The epithelial sodium/proton exchanger, NHE3, is necessary for renal and intestinal calcium (re)absorption

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Pan W, Borovac J, Spicer Z, Hoenderop JG, Bindels RJ, Shull GE, Doschak MR, Cordat E, Alexander RT. The epithelial sodium/proton exchanger, NHE3, is necessary for renal and intestinal calcium (re)absorption. Am J Physiol Renal Physiol 302: F943–F956, 2012. First published September 21, 2011; doi:10.1152/ajprenal.00504.2010.—Passive paracellular proximal tubular (PT) and intestinal calcium (Ca2+/H+) fluxes have been linked to active sodium (re)absorption. Although the epithelial sodium/proton exchanger, NHE3, mediates apical sodium entry at both these sites, its role in Ca2+/H+ homeostasis remains unclear. We, therefore, set out to determine whether NHE3 is necessary for Ca2+/H+ (re)absorption from these epithelia by comparing Ca2+/H+ handling between wild-type and NHE3−/− mice. Serum Ca2+ and plasma parathyroid hormone levels were not different between groups. However, NHE3−/− mice had increased serum 1,25-dihydroxyvitamin D3. The fractional excretion of Ca2+ was also elevated in NHE3−/− mice. Paracellular Ca2+ flux across confluent monolayers of a PT cell culture model was increased by an osmotic gradient equivalent to that generated by NHE3 across the PT in vivo and by overexpression of NHE3. Ca2+ uptake after oral gavage and flux studies in Ussing chambers across duodenum of wild-type and NHE3−/− mice confirmed decreased Ca2+ absorption in NHE3−/− mice compared with wild-type mice. Consistent with this, intestinal calbindin-D9k, claudin-2, and claudin-15 mRNA expression was decreased. Microcomputed tomography analysis revealed a perturbation in bone mineralization. NHE3−/− mice had both decreased cortical bone mineral density and trabecular bone mass. Our results demonstrate significant alterations of Ca2+/H+ homeostasis in NHE3−/− mice and provide a molecular link between Na+ and Ca2+/H+ (re)absorption.

Ca2+/H+ homeostasis: paracellular transport

CALCIUM (Ca2+) homeostasis is maintained via the coordinated regulation of renal, intestinal, and bone physiology (39). Ingested Ca2+ is absorbed from the intestine into the blood, where it is either filtered by the glomerulus and reabsorbed along the course of the nephron or deposited into bone. Filtered Ca2+ that isn’t reabsorbed is lost in the urine. The excretion of urine with an inappropriately high amount of Ca2+ is referred to as hypercalciuria. This condition contributes to the development of osteoporosis and nephrolithiasis (49, 52), diseases of Ca2+ mishandling that have significant morbidity and socioeconomic impact.

The exact mechanism causing hypercalciuria is unknown. Both inappropriate intestinal uptake and failed renal tubular reabsorption of filtered Ca2+ have been implicated. Recently, Worcester and colleagues (50) demonstrated that in individuals with hypercalciuria the proximal tubule inappropriately failed to reabsorb Ca2+ filtered by the glomerulus. Ca2+ is reabsorbed from the proximal tubule via a passive paracellular process, with the active transcellular flux of sodium (Na+) serving as the driving force (35, 37). Under conditions of normal dietary Ca2+ content, the majority of ingested Ca2+ is absorbed from the small intestine by a similar process (28). There are two potential mechanisms whereby the active transcellular flux of Na+ could mediate passive paracellular Ca2+ absorption. One is via the removal of water that in turn concentrates luminal Ca2+, thereby generating a chemical gradient. The second mechanism utilizes water flux to drive Ca2+ flux via convection (10, 15, 44, 46, 51). This latter process is referred to as “solvent drag.” Regardless of the exact mechanism, both models suggest that the transporters facilitating transepithelial Na+ fluxes at these sites also control Ca2+ absorption.

