Increased urinary Na-Cl cotransporter protein in familial hyperkalaemia and hypertension

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Abstract

Background. Familial hyperkalaemia and hypertension (FHH), also termed pseudohypoaldosteronism type II, is a rare monogenic form of hypertension caused by mutations in the WNK1 or WNK4 kinases. In vitro expression of WNK4 reduces surface abundance and activity of coexpressed NaCl cotransporter (NCCT). This effect is lost in disease-producing WNK4 mutants. In two mice models of FHH, one expressing two extra copies of mutant WNK4 (Q562E) and another in which a mutant (D561A) WNK4 replaced wild-type WNK4, renal distal tubule hyperplasia with overexpression of NCCT was found. Currently no FHH human renal tissue is available to test for increased distal tubule surface abundance of NCCT. The availability of a unique large family with FHH and the Q565E WNK4 mutation enabled us to investigate this issue in an indirect manner.

Methods. Assuming that shedding of NCCT to the urine reflects its abundance in the distal tubule epithelium, we measured urinary NCCT protein in eight subjects of the FHH family and in eight unrelated controls by western blotting.

Results. Urinary NCCT protein was about four times higher in FHH than in controls [111.1 ± 40.5 versus 26.1 ± 16.4 densitometry units (P < 0.0001)]. No significant difference in urinary sodium and potassium concentrations was seen between FHH and controls.

Conclusions. The increased urinary NCCT in FHH most probably reflects increased NCCT abundance in the apical membrane of distal tubule cells in patients with FHH and the WNK4 mutation and points to the pathogenetic mechanism for the clinical phenotype of FHH and the WNK4 mutation, supporting results in transgenic mice with the same mutation and in knockin mice with another mutation.

Keywords: familial hyperkalaemia and hypertension; Na-Cl cotransporter; pseudohypoaldosteronism type II; urine proteomics; WNK4 kinase

Introduction

Familial hyperkalaemia and hypertension (FHH), also known as pseudohypoaldosteronism type II (PHA II), is a rare monogenic form of hypertension [1]. It was first described in a sporadic case by Paver and Pauline in 1964 [2]. The first family with FHH was described 12 years later [3]. FHH is an autosomal dominant disorder characterized by hyperkalaemia, hypertension and metabolic acidosis in the presence of normal renal function [4,5]. All these abnormalities are corrected by relatively low dose of thiazide diuretics [4,5]. The molecular basis for the disorder was found by reverse genetics to be mutations in two isoforms of the WNK [With No K (lysine)] kinase genes, WNK1 and WNK4 [6]. WNK1 mutations are intronic deletions and cause increased expression of the gene. WNK4 mutations are missense mutations that change the function of the gene [6]. The mechanism by which these mutations produce FHH is not clear. The efficacy of thiazides in FHH points toward the thiazide arget, the NaCl cotransporter (NCCT), as a potential molecule regulated by WNK kinase. Indeed, in in vitro experiments, expression of wild-type WNK4 with NCCT in frog oocytes caused a decrease in NCCT activity and surface abundance [7,8]. This decrease was abolished using FHH-producing WNK4 mutants. Similar results were recently obtained in Cos-7 cells [9]. Additional proposed mechanisms for WNK4 in the pathogenesis of FHH include inhibition of the renal outer medullary potassium (ROMK) channel by WNK4, as found in in vitro expression studies in frog oocytes, an inhibition that is further increased using FHH-producing WNK4 mutations [10]. WNK4 was also shown to augment the paracellular Cl⁻ transport, which was further stimulated by disease-producing mutants [11,12]. The hypothesis of increased abundance of NCCT in the apical membranes of renal distal tubule cells in humans
affected by FHH is supported by the recent findings in two mice models of FHH, one expressing two extra copies of the mutant Q562E WNK4 and another in which a mutant (D561A) WNK4 replaced wild-type WNK4. In both models, distal tubule hyperplasia with overexpression of NCCT was found [13,14]. Since no human FHH kidney tissue is available, this hypothesis cannot be tested directly. The availability of a unique large family with FHH and the Q565E WNK4 mutation [15,16] enabled us to test it indirectly, assuming that the overexpressed NCCT is shed in increased amounts to the urine [17]. This is in analogy with aquaporin-2, where it was found that its amount in the urine is proportional to its amount in the whole kidney and more specifically in the apical plasma membrane of renal tubular cells [18]. Therefore, we compared the amount of urinary NCCT in subjects with FHH and the Q565E WNK4 mutation to that of healthy controls.

