Sodium-Hydrogen Exchanger Regulatory Factor-1 Interacts with Mouse Urate Transporter 1 to Regulate Renal Proximal Tubule Uric Acid Transport

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Sodium-hydrogen exchanger regulatory factor-1-deficient (NHERF-1−/−) mice demonstrate increases in the urinary excretion of phosphate, calcium, and uric acid associated with interstitial deposition of calcium in the papilla of the kidney. These studies examine the role of NHERF-1 in the tubular reabsorption of uric acid and regulation of mouse urate transporter 1 (mURAT1), a newly described transporter that is responsible for the renal tubular reabsorption of uric acid. In primary cultures of mouse renal proximal tubule cells, uric acid uptake was significantly lower in NHERF-1−/− cells compared with wild-type cells over a large range of uric acid concentrations in the media. Western immunoblotting revealed a 56 ± 6% decrease in the brush border membrane (BBM) expression of mURAT1 in NHERF-1−/− compared with wild-type control kidneys (P < 0.05). Confocal microscopy confirmed the reduced apical membrane expression of mURAT1 in NHERF-1−/− kidneys and demonstrated mislocalization of mURAT1 to intracellular vesicular structures. Para-aminohippurate significantly inhibited uric acid uptake in wild-type cells (41 ± 2%) compared with NHERF-1−/− cells (8.2 ± 3%). Infection of NHERF-1−/− cells with adenovirus–green fluorescence protein–NHERF-1 resulted in significantly higher rates of uric acid transport (15.4 ± 1.1 pmol/μg protein per 30 min) compared with null cells that were infected with control adenovirus–green fluorescence protein (7.9 ± 0.3) and restoration of the inhibitory effect of para-aminohippurate (% inhibition 34 ± 4%). These findings indicate that NHERF-1 exerts a significant effect on the renal tubular reabsorption of uric acid in the mouse by modulating the BBM abundance of mURAT1 and possibly other BBM uric acid transporters.

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The sodium-hydrogen exchanger regulatory factor (NHERF) proteins represent a family of proteins that are abundantly expressed in the apical membranes of transporting epithelia, such as the renal proximal convoluted tubule and small intestine. These adaptor proteins have multiple PSD-95/Dlg/ZO-1 (PDZ) protein interactive domains and, in the case of NHERF-1 and NHERF-2, a C-terminal domain that binds to ezrin, radixin, moesin, and merlin (1–4). In mice, inactivation of the NHERF-1 gene, the founding member of this family, results in increased urinary excretion of phosphate as a result of decreased membrane abundance of the sodium-dependent phosphate co-transporter 2a (Npt2a), hypercalciuria as a result of the reciprocal changes in the plasma concentrations of 1,25 (OH)2 vitamin D, and the interstitial deposition of calcium primarily in the papilla of the kidney (5,6). We also observed that both male and female NHERF-1−/− mice have an increase in the urinary excretion of uric acid, the mechanism of which is unknown (6).

Accordingly, these experiments were designed to study the relation between NHERF-1 and the renal transport of uric acid. In cultured proximal tubule cells, we demonstrate a decrease in the uptake of uric acid NHERF-1 null cells. This was associated with a decrease in the brush border membrane (BBM) abundance of mURAT1 (7.8). Although mouse URAT1 (mURAT1) does not transport para-aminohippurate (PAH), our studies indicate that uric acid transport was inhibited by PAH in cultured wild-type proximal tubule cells but not in NHERF-1 null cells. Rescue of NHERF-1 null cells using adenovirus-mediated gene transfer significantly increased uric acid transport and restored the inhibitory effect of PAH. When considered together, these findings indicate an important role for NHERF-1 in regulating uric acid transport in renal proximal tubule cells and provide evidence that NHERF-1 interacts with mURAT1 as well as other uric acid transporters.

