of function) and also found increased WNK abundance and NCC activation (42). Unexpectedly, KS-CUL3 knockout mice did not display a FHHt phenotype but displayed severe polyuria (due to a complete loss of aquaporin 2 expression), reduced blood pressure when Na\(^+\) restricted, and, after several weeks, developed renal fibrosis and eventually chronic kidney disease (42). The effect of CUL3 disruption to increase WNK kinase abundance and NCC phosphorylation in KS-CUL3 knockout mice was most likely direct rather than a response to reduced extracellular fluid volume since it was also observed in salt-loaded mice. Overall, the data suggest that the FHHt-causing mutation has effects beyond simply reducing CUL3 abundance, and more studies are required to determine the precise mechanisms by which CUL3 regulates blood pressure. Despite differences in the details, in all the proposed models, the ultimate effect of CUL3 mutation is disruption of the CUL3-KLHL3-RING ligase E3 ubiquitin ligase complex, resulting in WNK kinase accumulation.

CUL3 clearly plays an important role in the regulation of blood pressure and may, at first glance, represent the best target in the pathway (Fig. 1), since FHHt-causing mutations in CUL3 lead to the most severe form of FHHt. However, in practice, CUL3 may be the most challenging target for which to develop pharmacological agents that lower blood pressure. As with targeting KLHL3, an agent that stabilizes CUL3 or its interactions with KLHL3 and/or KLHL2 would be required to stimulate degradation of WNK kinases. An alternative would be to inhibit removal of Nedd8 by inhibiting activity of a multiprotein complex termed the COP9 signalosome, which mediates deneddylation of CUL3 (for a recent review on COP9 signalosome function, see Ref. 14). However, these approaches might lead to increased activity of CUL3 against itself or the substrate adaptors, as seen with CUL3 Δ403–459, resulting in hypertension (and hyperkalemia). Furthermore, since we observed unpredictable pleiotropic effects in KS-CUL3 knockout mice, the likelihood of adverse effects beyond the kidney when targeting CUL3 is high. For example, cardiomyocyte-specific disruption of the COP9 signalosome subunit CSN8 in mice led to cardiac hypertrophy, which rapidly progressed to heart failure and premature death (61). A clearer understanding of the precise mechanism by which CUL3 Δ403–459 leads to such a specific disease phenotype could guide design of drugs that specifically lower blood pressure.

Conclusions

An understanding of the molecular basis of FHHt has revealed a complex pathway that regulates renal Na\(^+\) reabsorption, vascular tone, and K\(^+\) homeostasis. Many aspects of the regulation of this pathway, including responses to dietary manipulation and hormonal signaling, have been delineated (40), but many questions remain. The precise mechanism by which CUL3 mutations lead to WNK kinase accumulation is still unknown, and the roles of other CUL3 adaptors, including KLHL2, are unknown. Stimulating activity of the CUL3-KLHL3 E3 ligase complex to reduce WNK abundance is likely to be difficult, since both CUL3 activation and inhibition may increase WNK abundance. The roles of CUL3 in normal and pathological renal function beyond FHHt are largely unknown, and targeting it may be fraught with unforeseen consequences, as demonstrated by the phenotype of KS-CUL3 knockout mice. Finally, CUL3 serves as a scaffold for many substrate adaptors, and modulating CUL3 activity to specifically affect only KLHL3- or KLHL2-dependent substrates to only reduce Na\(^{+}\) transport may prove impossible. Inhibitors of SPAK, OSR1, and WNK kinases are under development and are more attractive targets, but their efficacy in models of hypertension has not been examined. While chronic renal adaptation as seen with thiazide-like diuretics and loop diuretics may be a limitation of inhibiting these kinases, this may be tempered by additional effects on vascular tone.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS

M.Z.F. prepared figures; M.Z.F. and J.A.M. edited and revised manuscript; M.Z.F. and J.A.M. approved final version of manuscript; J.A.M. drafted manuscript.

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