Subjects and methods

Subjects

Two groups of subjects were studied, eight control subjects and eight subjects from the large family with FHH and Q565E WNK4 mutation [15,16] (subject III-3, IV-3, IV-4, V-3, V-4, V-6, V-7, V-13) (Figure 1 [16]). FHH was established in all subjects by typical clinical features of hyperkalaemia and hypercalciuria, together with the characteristic genotype as described previously [16]. Their basal serum electrolytes were similar to those of all the affected family members (K 5.6 ± 0.2 mmol/l, Na 140.4 ± 2.2 mmol/l, Cl 109.0 ± 1.5 mmol/l, HCO3⁻ 19.5 ± 1.4 mmol/l [16]. Five subjects were treated by hydrochlorothiazide when studied, since they were hypertensive. During the study all subjects were normotensive.

Detection of NCCT in Urine

Spot urine was collected and a sample was used for determination of electrolytes, creatinine and osmolarity. Fifty milliliters of urine was kept on ice with a mixture of protease inhibitors (protease inhibitor cocktail tablets, purchased from Roche). Urine was centrifuged at 17 000 g for 15 min at 4°C to remove all cell debris, and the pellet was discarded. The supernatant was centrifuged at 200 000 g for 80 min at 4°C. The resulting pellet was suspended in 500 µl of sucrose buffer [250 mM sucrose; 10 mM triethanolamine; 1 µg/ml leupeptin and 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF)] and added to Laemmli buffer with no dithiothreitol (DTT) [final concentration: Tris 50 mM pH 6.8; glyceral 10%; sodium dodecyl sulphate (SDS) 2%; bromo phenol blue 0.05%]. Samples were subjected to 8.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis. The amount of resuspended pellet loaded on each lane was calculated to represent urine that contained 3 mg of creatinine. Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane. The filter was blocked with 2% bovine serum albumin (BSA) in TBST (Tris 20 mM, NaCl 150 mM, tween-20 0.05%, pH 7.4) and incubated with rabbit anti-human NCCT polyclonal antibody kindly provided by Dr MA Knepper [19]. Filter was washed with TBST and incubated with secondary horseradish peroxidase (HRP) conjugated antibody. Visualization was achieved according to the manufacturer’s instructions. The intensity of the resulting bands was measured by densitometry.

Expression of results

Samples from the 16 subjects (8 controls and 8 FHH patients) were loaded on two gels, each containing both controls and FHH samples. Two of the samples (one control and one FHH) were loaded on both gels. Densitometry values were determined and the identical samples in the two gels were used for comparison of the two gels. The identical samples were very similar (not shown); thus, the gel shown in Figure 1A represents results of both gels with no normalization. Data are presented as mean ± SD. For comparison between means, Student’s t-test was performed. Two tailed P-values were reported. P < 0.05 was considered significant.

The study was approved by the Ethics Committee of the Sheba Medical Center, adhering to the Helsinki Declaration. Informed consent was obtained from all subjects studied.

