A New Kindred With Pseudohypoaldosteronism Type II and a Novel Mutation (564D>H) in the Acidic Motif of the WNK4 Gene

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Abstract—We identified a new kindred with the familial syndrome of hypertension and hyperkalemia (pseudohypoaldosteronism type II or Gordon’s syndrome) containing an affected father and son. Mutation analysis confirmed a single heterozygous G to C substitution within exon 7 (1690G>C) that causes a missense mutation within the acidic motif of WNK4 (564D>H). We confirmed the function of this novel mutation by coexpressing it in Xenopus oocytes with either the NaCl cotransporter (NCCT) or the inwardly rectifying K-channel (ROMK). Wild-type WNK4 inhibits 22Na+ flux in Xenopus oocytes expressing NCCT by ≈90% (P<0.001), whereas the 564D>H mutant had no significantly inhibitory effect on flux through NCCT. In oocytes expressing ROMK, wild-type WNK4 produced >50% inhibition of steady-state current through ROMK at a +20-mV holding potential (P<0.001). The 564D>H mutant produced further inhibition with steady-state currents to some 60% to 70% of those seen with the wild-type WNK4. Using fluorescent-tagged NCCT (enhanced cyan fluorescent protein–NCCT) and ROMK (enhanced green fluorescent protein–ROMK) to quantify the expression of the proteins in the oocyte membrane, it appears that the functional effects of the 564D>H mutation can be explained by alteration in the surface expression of NCCT and ROMK. Compared with wild-type WNK4, WNK4 564D>H causes increased cell surface expression of NCCT but reduced expression of ROMK. This work confirms that the novel missense mutation in WNK4, 564D>H, is functionally active and highlights further how switching charge on a single residue in the acid motif of WNK4 affects its interaction with the thiazide-sensitive target NCCT and the potassium channel ROMK. (Hypertension. 2005;46:295-300.)

Key Words: kinase ■ mutation

The familial syndrome of hypertension and hyperkalemia, also known as pseudohypoaldosteronism type II (PHA2) or Gordon’s syndrome, was first described some 40 years ago.1 At least 3 different genetic loci have been identified for this monogenic syndrome with a further locus likely2–6 (OMIM 601844, and 605232). Recent collaborative work from the Lifton and Jeunemaitre laboratories has identified 2 of these loci as members of the novel family of serine–threonine kinases WNK4 and WNK1.
Methods

Mutation Analysis
Genomic DNA was isolated from peripheral blood cells using a standard commercial method (Genomic Wizard kit; Promega). PvuII digests of this gDNA was Southern blotted and hybridized to [32P]-labeled probes as described by Wilson et al.17 The 18 coding exons of WNK4 were individually polymerase chain reaction (PCR) amplified using Taq polymerase (Promega; primer sequences available on request). The PCR products were then gel purified before sequencing in both directions using an ABI 377 and Big Dye fluorescent chemistry (Applied Biosystems).

Cloning and cRNA Synthesis
Full-length cDNA for human ROMK2 was identified in an IMAGE clone (4611308) and then subcloned into pEGFP-C1 to produce an enhanced green fluorescent protein (EGFP) fusion with the N terminal of ROMK2. Full-length sequences for NCCT and WNK4 were PCR amplified from mouse kidney cDNA and cloned into pCDNA3. The wild-type sequence of WNK4 was mutated to produce E mutants using site-directed mutagenesis. The pcDNA3. The wild-type sequence of WNK4 was mutated to produce E mutants using site-directed mutagenesis. The wild-type sequence of WNK4 was mutated to produce E mutants using site-directed mutagenesis. The wild-type sequence of WNK4 was mutated to produce E mutants using site-directed mutagenesis. The wild-type sequence of WNK4 was mutated to produce E mutants using site-directed mutagenesis.

Expression in Xenopus Oocytes
Xenopus laevis eggs were harvested and defolliculated as detailed previously.13 cRNA (10 ng of either NCCT or ROMK) was injected in a total volume of 100 nl per oocyte, and for coinjections involving WNK4 or one of the mutants, an additional 10 ng of WNK4 cRNA was added to the injectate. Water-injected oocytes were used as controls throughout. Oocytes were then incubated in ND96 containing 2 mmol/L sodium pyruvate and 0.1 mg/mL gentamicin at 18°C for 2 to 3 days before use as detailed previously.13