The epithelial sodium/proton exchanger, NHE3, is principally expressed in the apical membrane of renal and intestinal epithelia (36). Its renal expression is predominantly in the proximal tubule (7). Intestinal expression occurs throughout the small and large intestine (36). In both tissues, it mediates significant transepithelial Na+ flux and consequently provides the osmotic driving force for water flux. Consistent with this, NHE3 null mice (NHE3−/−) display increased intestinal luminal water content, diarrhea, and exhibit reduced water flux across the proximal tubule (33, 43). This later defect results in a decreased glomerular filtration rate because of tubuloglomerular feedback (33). NHE3 is also known to be regulated by the calciotropic hormones, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] and parathyroid hormone (PTH) (4, 9, 17, 22, 23, 53). These observations infer a role for NHE3 in Ca2+ homeostasis, potentially by providing the driving force for passive paracellular Ca2+ flux. We, therefore, set out to assess whether NHE3 participates in Ca2+ homeostasis in this fashion.

MATERIALS AND METHODS

Cell culture studies. Opossum kidney (OK) cells were purchased from ATCC. Cells were grown in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Experiments were performed in the absence of antibiotics. Transepithelial electrical resistance (TEER) measurements were made daily after plating 3 × 105 cells on 12-mm Transwell permeable supports (Corning, Lowell, MA) using a MILLICELL-ERS instrument (Millipore, Billerica, MA). Ca2+ flux assays were performed 5 days after plating on 24-well inserts (Corning) when the cells had formed confluent monolayers. After washing the cells with PBS, they were incubated in radiation buffer: 10 mM HEPES, pH 7.4, 135 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and 10 mM glucose. The
volume of buffer in the apical compartment was 250 μl and in the basolateral compartment was 1,000 μl. First, a 10-μl sample was removed from the basolateral compartment for time 0. Next, the apical solution was replaced with an equal volume of radiation buffer that had been supplemented with 25 μCi/ml of 45Ca2+ (PerkinElmer, Boston, MA). Samples were then collected from the basolateral compartment 4, 6, 8, and 10 min later. The rate of flux was linear within this time course. A sample of the solution added to the apical compartment was also obtained, before addition, to assess total counts. Radioactivity of the samples was measured with a LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Brea, CA). Ca2+ flux was then calculated as the rate of 45Ca2+ appearance in the cold/basolateral side (cpm/min) divided by the specific activity of radioactivity in the hot side (cpm/mmol of Ca2+). This was then normalized to the surface area of the membrane. Ouabain octahydrate and ruthenium red were purchased from Sigma (Sigma-Aldrich Canada, Oakville, ON) and KB-R7943 mesylate from Tocris (Tocris Bioscience, Ellisville, MO). For experiments employing an osmotic gradient, alterations to the radiation buffer were as follows: the buffer in the apical compartment was constant and consisted of 10 mM HEPES, pH 7.4, 140 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, and 70 mM mannitol; the buffer in the basolateral compartment was as above; however, the appropriate amount of mannitol necessary to achieve an increased osmolarity of 12.40, or 100 mosmol/l was added in addition to the baseline amount. For experiments employing a Ca2+ concentration gradient, alterations to the buffer were as follows: the buffer in the basolateral compartment was constant and consisted of 10 mM HEPES, pH 7.4, 135 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, and 70 mM mannitol; the buffer in the apical compartment was either the same as the basolateral compartment (0 mM difference) or was the same except the concentration of CaCl2 was increased from 1.0 to 1.2 mM (0.2 mM difference).

OK cells were stably transfected with a rat NHE3 construct containing three sequential HA tags (YPYDVPDYAS) in the first extracellular loop (NHE33HA3) (1). Stable cell lines (OK-NHE33HA3) were selected by cloning via limiting dilution in the presence of 750 μg/ml G418 and screened by immunofluorescence of the HA-tagged NHE3 (1). As a control, in parallel, cell lines stably expressing the empty vector pcDNA3.1(+) were generated by the identical procedure. Paracellular 45Ca2+ flux studies of these cell lines were completed in identical fashion to those of wild-type OK cells as detailed above. For studies performed in the absence of sodium, both the apical and basolateral buffers consisted of 10 mM HEPES, pH 7.4, 140 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and 10 mM glucose.

