Longitudinal study of urinary excretion of phosphate, calcium, and uric acid in mutant NHERF-1 null mice

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1Department of Medicine, 2Department of Physiology, University of Maryland School of Medicine, and 3Medical Service, Department of Veterans Affairs Medical Center, Baltimore; 4Harbor Hospital Center, Brooklyn, Maryland; and 5Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina

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Weinman, Edward J., Viresh Mohanlal, Nicholas Stoycheff, Fengying Wang, Deborah Steplock, Shirish Shenolikar, and Rochelle Cunningham. Longitudinal study of urinary excretion of phosphate, calcium, and uric acid in mutant NHERF-1 null mice. Am J Physiol Renal Physiol 290:F838–F843, 2006. First published October 25, 2005; doi:10.1152/ajprenal.00374.2005.—NHERF-1 binds numerous renal protein targets, including the proximal tubule transporters Na+/H+ exchanger 3 (NHE3) and Na+-phosphate cotransporter 2a (Npt2a). Young NHERF-1−/− male mice display defective targeting of Npt2a to apical membranes in the renal proximal tubule and manifest hypophosphatemia and increased urinary excretion of phosphate. The present studies describe the changes in the urinary excretion of phosphate, calcium, uric acid, and sodium in male and female wild-type and NHERF-1 null mice over a time period from 12 to 54 wk of age. Young male and female NHERF-1−/− mice demonstrated increased urinary excretion of phosphate and urine phosphate/creatinine ratios. There was an age-related decline in the phosphate/creatinine ratio in mutant mice such that there were no differences between wild-type and NHERF-1−/− by 24 to 30 wk of age despite the continued presence of hypophosphatemia. Male and female NHERF-1 null mice also demonstrate increased urinary calcium/creatinine and uric acid/creatinine ratios compared with wild-type controls. These studies indicate defects in the renal tubule transport of phosphate, calcium, and uric acid in NHERF-1−/− male and female mice that could account for the increased deposition of calcium in the papilla of null mice.

bone density and mineralization; hypophosphatemia; electrolyte excretion

INACTIVATION OF THE GENE encoding the sodium-phosphate co-transporter 2a (Npt2a, NaPi IIa) results in increased urinary excretion of phosphate and calcium and a striking bone phenotype characterized by decreased bone density and mineralization (2). Interestingly, by mechanisms not well understood, the changes in bone resolve as the animals age. It has not been established, however, whether the changes in mineral excretion persist. Like the Npt2a null mouse, targeted gene deletion of the sodium-hydrogen exchanger regulator factor-1 (NHERF-1) results in animals with increased urinary excretion of phosphate and calcium (14). The present experiments were undertaken to determine whether there were age-related changes in the urinary excretion of phosphate, calcium, uric acid, and sodium in NHERF-1−/− male and female animals. The results indicate that young male and female NHERF-1 null mice have increased urinary excretion of phosphate but that there is an age-related partial correction in the defect in phosphate transport. Both male and female NHERF-1 null mice also have increased urinary excretion of calcium and uric acid but, in contrast to the defect in phosphate excretion, the abnormalities persist throughout the period of observation up to 1 yr of age. The transient as well as the persistent changes in urinary mineral and electrolyte excretion in these mice predict urine prone to the formation of calcium and/or uric acid stones. Analysis of the mutant mice confirmed these predictions, thereby highlighting a potential mechanism for the formation of renal stones in the hypophosphaticemic animals.

MATERIALS AND METHODS

Male and female NHERF-1 (−/−) mice that had been bred into a C57BL/6 background for six generations and parental wild-type male and female inbred control C57BL/6 mice were studied from age 12 to 54 wk (14). Throughout the study period, the animals were fed a normal rodent chow diet and had free access to water. At ~6-wk intervals, animals were individually housed in a metabolic cage for 48 h. After a 24-h period of acclimatization, 24-h urine collections were obtained. At the end of the study period, the animals were killed, blood was collected, and kidneys were harvested for von Kossa staining. Blood sodium, potassium, calcium, and phosphate concentrations, and urine concentrations of creatinine, calcium, uric acid, and phosphate were determined using an autoanalyzer.

