Incomplete distal renal tubular acidosis from a heterozygous mutation of the V-ATPase B1 subunit

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Zhang J, Fuster DG, Cameron MA, Quiñones H, Griffith C, Xie X, Moe OW. Incomplete distal renal tubular acidosis from a heterozygous mutation of the V-ATPase B1 subunit. Am J Physiol Renal Physiol 307:F1063–F1071, 2014. First published August 27, 2014; doi:10.1152/ajprenal.00408.2014.—Congenital distal renal tubular acidosis (RTA) from mutations of the B1 subunit of V-ATPase is considered an autosomal recessive disease. We analyzed a distal RTA kindred with a truncation mutation of B1 (p.Phe468fsX487) previously shown to have failure of assembly into the V1 domain of V-ATPase. All heterozygous carriers in this kindred have normal plasma HCO3− concentrations and thus evaded the diagnosis of RTA. However, inappropriately high urine pH, hypocitraturia, and hypercalciuria were present either individually or in combination in the heterozygotes at baseline. Two of the heterozygotes studied also had inappropriate urinary acidification with acute ammonium chloride loading and an impaired urine-blood Pco2 gradient during bicarbonaturia, indicating the presence of a H+ gradient and flux defects. In normal human renal papillae, wild-type B1 is located primarily on the plasma membrane, but papilla from one of the heterozygote who had kidney stones but not nephrocalcinosis showed B1 in both the plasma membrane as well as diffuse intracellular staining. Titration of increasing amounts of the mutant B1 subunit did not exhibit negative dominance over the expression, cellular distribution, or H+ pump activity of wild-type B1 in mammalian human embryonic kidney-293 cells and in V-ATPase-deficient Saccharomyces cerevisiae. This is the first demonstration of renal acidification defects and nephrolithiasis in heterozygous carriers of a mutant B1 subunit that cannot be attributable to negative dominance. We propose that heterozygosity may lead to mild real acidification defects due to haploinsufficiency. B1 heterozygosity should be considered in patients with calcium nephrolithiasis and urinary abnormalities such as alkaliniuria or hypocitraturia.

V-ATPase; haploinsufficiency; kidney stones; distal renal tubular acidosis

RENAL TUBULAR ACIDOSIS (RTAs) are tubulopathies where non-volatile acid accumulates because of decreased tubular secretion of acid by the proximal or distal nephron, which can result from congenital or acquired causes (19, 31). Distal H+ excretion requires carbonic anhydrase to generate an acid-base pair (H+ and HCO3−) to furnish substrate for acid-base transporters. Luminal translocation of H+ from the cell into the urine is mediated by V-ATPase, and concurrent basolateral exit of HCO3− into the plasma occurs via anion exchanger 1 (AE1) (68). It is not mere coincidence that congenital forms of distal RTA (dRTA) in humans result from inactivating mutations of carbonic anhydrase II enzyme (23, 53, 55), the B1 or a4 subunits of V-ATPase (60, 67), or the AE1 Cl−/HCO3− exchanger (6, 7, 11).

These candidate genes were identified using classical genetics on kindreds derived from probands initially identified by clinicians. In general, phenotypic information is limited to clinical diagnosis of RTA based on hypobicarbonatemia without elevation of plasma anion gap or creatinine and concomitant unduly alkaline urine pH. The disorder may be brought to clinical attention by complications such as growth retardation, nephrocalcinosis, and nephrolithiasis (31). Most clinical data are limited to plasma HCO3− concentration and spot urinary pH with no further phenotypic characterizations.

Congenital dRTA usually follow autosomal recessive inheritance with the exception of some mutations of AE1, which are autosomal dominant. The basis for the recessive nature is usually attributed to adequacy of the normal functioning transporters provided by the single wild-type allele, or haplosufficiency. In the case of AE1-related autosomal dominant dRTA (6, 11, 25, 29, 57, 69), in vitro data have suggested that mutant AE1 may prevent wild-type AE1 from reaching the membrane via heterodimerization or by mistargeting of the mutant to the apical membrane, thereby nullifying apical H+ secretion (13, 49, 50, 64). This dominant model by mutant AE1 remains to be proven in vivo. Other than these AE1 mutations, the accepted paradigm is that one needs two mutant alleles to produce dRTA. However, the foundation of this paradigm is based on the absence of overt clinical diagnoses in heterozygotes.