Materials and Methods

Male NHERF-1−/− mice (F.129-Slc9a3r1tmSsl/Ssl) that were bred into a C57BL/6 background for six generations and parental wild-type...
inbred control C57BL/6 mice that were aged 12 to 16 wk were used in these experiments (5). The Institutional Animal Care and Use Committee at the University of Maryland, School of Medicine, approved all animal protocols and procedures. Primary cultures of renal proximal tubule cells were prepared as described previously (9). Briefly, kidneys were harvested and the cortices were dissected, minced, and digested using 1% collagenase type II (Worthington, Biochemical Corp., Lakewood, NJ) and 0.025% soy bean trypsin inhibitor. The cells were sedimented on 45% Percoll, and the layer that contained proximal tubule cells was aspirated. After washing several times with DMEM/F-12 medium to remove the Percoll, the cells were resuspended and plated on Matrigel-coated cell culture dishes in DMEM/F-12 medium that contained 50 U/ml penicillin, 50 µg/ml streptomycin, 10 ng/ml EGF, 0.5 µM hydrocortisone, 0.87 µM bovine insulin, 50 µM prostaglandin E1, 50 mM sodium selenite, 50 µg/ml human transferrin, and 5 µM 3,3',5-triiodo-l-thyronine. The cells were grown at 37°C in 5% CO2 until confluent.

The cellular accumulation of 14C-uric acid at 30 min at room temperature was determined as a measure of uric acid uptake. The transport medium contained (in mM) 137 NaCl, 5 KCl, 0.1 K2HPO4, 1 CaCl2, 1.8 MgSO4, 20 glucose, and 10 HEPES (pH 7.4). The uptake was terminated by washing the cells with ice-cold transport medium that contained 1 mM probenecid. The cells were then solubilized in 1% Triton X-100 for 30 min of centrifugation at 10,000 g. The supernatant was separated by 8% SDS-PAGE, transferred to nitrocellulose. Western immunoblotting was performed using previously characterized antibodies specific for mURAT1 (8). Individual bands were quantified using laser densitometry, and measurements were corrected for minor differences in protein loading by averaging six constant bands on Ponseau S–stained gels. Perfusion-fixed kidneys were used for immunohistochemical analyses as described previously (13). mURAT1 antibody at a concentration of 10 µg/ml and goat anti-rabbit secondary antibody coupled to Alexa 488 dyes (Molecular Probes, Eugene, OR) diluted 1:200 were used to localize mURAT1.

Membrane preparations from the cultured cells were obtained as described previously (9). In brief, cells were detached by scraping and resuspended in 1.5 ml of buffer that contained 50 mM Tris (pH 7.4), 0.1 mM EDTA, 0.1% β-mercaptoethanol, and Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). The cells were then disrupted by three 20-s pulses with a probe sonicator followed by 10 min of centrifugation at 1000 × g to remove large particulates and ultracentrifuged for 1 h at 100,000 × g. The pellet was resuspended in 0.1% (wt/vol) SDS and prepared for electrophoresis by the addition of Laemmli buffer. Western immunoblotting was performed using antibodies specific for mURAT1. Protein concentrations were determined, and the gel lanes were loaded equally (generally, 5 to 10 µg/lane). Individual bands were quantified using laser densitometry, and the measurements were corrected for minor differences in protein loading as described.

Co-immunoprecipitation of mURAT1 and NHERF-1 was performed using kidney cortex lysates. Mouse kidneys were harvested and placed in ice-cold IP buffer (100 mM NaCl, 20 mM sodium phosphate buffer [pH 7.4], and a cocktail of protease inhibitors; Roche 836153). The capsule was removed, and the cortex was dissected and homogenized in iced IP buffer. After homogenization, Triton X-100 was added to a final concentration 1% (IP+ Buffer). The homogenate was sheered with a 1-ml tuberculin syringe and placed on a rotary mixer for 30 min at 4°C to complete lysis. Samples were centrifuged at 12,000 × g for 30 min to remove insoluble fractions, and the supernatants were transferred to a new tube that contained washed Sepharose-A CL-4B beads for preclearing for 2 h. After removal of the beads, 15 ul of mURAT1 or NHERF-1 polyclonal antibodies was added to the supernatant and incubated overnight at 4°C on the rotary mixer. mURAT1 and NHERF-1 and associated proteins were immunoprecipitated using Protein A Sepharose CL4B beads freshly prepared in IP+ Buffer. The beads were washed three times with IP buffer by centrifugation at <3000 × g for 1 min, after which an equal volume of 2× Laemmli sample buffer was added and boiled for 5 min. The beads were removed, and the supernatant was separated by 8% SDS-PAGE, transferred to nitrocellulose membranes, and probed with mURAT1 and NHERF-1 antibodies.