Results

The characteristics of the subjects studied are shown in Table 1. The gender distribution in FHH and control subjects was identical. The FHH subjects and control subjects did not differ significantly in their age and BMI. Measurement
of urinary electrolytes revealed that FHH subjects had lower urinary sodium content than the control subjects (8.6 ± 6.0 versus 13.6 ± 4.0 mmol/mmol creatinine), but this was statistically insignificant (P = 0.07). FHH subjects had also lower urinary potassium concentration than controls (4.4 ± 1.3 versus 6.5 ± 2.6 mmol/mmol creatinine), which is not statistically significant (P = 0.06). In our previous studies of the family [15,16], similar urinary sodium and potassium levels were found in FHH and their unaffected family members that served as controls. In these studies, electrolytes were determined in urinary 24 h collection [15] or in first morning urinary samples [16]. In addition, in these studies no subject was on thiazide treatment [15,16]. Urinary osmolarity in the FHH subjects was similar to that of the controls. The measurement of urinary NCCT is shown in Figure 1A. The NCCT bands shown by the arrow have a molecular weight smaller than the 175 kDa marker. They are of the same molecular weight (about 160 kDa) as that reported by Pisitkun et al. for human urinary NCCT [19]. The bands are easily seen in the lanes of the FHH subjects. In the controls, they are clearly seen in subjects 2 and 8. The upper bands that are seen in control subjects 4 and 5 are irrelevant since they are clearly seen in subjects 2 and 8. In the controls, electrolytes were determined in urinary 24 h collection [15] or in first morning urinary samples [16]. In addition, in these studies no subject was on thiazide treatment [15,16]. Urinary osmolarity in the FHH subjects was similar to that of the controls. The measurement of urinary NCCT is shown in Figure 1A. The NCCT bands shown by the arrow have a molecular weight smaller than the 175 kDa marker. They are of the same molecular weight (about 160 kDa) as that reported by Pisitkun et al. for human urinary NCCT [19]. The bands are easily seen in the lanes of the FHH subjects. In the controls, they are clearly seen in subjects 2 and 8. The upper bands that are seen in control subjects 4 and 5 are irrelevant since they also appear while using the secondary antibody only (not shown). The amount of NCCT in the urine of FHH they also appear while using the secondary antibody only (not shown). The amount of NCCT in the urine of FHH is more than four times higher abundance of urinary NCCT than controls (Figure 1A and B). Densitometry shows that the mean abundance of NCCT of control subjects (11.1 ± 3.4 arbitrary densitometry units, P = 0.0001). There is almost no overlap between individual data in the two groups. Although the groups were small, it seems that thiazide therapy did not affect urinary NCCT [(100.8 ± 48.2 versus 128.1 ± 20.2 densitometry units, P = 0.40) in FHH subjects, on or off thiazide therapy, respectively].

### Discussion

The progress in urine proteomics with its capacity to detect very low concentrations of urinary proteins enabled advances in the study of physiological as well as pathological states [17]. An important example was the finding that urinary aquaporin-2 can be measured in the urine. Since it is shed from principal cell membranes into the urine, changes in the urinary concentration of aquaporin-2 reflect the activation of vasopressin on the kidney, which increases insertion and consequently the number of aquaporin-2 molecules in the apical membrane of the cortical collecting duct [20]. One of the proteins that can be determined in the urine is NCCT which, like aquaporin-2, is an apical membrane protein and has a crucial role in FHH. Indeed in rats urine, NCCT as well as other Na+ transporters can be detected [21]. NCCT was also detected in human urine [19]. It is inserted in urinary exosomes that are supposed to represent vesicles released from the apical plasma membrane of renal tubule epithelial cells [17]. Determination of NCCT in the urine can be utilized to perform quantitative measurement of NCCT in FHH subjects in comparison to controls.

Using an immunoblot assay, we have found a marked increase in the abundance of urinary NCCT in FHH subjects compared to controls. FHH subjects had a mean of about four times higher abundance of urinary NCCT than controls with almost no overlap between individual data in the two groups. Although FHH subjects had lower (statistically insignificant) urinary sodium and potassium concentrations, it does not seem that this was the cause for the marked increase in urinary NCCT in FHH. In no group of subjects was there a correlation between individual urinary NCCT values and urinary sodium or potassium. The difference in urinary NCCT is apparently the result of the WNK4 mutation that causes the clinical phenotype. It probably reflects increased shedding of NCCT into the urine. In analogy with the finding in the study of aquaporin-2 [18,20], we may assume that distal tubule apical cell membranes of FHH subjects have an increased abundance of NCCT. In support of such a mechanism are the findings in the recent report that transgenic mice which have two extra copies of the FHH-producing mutant Q562E WNK4 gene (the same mutant, Q565E, affecting the currently reported FHH subjects), have in