The clinical diagnosis of dRTA depends on the stringency of the definition and how one sets the threshold for defining renal acid underexcretion. Since metabolic acidosis is not a dichotomous trait, a forme fruste of dRTA can conceivably eclipse the definition and how one sets the threshold for defining renal acidification. Interestingly, a considerable fraction of the literature on this type of incomplete dRTA actually stems from patients with kidney stones. While most patients with calcium oxalate stones can maximally acidify their urine, up to 30% of patients with calcium phosphate stones have reduced ability to lower their urine pH (2, 22,
Impaired maximal acidification is also more common in recurrent stone formers and those with bilateral disease (2, 12, 15, 30, 43, 44, 54, 61, 63). No one investigated the basis for these subtle acidification anomalies.

Distal apical H+ secretion is mediated by V-ATPase (5, 27), which belongs to a family of ATP-energized H+-translocating ion pumps (18). V-ATPases acidify intracellular organelles, which is essential for receptor-mediated endocytosis, lysosomal hydrolysis, intracellular protein targeting, processing of hormones, and uptake and storage of neurotransmitters. In specialized cells, plasma membrane-situated V-ATPases are instrumental in the regulation of bone resorption, synthesis, and secretion of hormones, and uptake and storage of neurotransmitters. V-ATPases are complex proteins composed of at least 13 different subunits organized into 2 distinct functional domains: a peripheral catalytic sector 650-kDa V1 domain (A6B2C1D1E1F1G1H1) and an intramembranous 260-kDa proteolipid V0 domain [a,d]c[α,ε] (24). In mammals, B1 is restricted to epithelia of the inner ear, epididymis, and distal renal tubule. Mutations in the B1 subunit gene ATP6V1B1 are responsible for autosomal recessive dRTA with deafness (19, 28, 56, 60). The mechanisms by which some B1 subunit mutations lead to V-ATPase dysfunction were unknown until recently, when the majority of mutant B1 subunits were shown to fail to incorporate into the V1 subunit (20, 73).

We posed the simple but fundamental question of whether heterozygous carriers of mutant B1 have any detectable renal acidification abnormalities. In addition, if they do, is there evidence for negative dominance? We took a family known to harbor an inactivating truncation mutation of B1 (21) and studied the heterozygous members for abnormalities in renal acidification, prompted by the fact that one of the heterozygotes developed severe kidney stones. Despite the fact that none of the heterozygotes carry the clinical diagnosis of RTA, we obtained evidence that the heterozygotes have abnormal baseline steady-state urinary chemistry, and two heterozygotes showed abnormal urinary acidification upon provocation. Because of the previous postulation of negative dominance of the mutant B1 subunit over the wild-type subunit (73), we used in vitro biochemical, mammalian cell expression, and yeast complementation approaches to examine for this possibility. We conclude that the affected heterozygotes in our kindred are in a state of haploinsufficiency where the pump from the normal allele can sustain normal clinical chemistry but the burden of heterozygosity in this family is incomplete dRTA. Individuals with incomplete dRTA or calcareous nephrolithiasis with distinct chemical abnormalities should be considered as potential heterozygous carriers of mutant B1 alleles.

METHODS

Patient experiments. Subjects were recruited from University of Texas Southwestern Medical Center clinics with approval by the Institutional Review Board, and all participants gave informed consent. Normal subjects comprised staff and students on campus. A QuikChange Multi Site-Directed Mutagenesis Kit according to the manufacturer’s instructions (Agilent Technologies). All constructs were verified by sequencing. Human embryonic kidney (HEK)-293 cells [American Type Culture Collection (ATCC)] were cultured with 10% (vol/vol) FBS, penicillin (100 units/ml), and 1% Triton X-100, 100 mM NaCl, 50 mM Tris-HCl (pH 7.0, and the urine was then collected every 15 min for three urine collections for PCO2 and pH.