Real-time PCR using appropriate primers was used to quantify URAT1 mRNA. Total RNA from kidney cortex was extracted using RNaqueous-4PCR Kit (Ambion, Austin, TX). First-strand cDNA was purified from the RNA using Invitrogen’s SuperScriptIII First Strand

Figure 1. The uptake of 1500 µM uric acid was measured as function of time. Data are means ± SEM of four to eight determinations.
Synthesis System for RT-PCR (Carlsbad, CA) followed by real-time PCR using the iCycler with SYBR Green Supermix (Bio-Rad, Hercules, CA). Reaction conditions were as follows: 95°C for 3 min followed by 40 cycles of amplification (95°C for 30 s, 62°C for 60 s, and 72°C for 60 s) with final extension at 72°C for 3 min followed by melting curve analysis to verify specificity of the primers. Reverse transcription–PCR (RT-PCR) products were verified for correct size by fractionation on 1% agarose gels. The sense and antisense primers for mURAT1 were 5′-agaacctgtaagctggtgctcag-3′ and 5′-ggcctcagcgcacacg-3′, respectively. The internal control was m18S rRNA using the sense primer 5′-gcaattatccacctgaagc-3′ and the antisense primer 5′-gcctcacaacaactc-3′. Relative expressions were calculated according to the IQ-cycler manual.

Statistical Analyses
Values are presented as mean of means ± SEM. Statistical comparisons were performed using Peritz ANOVA (14).

Results
Our previous studies indicated that NHERF-1−/− mice have a significant increase in the urinary excretion of uric acid compared with wild-type control animals (6). To study the role of NHERF-1 on uric acid uptake in renal proximal tubule cells, we prepared primary cultures of proximal tubule cells using wild-type and NHERF-1 null mouse kidneys. As shown in Figure 2, the uptake of 14C-uric acid in NHERF-1 null renal proximal tubules was significantly lower than in wild-type cells over a range of media concentrations of uric acid from 1500 to 3500 μmol. The renal transport of uric acid in renal cells seems to involve several transporters acting in concert (15). URAT1 was recently identified as a major protein responsible for the transport of uric acid across the apical membrane of renal proximal tubule cells (7,8). For determination of whether mURAT1 interacts with NHERF-1, mURAT1 was immunoprecipitated from lysates from wild-type mice kidneys (left), and the precipitates were immunoblotted for NHERF-1 and mURAT1. The reverse experiment is shown on the right. Molecular weight markers (kD) are shown.

Figure 2. The relation between uric acid uptake as a function of the media concentration of uric acid in primary cultures of renal proximal convoluted tubule cells from wild-type (□) and sodium-hydrogen exchanger regulatory factor-1–deficient (NHERF-1−/−) mice (○). Data are means ± SEM of four to 11 determinations. *P < 0.05.

Figure 3. Mouse urate transporter 1 (mURAT1) was immunoprecipitated from lysates from wild-type mice kidneys (left), and the precipitates were immunoblotted for NHERF-1 and mURAT1. The reverse experiment is shown on the right. Molecular weight markers (kD) are shown.

We next determined the profile of selected drugs and organic anions that are known to inhibit uric acid transport. In these experiments, the uptake of 1500 μM uric acid in the absence of inhibitors averaged 5.6 ± 0.2 pmol/μg protein per 30 min in wild-type cells and 3.7 ± 0.3 in NHERF-1−/− cells. As summarized in Table 1, probenecid, pyrazinamide, and benz bromarone significantly inhibited the uptake of uric acid in cultured
effect of PAH was nearly obliterated in NHERF-1−/− proximal tubule cells. These findings suggest the possibility that NHERF-1 affects the activity and/or BBM abundance of other uric acid transporters in addition to mURAT1.