For 22Na flux studies, oocytes were placed for 24 hours in Cl- free ND96 solution containing 96 mmol/L sodium isethionate, 2 mmol/L potassium gluconate, 1.8 mmol/L calcium gluconate, 1 mmol/L magnesium gluconate, 5 mmol/L HEPES, 2.5 mmol/L sodium pyruvate, and 5 mg/L gentamicin. Thirty minutes before addition of uptake medium, oocytes were added to ND96 (Cl- and K+ free) with inhibitors (1 mmol/L ouabain, 100 μmol/L amiloride, and 100 μmol/L bumetanide), according to the protocol of Gamba et al.14 Oocytes were then transferred to isotonic uptake medium (58 mmol/L NaCl, 38 mmol/L N-methyl-D-glucamine, 2 mmol/L KCl, 1.8 mmol/L CaCl2, 1 mmol/L MgCl2, and 5 mmol/L HEPES, with inhibitors, pH 7.4) containing 22Na at a final concentration of 2.5 μCi/mL and incubated in a gently shaking incubator at 30°C for 1 hour. Oocytes were then washed 5× with 6 mL ice-cold aliquots of isotonic uptake medium and the oocytes counted individually in a gamma counter (Perkin-Elmer Cobra 5003). Thiourea sensitivity was shown by abolition of 22Na uptake with 100 μmol/L hydrochlorothiazide (data not shown).

Two-electrode voltage clamp (TEVC) recording used microelectrodes filled with 3 mol/L KCl (1 to 3 mol/L/L) for voltage sensing and current passing. External voltage and current electrodes consisted of fine, chloride-coated silver wires. Oocytes were held in a small chamber and perfused continuously with ND96 (2 ml/min) at room temperature (18°C to 20°C). After impaling, oocytes were held at a holding potential of −60 mV. Current voltage (I-V) plots were obtained from voltage step protocols ranging from −140 mV to +140 mV in 20-mV increments. Oocytes were held at each voltage step for 500 ms, with 100-ms intervals between the voltage steps. For the I-V plots, the steady-state current at each voltage step was used. Initially, oocytes were clamped at −60 mV in ND96, and when stable, the perfusing solution was switched to high-K+ ND96 and I-V plots obtained. The high-K+ ND96 was changed to high-K+ ND96 containing 2 mmol/L BaCl2, and a second set of I-V plots obtained.

Results

Clinical Details
A 22-year-old male presented with migraine and was found to have hypertension and hyperkalemia. No family history was available, in particular for his grandparents. He was treated initially with a low potassium diet and 10 mg of propranolol per day with some effect. Investigations at 26 years (then off treatment) showed a blood pressure of 150/95, plasma sodium 137 mmol/L, potassium 8.2 mmol/L, bicarbonate 16.2 mmol/L, chloride 119 mmol/L, urea 4.1 mmol/L, and creatinine 106 μmol/L. A Synacthen test was normal (cortisol rising from baseline 193 to 603 nmol/L at 1 hour), recumbent renin <0.2 pmol/ml per hour, and serum albumin aldosterone levels were 364, 420, and 676 pmol/L. A diagnosis of PHA2 (Gordon’s syndrome) was made, and he responded to 0.5 mg of cyclopenthiazide per day, achieving a normal blood pressure and plasma potassium a week later. He remains normotensive with normal plasma potassium at age 38 years.

The patient had 2 children who were screened for the condition after the diagnosis had been made. His daughter had normal blood pressure and plasma potassium on several occasions (between 2 and 12 years of age). The 4-year-old boy had been born prematurely at 36 weeks gestation but had had no neonatal illnesses and was symptomatic. His weight was on the second centile and height 25th centile for age, and his blood pressure was 100/60. Investigations showed 142 mmol/L plasma sodium, 6.7 mmol/L potassium, 13 mmol/L bicarbonate, 110 mmol/L chloride, and 48 mmol/L creatinine. Plasma renin was 0.6 pmol/ml per hour (normal 2.8 to 4.5) and aldosterone <69 pmol/L (normal 330 to 830). These results were considered consistent with PHA2, and he was treated with hydrochlorothiazide with a prompt (within a week) normalization in his plasma potassium. At 14 years of age, he remains well on 1.25 mg of
bendroflumethiazide per day, normotensive, with normal plasma potassium (4.7 mol/L).

**Genetic Screening**
Southern blots of *Pvu*II digests of genomic DNA from the 4 pedigree members showed no hybridization differences to normotensive control samples. To screen the WNK4 gene, individual exons were PCR amplified and the products directly sequenced. This revealed a single missense mutation in exon 7 of WNK4 in the affected father and son, which converts an aspartic acid residue within the highly conserved acidic motif of WNK4 to a histidine (564D>H; Figure 1). The father and son were heterozygous for this mutation in keeping with the autosomal dominant inheritance seen in other pedigrees with Gordon’s syndrome. There were no other mutations detected, and all of the unaffected mother’s and daughter’s WNK4 exons showed wild-type sequence.