For immunofluorescence and immunoprecipitation, cells were lysed in buffer [containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.5), 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 100 μg/ml PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotonin, and 2 μg/ml pepstatin A], and lysates were cleared by centrifugation (14,000 g for 30 min). Precipitating antibodies (1:200 dilution) followed by prewashed protein G-agarose (50% slurry) were added (4 h at 4°C). After being washed with lysis buffer (3 times), precipitated antibodies (1:200 dilution) followed by prewashed protein G-agarose (50% slurry) were added (4 h at 4°C). After being washed with lysis buffer (3 times), precipitated antibodies were assessed enzymatically (Boehringer-Mannheim Biochemicals, Indianapolis, IN).
Proteins were eluted from the beads with 2.5× loading buffer [2.5 mM Tris-Cl (pH 6.8), 2.5% SDS, 2.5% β-mercaptoethanol, and 25% glycerol], heated (95°C for 2 min), resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with the stated primary antibodies.

For immunocytochemistry, HEK-293 cells were fixed 48 h after transfection with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked by 3% BSA and 10% goat serum for 1 h. Specimens were incubated with anti-FLAG mouse monoclonal antibody. Fluorescence images were acquired through a Zeiss ×100 objective lens using a Zeiss LSM-510 laser scanning confocal microscope.

Microsomal vesicles were isolated from HEK-293 cells (two 10-cm dishes) as previously described (32). The cell pellet was resuspended in ice-cold hypotonic lysis buffer [10 mM HEPES (pH 7.2), 5 mM diisopropyl fluorophosphate, 10 μg/ml pepstatin A, 10% (vol/vol) aprotinin, and 10 mg/ml benzamidine] and then homogenized by 10 strokes in a tight fitting Dounce followed by 15 strokes after the addition of an equal volume of sucrose buffer (500 mM sucrose and 10 mM HEPES, pH 7.2). The lysate was centrifuged (600 g for 15 min), the secondary supernatant was centrifuged again (100,000 g for 45 min), and the pellet was resuspended in buffer (250 mM sucrose and 10 mM HEPES, pH 7.2) at 2–6 mg/ml protein. Vesicular H+ pumping was assessed by measurements of acidine orange absorbance quenching in a SLM-Amino DW2-C dual wavelength spectrophotometer (change in absorbance at 492–540 nm) (14, 72). Vesicles (300 μg) were diluted into 1.6 ml assay buffer [200 mM NaCl, 30 mM Na-tricine (pH 7.5), 3 mM MgCl2, 0.5 mM EDTA, and 6 μM acidine orange]. Reactions were initiated by the addition of ATP (final concentration: 1.3 mM) and valinomycin (final concentration: 1 μM) and were terminated by the addition of 1.6 μM of H+ ionophore 1799.

Yeast transformation and growth selection. COOH-terminally HA-tagged expression constructs in p426TEF or p425TEF vectors (35) were transformed into Saccharomyces cerevisiae strain YBR127C (genotype MATa/MATalpha his3Δ1/α leu2Δ0/α met15Δ0/Δ ura3Δ0 ura3Δ0 VMA2/Δ VMA2, ATCC) and grown at 30°C in YEPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l d-glucose, and 100 mg/l adenine hemisulfate) or synthetic minimal medium: 1% (wt/vol) glucose, and 100 μg/ml adenine hemisulfate, and 50 mM MES, pH 5.5). All yeast media contained 200 μg/ml geneticin. Transformation was performed as previously described by Gietz et al. (52). Single colonies were isolated on pH 5.5 SD–UL plates, and the growth of transformants was assessed on YEPD plates buffered to pH 5.5 with 50 mM MES or pH 7.5 with 50 mM MOPS.