In separate groups of animals (9–12 wk of age) maintained under the same conditions, blood was collected for determination of 1,25(OH)2-vitamin D levels and parathyroid hormone (PTH) concentrations. 1,25 (OH)2 vitamin D was assayed by RIA using a125I-1,25 (OH)2 vitamin D assay kit. Mouse PTH was assayed by ELISA. Von Kossa staining for mineral in 54-wk-old kidneys was performed using coronally spliced kidneys immersed in 10% neutral buffered formalin and dehydrated with 70% ethanol (3). Seven-micrometer sections from paraffin-embedded kidneys were cut followed by application of 5% silver nitrate and exposure to UV light for 30 min. Data are expressed as means ± SE and were analyzed using the Student’s t-test.

RESULTS

A cohort of male and female wild-type and NHERF-1 null mice was studied from age 12 wk to ~1 yr. At the time of death, wild-type and NHERF-1−/− males weighed 40.1 ± 2.8 and 39.2 ± 2.2 g, respectively [P = not significant (NS)]; and females 26.7 ± 1.6 and 26.8 ± 0.8 g, respectively (P = NS). It was not feasible to monitor food intake, but absolute sodium...
excretion was monitored as a surrogate measure. In the final urine collections at 54 wk of age, the urinary excretion of sodium averaged 221 ± 85 and 257 ± 59 μeq/24 h in wild-type and NHERF-1−/− males (P = NS) and 255 ± 75 and 229 ± 61 μeq/24 h in wild-type and NHERF-1−/− females (P = NS). Moreover, as shown in Table 1 and Fig. 1D, there were no significant differences in the urine sodium/creatinine ratio between wild-type and NHERF-1−/− mice of either gender over the time course of these observations.

The ratios of the urine concentration of phosphate, calcium, uric acid, and sodium to creatinine are summarized in Table 1 and the trends are shown graphically in Fig. 1. The urine phosphate/creatinine ratio was significantly higher in young male and female NHERF-1−/− mice compared with wild-type controls. In animals 12 to 14 wk of age, the urinary excretion of phosphate averaged 1.4 ± 0.5 and 3.7 ± 0.6 mg/24 h in wild-type and NHERF-1−/− male mice (P < 0.05) and 1.0 ± 0.3 and 3.1 ± 0.6 mg/24 h in wild-type and NHERF-1−/− female mice (P < 0.05). There was an age-related decline in the urine phosphate/creatinine ratio in both male and female NHERF-1 null mice such that by the age of 30 wk, the urine phosphate/creatinine ratio was not significantly different from wild-type mice. At the time of death at age 54 wk (Table 2), serum phosphate concentrations averaged 7.2 ± 0.2 and 6.2 ± 0.3 mg/dl in wild-type and NHERF-1−/− male animals respectively (P < 0.05), and 7.0 ± 0.4 and 6.0 ± 0.1 mg/dl in wild-type and NHERF-1−/− female animals, respectively (P < 0.05). In the final urine collections at 54 wk of age, the urinary excretion of phosphate averaged 2.1 ± 0.6 and 3.22 ± 0.18 mg/24 h in wild-type and NHERF-1−/− male mice and 2.2 ± 0.2 and 3.2 ± 0.5 mg/24 h in wild-type and NHERF-1−/− female mice. Although the urinary excretion of phosphate tended to be higher in both male and female NHERF-1 null animals, the differences were not statistically significant.

The urine calcium/creatinine ratio was significantly higher in male and female NHERF-1−/− animals compared with wild-type control mice. By contrast to phosphate, the increased calcium excretion showed no tendency to correct with age and remained significantly higher in NHERF-1 null mice up to a period of 1 yr. In the final urine collections at 54 wk of age, the urinary excretion of calcium averaged 0.07 ± 0.03 and 0.24 ± 0.06 mg/24 h in wild-type and NHERF-1−/− males (P < 0.05) and 0.07 ± 0.03 and 0.23 ± 0.05 mg/24 h in wild-type and NHERF-1−/− females (P < 0.05). The increase in calcium and phosphate excretion in the younger mice raised the possibility of hyperparathyroidism. Accordingly, serum PTH levels were determined in mice 9 to 12 wk of age (Table 3). There was a tendency for PTH to be lower in male and female NHERF-1−/− mice compared with wild-type controls but these differences failed to reach statistical significance. These data clearly show that the NHERF-1 null mice did not display hyperparathyroidism. We also measured 1,25 (OH)2 vitamin D concentrations in female mice. Due to the volume of serum required, samples from two to four animals were pooled for each assay. The 1,25 (OH)2 vitamin D concentrations were significantly higher in NHERF-1 null female mice (3.18 ± 0.63 pg/ml) compared with wild-type mice (10.1 ± 4.4 pg/ml; P < 0.05).