**Functional Studies**
To establish that this WNK4 mutation is the disease-causing mutation in our pedigree, we investigated its function first by coexpressing it with NCCT to assess its effect on this NCCT. *Xenopus* oocytes injected with NCCT cRNA demonstrated a 3- to 5-fold increase in $^{22}$Na$^+$ uptake over water-injected controls (Figure 2). The coinjection of cRNA for wild-type WNK4 with NCCT caused almost complete inhibition of this flux. However, coinjection of cRNA for the mutant 564D>H WNK4 produced no significant inhibition of the $^{22}$Na$^+$ flux, which was not significantly different from NCCT alone. This behavior of the mutant 564D>H protein is identical to that seen with previously reported WNK4 mutation, 562Q>E (Figure 2).

We next assessed the effect of the 564D>H mutation on the interaction of WNK4 with the ROMK K-channel in voltage-clamped oocytes injected with cRNA for the ROMK2 isoform of ROMK. Expression of ROMK alone produced the expected inwardly rectifying I-V plot, which was substantially inhibited by coinjection of wild-type WNK4 cRNA (Figure 3A). Coinjection of cRNA for the 564D>H WNK4 mutation produced significant reduction in the steady-state current through ROMK, which was also seen after coinjection of ROMK with the 562Q>E WNK4 mutation (Figure 3B).

The effects of previously reported WNK4 mutations with NCCT and ROMK have been explained by a reduction in their surface expression. Therefore, we coexpressed the 564D>H WNK4 mutation in oocytes expressing either ROMK as a green fusion protein (EGFP-ROMK) or NCCT as a blue fusion protein (ECFP-NCCT). These experiments confirm that surface expression in *Xenopus* changes in parallel with the function of the native proteins when they are coexpressed with wild-type WNK4 or the 564D>H mutation (Figures 4 and 5).

**Discussion**
We report here a novel missense mutation in WNK4 (564D>H), which shows the same functional properties as other nonconservative mutations reported within the acidic motif. The striking feature of these missense mutations is their divergent behavior versus NCCT and ROMK. Although wild-type WNK4 reduces surface expression of both these proteins, the mutations behave as loss-of-function mutations versus NCCT but gain-of-function mutations versus ROMK. Coexpression of the 564D>H mutant actually had no significant effect on ECFP-NCCT fluorescence, whereas it produced significantly greater suppression of EGFP-ROMK than wild-type WNK4 (Figure 5). This divergence has led to
speculation that WNK4 acts as gatekeeper molecule to balance salt homeostasis via NCCT and K secretion through ROMK.12

The hyperkalemia seen in Gordon’s syndrome distinguishes it from other monogenic hypertension syndromes, which characteristically cause hypokalemia. The degree of hyperkalemia can be dramatic, with levels of >8 mmol/L reported in untreated individuals.15,16 Its origin was puzzling, although increased NCCT activity in the distal convoluted tubule would be expected to reduce NaCl entering the collecting duct, hence reducing electrogenic Na+ uptake and subsequent K+ secretion through ROMK. Based on the 6- to 7-fold shift in sensitivity to thiazides reported in subjects with Gordon’s, in vivo activation of NCCT appears to be substantial.8 The studies in Xenopus also suggest a similar difference in terms of NCCT expression between wild-type WNK4 and mutants carrying typical disease mutations such as 564D>H (Figures 4 and 5). However, reduction in Na+ uptake through epithelial Na+ channel (ENaC) may provide insufficient K+ conservation alone to cause hyperkalemia. Even in the presence of pharmacological inhibition of ENaC with amiloride, hyperkalemia is unusual in subjects who have normal renal function. This has prompted the search for a direct effect of WNK4 on ROMK function,12 and as we have shown here, WNK4 very substantially inhibits ROMK expression with further suppression by 564D>H and another acidic motif mutation 562Q>E.

Loss-of-function mutations in ROMK itself are associated with hyperkalemia, although it is only seen transiently in the antenatal period (OMIM 241200 or type 2 Bartter’s).17,18 Subjects also show salt wasting and hypotension outside the antenatal period because reduced secretion of K+ through ROMK in the thick ascending limb (TAL) directly limits operation of the Na,K,2Cl-cotransporter. WNK4 mutations that cause profound suppression of ROMK expression might be expected to cause a similar effect on loop function. There may be several reasons why this does not occur in subjects with Gordon’s syndrome. First, subjects with Gordon’s syn-

Figure 3. A, Typical I-V plots for oocytes expressing ROMK channels either alone or coexpressed with wild-type (WT) WNK4. B, In a different batch of oocytes the plateau current (μA) through ROMK at a holding potential of +20 mV in the presence of WT WNK4 or the 2 mutations (564D>H and 562Q>E). Data points are mean±SEM. Asterisk indicates significant difference from WT; P<0.01.