For immunoblot analysis, 10 optical density units of yeast were washed once with 1 mM EDTA-H2O and lysed in 2 M NaOH (10 min, 4°C). Proteins were isolated by trichloroacetic acid/acetone precipitation, resuspended in SDS sample buffer [25 mM Tris-Cl (pH 6.8), 9 M urea, 1 mM EDTA, 1% (wt/vol) SDS, 0.7 M β-mercaptoethanol, and 10% (vol/vol) glycerol], separated by SDS-PAGE, and analyzed by immunoblot analysis as described above.

RESULTS

Pedigree, clinical phenotype, and 24-h urine chemistry. Figure 1 shows the pedigree previously partially described (3, 21). The family members have advanced in age since the original publication (3), but the clinical diagnoses have not changed, with six family members carrying the diagnosis of dRTA. One heterozygote carrier presented with urolithiasis with a heavy stone load, which required surgical intervention (asterisk in Fig. 1). Interestingly, she also had unduly alkaline urine and unexplained hypocitraturia, which cannot be explained by unusual acid intake (urinary SO4 excretion: 31 meq/day) or alkali intake (urinary K+ excretion: 67 meq/day, estimated net gastrointestinal alkali absorption: 27 meq/day) (65). The combination of high urine pH and hypocitraturia suggest defective urinary acidification. This prompted us to screen the rest of the family. Stone risk profiles from 24-h or spot urines are shown in Fig. 1. Although none of the heterozygotes carriers were previously recognized by their physicians as having dRTA, all of them showed some degree of urinary abnormalities, such as hypocitraturia and hypercalciuria, that cannot be attributable to secondary causes. Some of them also have urine pHs that are slightly alkaline, but since these were obtained on outpatient random diets, one cannot definitely conclude on abnormality. We could not identify asymptomatic stones in the rest of the family members because the subjects declined imaging experiments.
**Incomplete dRTA and Heterozygous Mutation of V-ATPase B1 Subunit**

**Summary**

This study investigates the metabolic consequences of a heterozygous mutation in the V-ATPase B1 subunit, focusing on the role of this mutation in acid-base homeostasis. The research is centered around a single family with a history of kidney stone formation and abnormal renal acidification.

**Key Findings**

1. **Metabolic Experiments**
   - The family consented to metabolic experiments, including the use of ammonium chloride to induce acid-base stress.

2. **Urine Parameters**
   - The study compared urine pH and bicarbonate concentrations among family members.
   - Heterozygous individuals showed signs of reduced urinary acidification compared to wild-type siblings.

3. **Immunohistochemistry**
   - Expression of the B1 subunit was assessed in renal tissue samples.
   - Abnormal cellular distribution of B1 was observed in heterozygotes.

4. **Intraoperative Biopsy**
   - Histological analysis revealed differences in the expression and localization of B1 in heterozygous vs. normal tissue.

**Discussion**

- **Heterozygous Mutation**
  - The p.Phe468fsX487 truncation affects the B1 subunit, leading to a dominant negative effect on renal acidification.

- **Clinical Implications**
  - The findings support the notion of incomplete dRTA in heterozygous carriers, which may require consideration in clinical settings.

**Conclusion**

The study provides insights into the metabolic and cellular consequences of a heterozygous V-ATPase B1 mutation, highlighting its clinical relevance in renal medicine.
for the wild-type B1 antigen, the V-ATPase activity of cotransfected cells was actually normal.

To further confirm this point, we kept the amount of wild-type B1 constant and gradually increased the truncated mutant and searched for the presence of interference. When transfected mutant B1 expression was increased, the transfected wild-type B1 expression appeared to be decreased (Fig. 4C). However, a similar effect was seen in the converse experiment, where increasing wild-type B1 expression decreased mutant B1 expression (Fig. 4C). When both B1 subunits are driven to extremely high levels by strong promoters in these transient expression experiments, the two subunits may be competing for the same limited translational machinery. We fathom that this is in fact the more likely explanation than a dominant negative effect of the mutant on translation of wild-type B1.

To further test this hypothesis, we examined whether transfection of exogenous mutant B1 affects the expression of native B1 and its ability to assemble. As shown in Fig. 5, exogenous mutant B1 did not affect the level of native B1 expression, assembly, or function. This speaks strongly against a dominant negative effect of mutant B1 on native wild-type B1.