The renal transport of uric acid is mediated by several different organic anion transport proteins as well as members of the multidrug resistance protein and galectin family of proteins (7). While the interaction between potential urate transporters and renal PDZ proteins has not been completely investigated, URAT1 was identified as a protein that interacted with the PDZ-containing protein, NHERF-3 (PDZK1) (4). Because NHERF-3 binds to and colocalizes with NHERF-1 at the apical membrane of renal proximal tubule cells, the urine uric acid/creatinine ratio was determined in wild-type and NHERF-1 null mice (5). In both male and female mutant mice,

Table 1. Ratios of the urine concentration of PO4, Ca, UA, and Na to creatinine as a function of age in WT and NHERF-1−/− (KO) male and female mice

<table>
<thead>
<tr>
<th></th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
<th>36</th>
<th>42</th>
<th>48</th>
<th>54</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTM</td>
<td>0.39±0.03</td>
<td>0.29±0.02</td>
<td>0.33±0.03</td>
<td>0.30±0.09</td>
<td>0.33±0.04</td>
<td>0.29±0.04</td>
<td>0.23±0.02</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td>KOM</td>
<td>0.65±0.07*</td>
<td>0.51±0.05*</td>
<td>0.59±0.08*</td>
<td>0.47±0.03*</td>
<td>0.52±0.05*</td>
<td>0.55±0.11*</td>
<td>0.61±0.10*</td>
<td>0.57±0.07*</td>
</tr>
<tr>
<td>WFTF</td>
<td>0.42±0.02</td>
<td>0.35±0.03</td>
<td>0.35±0.04</td>
<td>0.37±0.04</td>
<td>0.32±0.06</td>
<td>0.32±0.04</td>
<td>0.32±0.04</td>
<td>0.30±0.02</td>
</tr>
<tr>
<td>KOFR</td>
<td>0.75±0.10*</td>
<td>0.65±0.08*</td>
<td>0.64±0.09*</td>
<td>0.64±0.08*</td>
<td>0.62±0.05*</td>
<td>0.63±0.04*</td>
<td>0.61±0.04*</td>
<td>0.60±0.05*</td>
</tr>
</tbody>
</table>

Results (means ± SE) are expressed as the ratio of the urine concentration of phosphate (PO4), calcium (Ca), uric acid (UA), or sodium (Na) to creatinine. All groups contained a minimum of 6 animals. Male (M) and female (F) wild-type (WT) and NHERF-1−/− (KO) mice were housed in metabolic cages for collection of urine at 6-wk intervals from age 12 to 54 wk. *P < 0.05 vs. animals of the same sex and age.
Fig. 1. Urine phosphate/creatinine (A), calcium/creatinine (B), uric acid/creatinine (C), and sodium/creatinine (D) ratio plotted as a function of age (in wk) in male and female wild-type (○) and NHERF-1^-/- (●) mice. The differences in urine phosphate/creatinine ratios between wild-type and NHERF-1^-/- males and females were statistically significant (P < 0.05) until week 24. The differences in calcium/creatinine and uric acid/creatinine ratios between wild-type and NHERF-1^-/- males and females were statistically significant throughout the study period. The differences in sodium/creatinine ratios were not statistically different.
DISCUSSION

The sodium-dependent phosphate cotransporter 2a (Npt2a, NaPi IIa) is expressed in the apical membrane of the renal proximal convoluted tubule and is responsible for the reabsorption of ~80% of the phosphate filtered at the glomerulus (9). Moreover, Npt2a is the major physiologically regulated phosphate transporter in the kidney and its abundance in the brush-border membrane (BBM) of the kidney is increased in response to restriction of the dietary intake of phosphate and decreased in response to PTH (9). Young Npt2a<sup>−/−</sup> mice are characterized by an increase in the urinary excretion of phosphate and calcium associated with increased serum 1,25(OH)<sub>2</sub> vitamin D levels and decreased PTH concentrations (2). Recent studies established that Npt2a, through a COOH-terminal PDZ-binding sequence, associates with a number of renal PDZ domain-containing proteins including NHERF-1 (6, 8). In the absence of NHERF-1, Npt2a fails to appropriately target to the BBM (8, 14). Like the Npt2a null mouse, young male NHERF-1<sup>−/−</sup> mice manifested increases in the urinary excretion of phosphate associated with mild hypophosphatemia and increases in the urinary excretion of calcium (14). The present studies examined the urinary excretion patterns of phosphate, calcium, and uric acid over time in male NHERF-1<sup>−/−</sup> mice and provide the first description of urinary mineral and electrolyte excretion in female NHERF-1<sup>−/−</sup> animals.

