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

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Submitted 2 March 2016; accepted in final form 7 April 2016

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.

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likelihood that target blood pressure targets will be lowered in the wake of the SPRINT trial, the need for the development of new antihypertensive agents may therefore be even greater. This perspective will examine the cullin-with no lysine (WNK)-STE20 (sterile 20)/SPS-1-related proline/alanine-rich kinase (SPAK)/oxidative stress response kinase (OSR1) pathway, which has been intensely investigated for almost 15 yr, as a potential source of novel antihypertensive agents.

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 WNK4 abundance by targeting it for proteasomal degradation. CUL3 acts as a protein scaffold for the complex, with KLHL3 serving as a substrate-binding adaptor. In FHHt, mutations in CUL3, KLHL3, or WNK4 prevent WNK4 degradation, and the resulting increase in WNK4 abundance leads to hyperactivation of NCC. Mutations in WNK1 also lead to increased WNK1 abundance, but in most cases this may be independent of defective WNK1 degradation (see below). Some details regarding the precise mechanisms by which FHHt-causing mutations lead to dysregulation of NCC are still controversial, in particular for CUL3, but this overall scheme is well accepted. Knowledge of the mechanisms by which this pathway regulates blood pressure points to potential strategies to develop novel antihypertensives, which will be discussed based on proximity to NCC.

![Fig. 1. Model of Na\(^{+}\)-Cl\(^{-}\) cotransporter (NCC) regulation by the cullin-3 (CUL3)/kelch-like 3 (KLHL3)-with no lysine (WNK)-STE20/SPS-1-related proline/alanine-rich kinase (SPAK)/oxidative stress response kinase (OSR1) pathway. NCC is phosphorylated at several residues by the kinases SPAK and OSR1, which leads to its activation. SPAK and OSR1 are phosphorylated and activated by WNK kinases. 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 abundance stimulates NCC, leading to NaCl retention, whereas reduced WNK kinase abundance decreases NCC activation, reducing NaCl retention. Only WNK4 is shown since current data suggest it is the major WNK regulating NCC along the distal convoluted tubule, although WNK1 may also play a role under certain conditions.](http://ajprenal.physiology.org/)

**SPAK/OSR1**

SPAK and OSR1 are closely related kinases that can phosphorylate, and thus activate, cation co-transporters in vitro (13, 55, 56). In renal epithelia, they are localized to the apical membrane, where they phosphorylate and activate NKCC2, the target of loop diuretics expressed along the thick ascending limb, and also NCC. While SPAK and OSR1 have not been reported to be mutated in FHHt, a possible role for SPAK in human hypertension has been shown by association studies that identified several linked polymorphisms in the SPAK gene (16, 71). Some followup studies confirmed these findings in other races or populations (2, 8, 35, 84), but others did not (9, 75). A recent meta-analysis confirmed the association of one polymorphism, rs3754777 (74), and a recent in vitro study showed that it increased SPAK mRNA abundance and subsequently NCC activation (36). Disruption of SPAK or OSR1 in mice results in a decrease in blood pressure at baseline or after NaCl restriction and mild reductions in plasma K\(^{+}\) concentration (20, 41, 78). It should be noted that OSR1 was disrupted specifically along 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 also expressed in the
vasculature, and SPAP knockouts and OSR1 heterozygotes display reduced phosphorylation of NKCC1, which in SPAP knockout mice was correlated with impaired responses to the selective α1-adrenergic agonist phenylephrine. Thus, in addition to stimulating renal Na\(^+\) reabsorption, SPAP and OSR1 may play roles in hypertension by increasing vascular tone. It is also worth noting that interactions of SPAP 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 SPAP 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 SPAP inhibitors (29). The first, IS-14279, rapidly reduced NCC phosphorylation in mice but did not affect SPAP/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 SPAP 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 SPAP, which makes them attractive targets for modification to enhance their specific effects on SPAP, without inhibiting other kinases. While these data are promising, there are weaknesses with targeting SPAP and OSR1. First, out inhibiting other kinases. While these data are promising, modification to enhance their specific effects on SPAP, with-

**WNK1 and WNK4**

Mutations in WNK kinases are proposed to cause FHHt by increasing their abundance, which leads to inappropriate activation of NCC (Fig. 2). Mutations in WNK1 are intronic, leading to aberrant expression of the kinase-active form of WNK1 along the DCT, where its expression levels are normally low (67). WNK4 mutations are missense mutations located close to one of two coiled-coiled motifs. Some of these mutations have been shown to disrupt interactions between WNK4 and KLHL3 (48, 70), which would be predicted to
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 hypomorphic 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...
mechanisms by which CUL3 regulates blood pressure. Despite
dance, and more studies are required to determine the precise
mutation has effects beyond simply reducing CUL3 abun-
dosed mice. Overall, the data suggest that the FHHt-causing
electrolyte handling, these mice also displayed a vascular
defect, characterized by increased arterial stiffness and en-
hanced agonist-mediated contraction in vivo. The overall con-
clusion of Kurz and colleagues was that FHHt due to
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 func-
tion) 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
at sites involved in splicing of exon 9 and lead to its loss (4).
This 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 contro-
versial. 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-terminus
(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. There-
fore, 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). Sig-
mund 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 en-
hanced agonist-mediated contraction in vivo. The overall con-
clusion of Kurz and colleagues was that FHHt due to
mutations is the result of reduced abundance of wild-type
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tion) 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 abun-
dance, 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 ob-
served 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 re-
vealed a complex pathway that regulates renal Na⁺ reabsorp-
tion, 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 limi-
tation of inhibiting these kinases, this may be tempered by
additional effects on vascular tone.

GRANTS

J. A. McCormick is funded by National Institute of Diabetes and Digestive
and Kidney Diseases Grant R01-DK-098141.

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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