To pursue this question and to determine whether the abnormalities in uric acid transport in NHERF-1 null proximal tubule cells were the result of the absence of the protein rather than the consequence of systemic factors that inhibit uric acid transport or developmental defects, we infected null cells with control adenovirus-GFP or adenovirus-GFP-NHERF-1. As summarized in Figure 6, the plasma membrane abundance of mURAT1 was significantly higher in NHERF-1−/− cells that were infected with adenovirus-GFP-NHERF-1 compared with adenovirus-GFP–infected cells (n = 4; P < 0.05). There was no significant difference in total mURAT1 measured in whole-cell homogenates between adenovirus-GFP and adenovirus-GFP-NHERF-1 cells. We next measured uric acid uptake at a media concentration of 3500 μM and also examined the effect of PAH. As summarized in Table 2, basal uric acid uptake averaged 7.9 ± 0.3 pmol/μg protein per 30 min in NHERF-1 null cells that were infected with control adenovirus-GFP and 15.4 ± 1.1 in cells that were infected with adenovirus-GFP-NHERF-1 (P < 0.01). Whereas PAH did not significantly inhibit uric acid uptake in NHERF-1 null cells that were infected with adenovirus-GFP (% inhibition 4.0 ± 2.9; NS), PAH significantly inhibited uric acid uptake by 34.0 ± 3.5% (P < 0.01) in adenovirus-GFP-NHERF-1–infected cells. Thus, infection of NHERF-1 null cells increased total uric acid uptake by 94 ± 9% (P < 0.05) and the PAH-resistant uptake of uric acid by 42 ± 6% (P < 0.05).

Discussion

We recently reported that NHERF-1−/− mice excrete increased amounts of uric acid compared with wild-type controls (6). These studies were initiated to define further the relation between this adaptor protein and uric acid excretion. It is generally agreed that uric acid undergoes bidirectional transport predominantly in the proximal convoluted tubule of the kidney (15). There is species variability in the net fluxes, however, with some animals, such as rabbit, demonstrating net secretion and others, such as human and rodents, exhibiting net reabsorption.

To study the role of NHERF-1 on uric acid transport by the kidney, we measured uric acid uptake in primary cultures of proximal tubule cells from the mouse. Although we could not exclude the possibility of a tracer effect, consequent to the conversion of intracellular uric acid to allantoin on initial rates of uptake, we measured uric acid uptake at 30 min, a time point that approximates an initial rate under the conditions of study. Our results indicated that there was a significantly lower uptake of uric acid in NHERF-1−/− cultured cells compared with wild-type controls, a difference that was particularly marked at higher media concentrations of uric acid. The importance of NHERF-1 in uric acid transport was supported by experiments in NHERF-1−/− cultured cells that were infected with adenovirus-GFP-NHERF-1. Uric acid transport was significantly higher in NHERF-1 null cells that expressed GFP-NHERF-1 than GFP alone. These findings indicate that the defect in uric acid uptake is not a consequence of developmental or the absence of NHERF-1.

Figure 4. Proteins from brush border membranes (BBM) and whole kidney lysates from wild-type (WT) and NHERF-1 null (KO) mice were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-mURAT1 antibody, and the intensities were quantified using laser densitometry. Representative immunoblots are shown. The bar graphs represent the average ± SEM of five experiments. The differences in the abundance of URAT1 in BBM but not kidney lysates between WT and NHERF-1−/− null mice was statistically significant (*P < 0.05).