| Table 1. Characteristics and urinary NCCT data of the groups of subjects studied |
|--------------------------------|---------------------|---|---|
| Variable                        | FH       | Control |   |
| Number                          | 8        | 8       |   |
| Age (year)                      | 46.5 ± 18.6 | 36.5 ± 6.4 | 0.17 |
| Gender (% males)                | 37.5     | 37.5    | 1.0 |
| BMI                             | 30.9 ± 5.3 | 26.6 ± 7.1 | 0.19 |
| Urinary Na (mmol/mmol creatinine)| 8.6 ± 6.0 | 13.6 ± 4.0 | 0.07 |
| Urinary K (mmol/mmol creatinine) | 4.4 ± 1.3 | 6.5 ± 2.6 | 0.06 |
| Urinary Mg (mmol/mmol creatinine)| 0.31 ± 0.06 | 0.27 ± 0.08 | 0.21 |
| Urine osmolarity (mOsmol/kg H2O) | 822 ± 276 | 777 ± 201 | 0.38 |
| NCCT in urine (densitometry units)| 111.1 ± 40.5 | 26.1 ± 16.4 | <0.0001 |
| Subjects on/off thiazides | 5/3 |   |   |
| NCCT in urine (densitometry units), FHH subjects on thiazides (n = 5) | 100.8 ± 48.2^a |   |   |

^aSimilar to NCCT in FHH subjects off thiazides (128.1 ± 20.2, P = 0.40).
addition to hyperkalaemia, hypercalciuria and hypertension, also marked hyperplasia of the distal convoluted tubule [13]. Using immunohistochemical techniques, it was found that hyperplasia is confined to the distal tubule cells and does not occur in other nephron segments. In this segment, distal convoluted tubule surface area and total NCCT expression are increased. Very recently, Yang et al. produced knockin mice expressing the FHH-producing D561A WNK4 mutant [14]. Like Lalioti et al. [13], Yang et al. found increased expression of NCCT in the renal distal nephron in their animal model [14]. In addition, they have shown increased phosphorylation of the Ste20-related kinases, SPAK (Ste20-related proline alanine-rich kinase) and OSR1 (oxidative stress response kinase [1]) in the mutant mice, establishing a role for the WNK4-SPAK/OSR1-NCCT cascade [22–24] in the production of FHH. Although the mechanism proposed by Yang et al. [14] is different than that proposed by Lifton and colleagues [13], which was based on the findings in frog oocytes, both groups find overexpression of NCCT in the distal convoluted tubule. Our findings of increased urinary NCCT in FHH support the notion that in the human disease, NCCT is overexpressed in this nephron segment.

These findings support the central role of the increased NCCT membrane abundance in the pathophysiology of FHH and diminish the importance of the role of paracellular chloride transport in the production of FHH [11,12,25]. Similarly in the transgenic mouse model, homozygous knockout of the NCCT gene reversed the phenotypic abnormalities, indicating that the effect of WNK4 on NCCT only is sufficient to produce hyperkalaemia, hypercalciuria and hypertension [13]. In addition, in both mice models [13,14], mutant mice did not differ from wild-type mice regarding ROMK renal abundance and no increased paracellular chloride flux was found in the knockin mutant [14].

It is interesting to note that in Gitelman's syndrome, which features hypokalaemia, metabolic alkalosis and hypertension, is a mirror image of FHH and is caused by inactivating mutations of NCCT, immunoblotting of the kidneys showed that patients had reciprocal decreased amounts of urinary NCCT [26].

Our finding may shed light on additional pathophysiological mechanisms of action of WNK kinases. For example, the question whether the effect of WNK1 in the pathogenesis of FHH is mediated solely by the kidney or whether extrarenal effects are involved has not been settled [27]. Similar to WNK4, WNK1 stimulates and phosphorylates SPAK and OSR1 [22,23]. If patients with FHH and the WNK1 mutations have also increased urinary NCCT, it will suggest that these patients also have increased abundance of NCCT in their renal plasma membranes. On the clinical ground, FHH subjects with the WNK4 mutations have marked sensitivity to thiazides in reducing hypertension, hyperkalaemia and hypercalciuria [15,16]. Regarding FHH subjects with the WNK1 mutation, no data supporting the notion that they have increased sensitivity to thiazides were reported (28).

Finally, the mechanism by which the presumed increased abundance of renal cell membrane NCCT is associated with increased sensitivity to thiazides is not known. In the current study, thiazide therapy did not change urinary and presumably renal abundance of NCCT. A major limitation of the study is that only three of the eight FHH subjects were off thiazides. Further studies are necessary to confirm these results.

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Conflict of interest statement. No author has a conflict of interest to declare.

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