Young male and female NHERF-1<sup>−/−</sup> mice have increases in the urine excretion of phosphate and the phosphate/creatinine ratio compared with age-matched wild-type controls. There is, however, a progressive age-related decrease in the urine phosphate/creatinine ratio in both male and female NHERF-1 null animals such that by 6 mo of age, differences between wild-type and mutant mice are no longer evident. The pattern of change is similar in males and females. It is noteworthy, however, that despite the correction in the urine phosphate/creatinine ratio, both male and female null mice are mildly hypophosphatemic when tested at 1 yr of age. Moreover, the urinary excretion of phosphate in null mice age 54 wk is higher (albeit not statistically significantly) than age-

Table 2. Serum concentrations of Na, K, Cl, Ca, and PO<sub>4</sub>

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>Na, mM/l</th>
<th>K, mM/l</th>
<th>Cl, mM/l</th>
<th>Ca, mg/dl</th>
<th>PO&lt;sub&gt;4&lt;/sub&gt;, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTM 54</td>
<td>148±1</td>
<td>5.9±0.2</td>
<td>106±1</td>
<td>10.8±0.2</td>
<td>7.2±0.2</td>
</tr>
<tr>
<td>KOM 54</td>
<td>149±3</td>
<td>5.7±0.3</td>
<td>108±1</td>
<td>10.3±0.2</td>
<td>6.2±0.3*</td>
</tr>
<tr>
<td>WTF 54</td>
<td>149±2</td>
<td>5.8±0.3</td>
<td>110±3</td>
<td>9.9±0.2</td>
<td>7.0±0.4</td>
</tr>
<tr>
<td>KOF 54</td>
<td>149±1</td>
<td>5.1±0.2</td>
<td>112±1</td>
<td>10.0±0.1</td>
<td>6.0±0.1*</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE; n = 6 in all groups. *P < 0.05 vs. animals of the same sex and age. Serum concentrations of Na, K, Cl, Ca, and PO<sub>4</sub> in WT and NHERF-1<sup>−/−</sup> (KO) M and F animals.

Table 3. Parathyroid hormone and 1,25 (OH)<sub>2</sub> vitamin D concentrations

<table>
<thead>
<tr>
<th></th>
<th>WTM</th>
<th>KOM</th>
<th>WTF</th>
<th>KOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH</td>
<td>54.8±9.4 (7)</td>
<td>38.5±10.1 (7)</td>
<td>52.7±4.8 (6)</td>
<td>36.2±10.2 (8)</td>
</tr>
<tr>
<td>1,25 (OH)&lt;sub&gt;2&lt;/sub&gt; vitamin D</td>
<td>ND</td>
<td>ND</td>
<td>10.1±4.4 (4)</td>
<td>31.8±6.3 (6)*</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE. Numbers in parenthesis are the number of samples analyzed. Serum parathyroid hormone (PTH) (pg/ml) and 1,25(OH)<sub>2</sub> vitamin D (pg/ml) were determined in WT and NHERF-1<sup>−/−</sup> (KO) M and F animals 9–12 wk of age. *P < 0.05 vs. animals of the same sex and age. ND, test not performed.
matched controls. When considered together, these findings indicate that the urinary excretion of phosphate is appropriately high in these animals and that there are persistent alterations in the renal tubular transport of phosphate. These results may also indicate other adaptive responses not mediated by the NHERF-1 interaction with Npt2a.