Examination for evidence of negative dominance in yeast. To thoroughly refute the dominant negative hypothesis, we used a second system to test for dominance. Yeast provides an ideal system for studying transporters in general because of the...
ease in obtaining definitive genetic null cells and the ability to
use growth as a functional screen; thus, this host has been used
extensively for studying V-ATPase subunits (26, 48). Therefore,
an independent test of negative dominance by the mutant
B1 subunit is H+/H1001pumping function in yeast, which is critical
to confer the ability to grow in pH 7.5. As previously shown,
human B1 complements the yeast homolog well in a growth
assay (21). In null yeast, the presence of mutant B1 did not
affect the expression level of B1 or the ability of wild-type B1
to support growth in pH 7.5 (Fig. 6A). This provides additional
evidence showing that the truncated mutant protein exerts no
significant effects on wild-type B1. We further examined
whether there was any evidence of negative dominance in 12
other known human mutations to complete an exhaustive
search using the yeast model. None of these mutants affected
the ability of wild-type B1 to complement pump function in
null yeast, and none of these mutations affected the level of B1
expression (Fig. 6B).

**DISCUSSION**

Inactivating mutations of the V1 subunit B1 and the V0
subunit a4 are associated with congenital autosomal recessive
dRTA with sensorineural hearing loss (28, 56, 58, 60, 67). The
mechanism of dysregulated acidification from B1 mutations
was unclear until recently, when disrupted V-ATPase assembly
in vitro was described as one mechanism for seven known
ATP6V1B1 missense mutations (20, 73). The finding of abnor-
mal acidification in the heterozygous carriers in this kindred
was unexpected but unequivocal. The question is why these
subjects should be abnormal given that they have one normal
copy of B1 and, as far as we know, B1 expression is biallelic.
The current stoichiometric model of V1 (A3B3C1D1E3F1G3H1)
confers a 0.875 (1– 0.53) probability of incorporating at least
one abnormal B1 subunit. A heterogeneous wild-type/mutant
B1 can conceivably affect ATP hydrolysis by A3B3 and impair
pump function. However, we cannot find evidence showing
that the truncated B1 is capable at all of incorporation into V1.
However, mutant B1 can theoretically affect the synthetic
pathway of wild-type B1 if both alleles are translated together.
Based on the observation that overexpressed mutant B1 sub-
units can lower Na+/H1001-independent cell pH recovery after acid
load in a rat IMCD cell line, Yang and coworkers (73)
proposed a dominant negative mechanism of transfected mu-
tant B1 subunits over native wild-type B1 subunits. However,
in the background of native B1, the effect of the translected

**Fig. 5.** Effect of TM B1 on native B1 in HEK-293 cells. TM B1 was
transfected into HEK-293 cells, and its effects on native B1 expression and
assembly with other subunits were studied. After cell lysis, transfected heter-
ologous TM B1 was removed by immunodepletion with anti-HA antibody
beads, and native B1 was immunoprecipitated with anti-B1. The immunocom-
plex was resolved by SDS-PAGE and immunoblotted for the antigens stated.
A, top: immunoblot of transfected TM B in cell lysates with anti-HA. Middle,
immunoblot of lysates after capture of TM B by immunoprecipitation (IP) for
TM B HA, native B, A, and E subunits that are part of V1. Bottom, immunoblot
of the native B immunocomplex by anti-B. The complex was immunoblotted
for B, A, and E subunits, which are parts of V1. B: endosomal V-ATPase
activity in untransfected, vector-transfected, and TM B-transfected HEK-293
cells.