Figure 4. Representative confocal images of oocytes expressing EGFP-ROMK (green) or ECFP-NCCT (blue) alone or coexpressed with wild-type (WT) or mutant WNK4 (564D>H).
drome may be able to cope with the large amount of NaCl exiting the loop if ROMK expression were reduced here because of NCCT activation in the adjacent segment of the nephron. Alternatively, the selective expression of WNK4 within the distal convoluted tubule (DCT) and not TAL could explain why ROMK expression is preserved in the TAL. Another explanation may be that WNK4 affects ROMK expression in an isoform-specific manner. In the rat, ROMK isoforms1–3 are differentially expressed along the nephron, with ROMK1 the predominant isoform in the collecting duct.19 It is also notable that hyperkalemia in type 2 Bartter’s usually occurs with subjects with gene deletions removing isoforms 2 and 3.17 Nevertheless, our studies and those reported previously by Kahle et al used the ROMK2 isoform,12 which, in the rat, is expressed in the TAL and collecting duct.19

The reduced surface expression caused by WNK4 could reflect either reduced insertion of NCCT/ROMK into the membrane or their increased removal. For ROMK, evidence using a dynamin mutant protein suggests that WNK4 may accelerate its removal by a clathrin-mediated degradation pathway.12 How WNK4 affects the components of NCCT trafficking has not been investigated and clathrin recognition motifs are not present in NCCT. The reduced expression of NCCT is also distinct from ROMK in requiring intact kinase activity. This is intriguing because evidence that phosphorylation either affects or is necessary for NCCT trafficking is sparse,20 and of course, the phosphorylation target for WNK4 may not be NCCT itself. The presence of disease-causing WNK4 mutations in the acidic motif or C-terminal coil–coil domain probably does not affect kinase activity and further suggests that protein–protein interactions are a necessary part of the NCCT-WNK4 interaction.7

A genome-wide search based on the Framingham cohort has reported a hypertension locus on chromosome 17, which actually sits directly over the WNK4 gene.21 These and other data have led to the suggestion that WNK4 dysfunction might be important in a general hypertensive population. Published pedigrees with Gordon’s syndrome are very infrequent in the literature, as is the case for other monogenic hypertension syndromes such as Liddle’s and apparent mineralocorticoid excess. However, WNK4 mutations causing milder phenotypes may exist. Mutation scanning of ~1000 Japanese patients with hypertension or renal failure has identified 3 further missense mutations in WNK4.22 No data were presented on their functional activity, and the exon 7 mutation they reported (556P>T) is outside of the acidic motif; in fact, none of the substitutions occur in residues that are species conserved. There are no reports of common functional polymorphisms within WNK4 in non-Japanese populations. Hence, there is no evidence at present to suggest that WNK4 contains common risk alleles for essential hypertension.

The discovery of a crucial regulatory role for WNKs in the distal nephron has come about through the molecular cloning of just 2 of the known loci for Gordon’s syndrome. Our new pedigree confirms that the disease mutations in WNK4 cluster in the acidic motif. Why this motif is important is presently unknown, but charge changes in this run of 10 amino acids profoundly disturb the function of WNK4. More work is needed to define the structure of the WNK4 protein as well as its interacting partners. Cloning the remaining loci for Gordon’s syndrome may provide further clues as to this regulatory process or even identify other interacting proteins.

**Perspectives**

Establishing the molecular basis for rare monogenic syndromes such as Gordon’s syndrome can shed important light on salt homeostasis in the kidney. Work in the last couple of years has identified mutations in the genes encoding 2 novel serine/threonine kinases, WNK1 and WNK4, as the cause of the Gordon’s phenotype of hypertension and hyperkalemia. The characteristic sensitivity of Gordon’s patients to thiazide diuretics is now explained by loss of the normal function of WNK4 to suppress expression of the thiazide-sensitive co-transporter (NCCT) in the apical membranes of cells lining the distal convoluted tubule; the resulting overexpression of NCCT causes salt retention and hypertension. Mutant WNK4 also causes a deficit in surface expression of the ROMK K$^+$ channel leading to impaired K$^+$ secretion in the collecting duct and hence hyperkalemia. The phosphorylation target(s) for WNK4 are not known and only the WNK4-NCCT interaction is definitely dependent on its kinase activity. Identifying the phosphorylation target will be an important direction for future research because WNK1 and WNK4 have closest homology to STE20 kinases. This suggests they may be involved in a mitogen-activated protein kinase signaling pathway that regulates surface expression of NCCT, ROMK, and probably other transporter molecules. However, phosphorylation is not the only effector mechanism for WNKs. Like several other kinases, they have direct effects through protein–protein interaction. In fact, the WNK4 mutations, including the novel one described here (564D>H), cluster in an acidic motif that is highly conserved across WNK1 and WNK4. How this acidic motif is involved in protein–protein interactions is an important and unresolved question.

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