Like the Npt2a null mice, both male and female NHERF-1−/− mice have increased urinary excretion of calcium compared with age-matched wild-type control mice. There is no tendency for the increased urinary excretion of calcium to correct over the 1 yr of observation. When tested at 9–12 wk, serum 1,25 (OH)2 vitamin D levels were higher and PTH levels tended to be lower in NHERF-1 null mice. This pattern is similar to the Npt2a null mice (2). It is suggested that the defective transport of phosphate in proximal tubule cells signals activation of the 1-hydroxylase enzyme that converts 25 OH vitamin D to the active 1,25 (OH)2 vitamin D form. As a consequence, gut reabsorption of calcium is stimulated and PTH secretion is suppressed. However, direct experimental evidence supporting the kidney as the sole source of the active vitamin D metabolite in this mouse model is still lacking and it is possible that extrarenal sites for vitamin D conversion are also activated by, as yet, unknown mechanisms. It is also possible that NHERF-1 directly affects renal transport proteins involved in renal calcium reabsorption given that NHERF-1 binds with an array of renal transporters and ion channels in addition to hormone receptors and signaling proteins (13). The major calcium channels in the kidney, TRPV5 and TRPV6, possess PDZ-binding sequences in their COOH termini and have the potential to bind NHERF-1 in vitro (Bindels R, personal communication). It has recently been reported that NHERF-2 binds to TRPV5 (10). The functional relationship between the TRPV5 and TRPV6 channels and their binding to NHERF-1 has not been fully explored, but in the distal nephron NHERF-1 and NHERF-2 are not expressed in the same cells (15, 16). A significant amount of calcium is reabsorbed in the thick ascending limb of Henle. Studies in rats, mice, and humans, however, have failed to localize NHERF-1 to this nephron segment (15–17). Thus, at the present time, it is uncertain whether the absence of NHERF-1 affects the activity or abundance of specific transporters and channels responsible for the renal tubular reabsorption of calcium.

The present studies also indicate an increase in the urinary excretion of uric acid in male and female NHERF-1 null mice and, like the increases in the urinary excretion of calcium, this defect persisted over the 1-yr time course of these studies. While the average uric acid/creatinine ratios were considerably higher in NHERF-1 null animals compared with controls, there was far more variability from animal to animal than observed for either calcium or phosphate. The reason for this variability is unknown at present. Uric acid transport in renal tubule cells involves the coordinated function of a number of anion exchangers and, possibly, members of the multidrug resistance protein and galactin families of proteins (7). Considerable interest has focused on URAT1 because the absence of this protein results in hypouricemia and increased urinary excretion of urate (4). URAT1 has a COOH-terminal PDZ binding motif, and recent studies have indicated that NHERF-3 is the major PDZ-binding partner of URAT1 (1). NHERF-3 is localized to the microvillar membrane of renal proximal tubule cells where it colocalizes with NHERF-1 (5). Moreover, NHERF-3 and NHERF-1 have the potential to bind one another (5). In the NHERF-1 null mouse, the abundance and cellular targeting of NHERF-3 appear to be the same as in wild-type mice but the potential interaction between NHERF-1 and NHERF-3 in the targeting and regulation of URAT1 and/or other urate transporters requires further investigation.

The urine sodium/creatinine ratios were monitored during the course of these experiments as sodium excretion affects the renal excretion of phosphate, calcium, and uric acid. Urinary sodium excretion was not significantly different between NHERF-1−/− and wild-type mice. The present experiments, then, provide the first longitudinal measurements of the urinary excretion patterns in male NHERF-1 null mice and the first documented excretion patterns in NHERF-1 null females. The increased excretion of phosphate in the young NHERF-1 null mice has been linked to the inability to effectively target Npt2a to the apical membrane of renal proximal tubule cells in the absence of NHERF-1 (14). However, our studies showed that the defects in the urinary excretion of phosphate corrected, at least in part, in both male and female NHERF-1−/− animals at ~6 mo of age. Currently, the mechanism of correction in phosphate excretion in the aging animals remains unknown but may involve other non-Npt2a mechanisms. Finally, we are also investigating the mechanisms underlying the persistent defects in the urinary excretion of calcium and uric acid observed in the NHERF-1 null mice. It is worth noting that the urinary excretion pattern seen in the NHERF-1 null animals, namely, increased excretion of calcium and uric acid, would predict urine prone to the formation of stones. Indeed, there was increased renal deposition of calcium that was particularly prominent in the papilla in the NHERF-1−/− kidneys compared with wild-type controls. It would be tempting to speculate that some forms of human nephrolithiasis are the consequence of NHERF-1 mutations in a manner analogous to stone formers with mutations in Npt2a (11, 12).

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