Figure 5. Representative confocal image of proximal tubules from wild-type kidneys (left) and images from NHERF-1 null kidneys (middle and right) stained with anti-mURAT1.
The uptake media contained 1500 nM of wild-type and NHERF-1 transfections in an anion exchanger named URAT1 was recently identified as resistant protein and galectin family of proteins (16). Mutations in OAT proteins as well as members of the multidrug transporter (OAT) proteins as members of the multidrug resistance protein and galectin family of proteins (16). Mutations in an anion exchanger named URAT1 were recently identified in humans with hypouricemia and increased urinary excretion of uric acid, suggesting that URAT1 is the long-sought uric acid transporter in the kidney (7,17). Human URAT1 has a C-terminal sequence of T-Q-F and seems to bind specifically to NHERF-3 using cellular assays (18). The C-terminal sequence of mouse URAT1 is T-R-L, a preferred sequence for the PDZ I domain of NHERF-1 (8). Indeed, in these studies, NHERF-1 is detected in mURAT1 immunoprecipitates from lysates of wild-type kidneys, confirming the in vivo binding of these two proteins. Quantitative immunoblotting of renal BBM vesicles indicates decreased abundance of mURAT1 in NHERF-1 KO mice compared with wild-type controls but no differences in total mURAT1 as determined from analysis of whole-kidney lysates. Laser confocal microscopy of kidneys confirmed the decrease in the apical membrane abundance of mURAT1 in NHERF-1 null kidneys and, in some cells, the detection of mURAT1 in submicrovillar vesicular structures in a pattern reminiscent of the cellular distribution of Npt2a in NHERF-1 KO kidneys (7). In cultured NHERF-1 KO proximal tubule cells, infection with adenovirus-GFP-NHERF-1 increased not only uric acid uptake but also the relative abundance of mURAT1 in the plasma membranes of these cells.

NHERF-1, in addition to binding to transporters, ion channels, hormone receptors, and signaling proteins, interacts with transcription factors, including TAZ, SRY-1, and β-catenin (3,4). To study the possibility that NHERF-1 regulates the mRNA for mURAT1, we performed quantitative RT-PCR in wild-type and NHERF-1 null kidneys. We detected no differences in mURAT1 mRNA relative to ribosomal 18S RNA, indicating that the effect of NHERF-1 on mURAT1 is posttranscriptional. In another mouse line in which the NHERF-1 gene was inactivated, there were significant structural alterations in gut epithelia, including malformation of microvilli (19). Analyses of renal epithelia in our NHERF-1 null mice, however, failed to show similar structural alterations (13). Moreover, although our mice demonstrate defects in the abundance of Npt2a and defective regulation of NHE3, there is not a generalized defect in proximal tubule function (5,11). When considered together, these findings indicate an important role for NHERF-1 in determining the cellular distribution of mURAT1, although the mechanism of this effect is unknown. NHERF-1 may act as a chaperone in a manner analogous to the adaptor protein CAL and CFTR, as a membrane retention signal to stabilize mURAT1 in the plasma membranes as postulated for the interaction between NHERF-1 and...
pyrazinamide, and benzbromarone was significantly less in decreases in uric acid transport in response to probenecid, take in cultured proximal tubule cells from wild-type mice. The was the finding that PAH significantly inhibited uric acid up-
mURAT1-expressing oocytes (8). A major difference, however, the mechanism for uric acid uptake in the mouse, we examined the effect of several agents and organic anions that are known to inhibit uric acid transport. In wild-type cells, the inhibitory activity or abundance of other uric acid transporters in the kidney. The supposition also arises from the observation that uric acid transport mediated by mURAT1 expressed in frog oocytes is not inhibited by PAH (8). In rat BBM, conversely, PAH strongly inhibits uric acid uptake (10,20). To detail further the mechanism for uric acid uptake in the mouse, we examined the effect of several agents and organic anions that are known to inhibit uric acid transport. In wild-type cells, the inhibitory profile to the compounds tested, including probenecid, pyrazinamide, and benzbromarone, was similar to that reported in mURAT1-expressing oocytes (8). A major difference, however, was the finding that PAH significantly inhibited uric acid uptake in cultured proximal tubule cells from wild-type mice. The decreases in uric acid transport in response to probenecid, pyrazinamide, and benzbromarone was significantly less in magnitude in NHERF-1−/− proximal tubule cells compared with wild-type cells. By contrast, the inhibitory effect of PAH was almost abolished in NHERF-1 null proximal tubule cells. NHERF-1 null cells that were infected with adenovirus-GFP-NHERF-1 demonstrated a 94% increase in uric acid uptake and a 42% increase in the PAH-resistant component of uric acid uptake. If the mURAT1 transporter truly does not accept PAH, then these differential findings would be consistent with an effect of NHERF-1 on not only URAT1 but also other BBM uric acid transporters. OAT4, OAT5, and Npt1 are other anion transporters that contain C-terminal PDZ domain binding motifs. Very recent studies suggested that OAT4 may not be expressed or is expressed poorly in rodents and that OAT5 is its functional equivalent (21). By contrast to OAT4, these studies indicate that OAT5 does not transport PAH (21). Npt1 is a voltage-driven OAT that transports both PAH and uric acid, but its functional interaction with NHERF-1 has not been examined (22). Study of the potential role of these other uric acid transporters, unfortunately, is hampered because of the lack of appropriate reagents, and it seems that more specific antimouse antibodies to potential uric acid transporters will need to be developed to investigate the role of NHERF-1 in regulating uric acid transporters other than mURAT1.