**Fig. 6.** Expression of WT and TM B1 in yeast. Expression
plasmids p425TEF and p426TEF are described in
METHODS. P425 and p426 denote plasmid only. WT and
various human B1 mutations are shown. A, left: the
yeast growth assay is permissive at pH 5.5 and restric-
tive at pH 7.5. A total of 5 × 107 yeast cells were
plated with serial 10-fold dilutions from left to right,
and growth was assessed after 4 days. Right, expres-
sion of WT and TM COOH-terminally HA-tagged
human B1 constructs in yeast. Equal amounts of yeast
lysates of 10 optical density units were loaded. Immu-
noblot analysis was performed with a monoclonal anti-HA
antibody. B: 12 other documented naturally occurring
human B1 mutations were tested in a similar fashion in
null yeast as described above. Left, yeast growth assay. Right, immunoblot.
protein is not easy to assay and certainly difficult to interpret. We reduced endogenous B1 to levels where we could see the activity of the transfected protein, and the initial results hinted at the possibility of negative dominance (Fig. 4A). However, any reduction in wild-type B1 activity was in fact seen only in the situation where both transfected proteins were driven to very high levels. Mutant B1 did not exert any dominant effect over wild-type B1 expression, assembly, or function (Figs. 5 and 6). To complete our search, we studied 12 naturally occurring human B1 mutations in null yeast and failed to find any dominant negative action for any of them. Although this was a single sample, the heterozygous carrier exhibited an abnormal pattern of staining of B1 in the renal medulla, which we interpret as unassembled mutant B1. The higher than expected intracellular staining of B1 in the renal medulla, which we interpret as unassembled mutant B1. The higher than expected intracellular staining likely represents the failure of mutant B1 to incorporate into V₁, as we have observed in cell culture (20). At the moment, the data in concert support the de facto conclusion that the clinical burden of heterozygosity in this family is likely due to haploinsufficiency.

We are cognizant of the fact that not all families with dRTA are linked to either ATP6V1B1 or ATP6V0A4 (56), suggesting possible additional loci and mechanisms for congenital dRTA. We cannot rule out the possibility that our clinical and metabolic findings can be related to other putative loci that modify urinary acidification. Nephrolithiasis or nephrocalcinosis are common in subjects with dRTA (4, 8, 9, 66) due to the combination of alkaline urine and hypocitraturia and, to some extent, hypercalciuria (8, 37, 39, 40), although abnormalities in stone inhibitors, such as glycosaminoglycans and nephrocalcin, have also been proposed (36). These urinary abnormalities promote all calcium stones but, in particular, calcium phosphate stones, which is compatible with human population data that show increased prevalence of renal acidification defect in calcium phosphate stone formers (22, 45, 46).

Although there is universal agreement that calcium stones are common in dRTA, the consensus is that dRTA is a rare cause of kidney stones. However, decades of patient data have provided unequivocal evidence for impaired renal acidification in the general stone-forming population without frank Mendelian dRTA. In 7 studies (12, 38, 41, 42, 59, 61, 70) amounting to >1,000 stone formers, abnormalities in urinary acidification have been found in an average of 17% (range: 6–26%) of all-comers. Whereas the first stone suffer may have a <10% chance of abnormal renal acidification (43), in recurrent stone formers, the frequency has been estimated from 7% to 100% in 7 studies totaling >200 patients (1, 2, 10, 34, 44, 54, 63). Abnormalities in acidification are also more common in bilateral stones, ranging in prevalence from 36% to 64% in a total of 228 patients (15, 44).

The clinical data suggest prevalent defective renal acidification even in the general stone-forming population and is staggeringly high in specific subgroups. In a small number of patients with the triple feature of bilateral recurrent calcium phosphate stones, Konnak and coworkers (30) found that all of them had defective renal acidification. Although these individuals may have acquired incomplete dRTA, they can also be heterozygous carriers of candidate genes. However, no genotypic data were presented in any of these studies mentioned above. In fact, based on the supposition, albeit unverified, that heterozygous carriers of the dRTA candidate genes are completely normal individuals, genetic causes for subtle defects in acidification in stone formers were likely never examined. If heterozygous careers of mutant candidate genes of dRTA harbor subclinical defects that elude routine clinical chemistry and confer a propensity for disease rather causing disease, then one will not succeed in recognizing inheritance patterns at all. There is a need for database of sequence variance in candidate genes for dRTA in the general stone-forming population.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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