### Table 2. The effect of infection of NHERF-1−/− cells with adenovirus-GFP or adenovirus-GFP-NHERF-1 on basal uric acid uptake and the inhibitory effect of PAH[a]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>−PAH</th>
<th>+PAH</th>
<th>% Inhibition by PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus-GFP</td>
<td>7</td>
<td>7.9 ± 0.3</td>
<td>7.6 ± 0.3</td>
<td>4.0 ± 2.9</td>
</tr>
<tr>
<td>Adenovirus-GFP-NHERF-1</td>
<td>7</td>
<td>15.4 ± 1.1</td>
<td>10.7 ± 0.8</td>
<td>34.0 ± 3.5</td>
</tr>
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</table>

[a]Effect of infection of cultured NHERF-1−/− proximal convoluted tubules cells with adenovirus–green fluorescence protein (GFP) or adenovirus-GFP-NHERF-1 on the uptake of uric acid (pmol/μg protein/30 min) was determined in the absence (−) or presence (+) of 1 mM PAH. The uptake media contained 3500 μM uric acid. Results are expressed as means ± SEM.

P < 0.05 versus cells infected with adenovirus-GFP.

P < 0.05 versus studies in the absence of PAH.

Npt2a, or as a determinant of the recycling of mURAT1 to the plasma membrane as documented for the relation between NHERF-1 and some G protein–coupled receptors such as the β2-adrenergic receptor (3,4).

Although there is a clear interaction between NHERF-1 and mURAT1, comparison of the changes in uric acid excretion in NHERF-1−/− mice with the alterations in the abundance of mURAT1 raises the possibility that NHERF-1 also affected the activity or abundance of other uric acid transporters in the kidney. We postulate that the demonstrated abnormalities in uric acid excretion in the NHERF-1 null mouse contribute to this process and that naturally occurring mutations in the NHERF genes could result in a phenotype that is prone to the development of kidney stones (25,26). Thus, NHERF-1 knockout mice could prove to be an invaluable experimental tool in dissecting the complex mechanisms of clinical nephrolithiasis.

### Conclusion

These studies indicate an important role for NHERF-1 in uric acid uptake in renal proximal tubules of mice and in determining the apical membrane abundance of mURAT1. When considered with our previous studies, these experiments also raise the possibility that NHERF-1 regulates other uric acid transporters in addition to mURAT1. These studies are of potential clinical relevance given that a significant percentage of people who form calcium oxalate stones have concurrent abnormalities in uric acid metabolism characterized by hyperuricosuria and/or hyperuricemia (23,24). Uric acid crystals have been demonstrated to act as a nidus for calcium oxalate stone formation. In addition, uric acid alters the solubility/precipitation product of calcium and oxalate in solution. The NHERF-1 null mouse excretes increased amounts of phosphate and calcium with significant deposition of calcium in the interstitium of papilla of the kidney (6). We postulate that the demonstrated abnormalities in uric acid excretion in the NHERF-1 null mouse could prove to be an invaluable experimental tool in dissecting the complex mechanisms of clinical nephrolithiasis.

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### Disclosures

None.
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