**Generation and characterization of NHE3−/− mice.** Heterozygous NHE3+/− mice were generated as described elsewhere (43). The pairing of heterozygotes resulted in the generation of wild-type and NHE3−/− mice that were used for our experimental purposes. Genotyping was performed by PCR as described (11). Standard pelleted chow (PicoLab Rodent Diet 5053; 20% wt/wt protein, 4.5% wt/wt fat, 0.81% wt/wt calcium, 1.07% wt/wt potassium, 0.30% wt/wt sodium; and 2.2 IU/g vitamin D3) and drinking water were available ad libitum. Wild-type and NHE3−/− mice were housed in metabolic cages, between 7 and 8 wk of age, for 24 h at a time (n = 7/group). Kidney, duodenum, jejunum, and right hind limb bones were collected from wild-type and NHE3−/− mice after the metabolic cage studies. The tissue was immediately snap frozen in liquid nitrogen and then stored at −80°C until utilized. All experiments were performed in compliance with the animal ethics board at the University of Alberta, Health Sciences Section (protocol 576).

**Characterization of Ca2+ homeostasis.** Total Ca2+ in serum and urine was determined using a colorimetric assay kit (Quanitchrome Calcium Assay Kit, BioAsay System, Hayward, CA) per the manufacturer’s instructions. Blood-gas analysis was performed with the RAPIDPoint 400/405 System from Siemens (Siemens Healthcare Diagnostics, Tarrytown, NY).

Table 1. *Real-time PCR primers and probes*

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<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin-DOK (calb1)</td>
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<td>GCTTAGAGCTCGAGATGGAG</td>
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<tr>
<td>PMCA1b</td>
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<td>CAGCATTCTCCTGAGATGTTGAC</td>
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<td>Trpv5</td>
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<tr>
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<td>GAPDH</td>
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See text for definitions.
Diagnosics, Deerfield, IL). Serum and urine creatinine were determined with a Creatinine Parameter Assay Kit (R&D Systems, Minneapolis, MN), following the manufacturer’s protocol. Fractional excretion of Ca\(^{2+}\) was determined by dividing the product of urine Ca\(^{2+}\) and serum creatinine by the product of free plasma Ca\(^{2+}\) and urine creatinine. Urine osmolality was measured with an Advanced Osmometer (model 3D3, Advanced Instruments, Norwood, MA). Urine pH was assessed by dipstick (Chemstrip 10, Roche Diagnostics, Laval, PQ). The plasma intact PTH level was determined with a mouse Intact PTH ELISA kit (Immutopics International, San Clemente, CA), and serum 1, 25(OH)\(_2\)D\(_3\) concentrations were determined by a \(\gamma\)-\(\beta\) radioimmunoassy kit (Immunodiagnostics Systems, Fountain Hills, AZ) per the manufacturer’s instructions.

**Real-time quantitative PCR.** Total mRNA was isolated from kidney and duodenum using TRIzol Reagent (Invitrogen, Carlsbad, CA) per the manufacturer’s instructions. After treatment with DNaseI (Amp Grade; Invitrogen), 1 \(\mu\)g of RNA was reverse transcribed by Random Primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). The cDNA was subsequently used to determine calbindin-D\(_{28K}\) (S100g), calbindin-D\(_{9K}\), the plasma membrane Ca\(^{2+}\)-ATPase (PMCA1b, Atp2b1), the sodium/calcium exchanger, member1 (NCX1, Slc9a1), transient receptor potential potential 5 (TRPV5, Trpv5), transient receptor potential potential 6 (TRPV6, Trpv6), claudin-2, claudin-12, claudin-15, claudin-16, and claudin-19 mRNA levels. As an internal control mRNA levels of the housekeeping gene GAPDH were determined. Expression levels were quantified by PCR (qPCR) on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes were made by IDT (Integrated DNA Technologies, San Diego, CA) or ABI (Applied Biosystems). The sequences are listed in Table 1.

**Fig. 1.** Paracellular Ca\(^{2+}\) flux \((J_{Ca})\) across opossum kidney (OK) cells. \(^{45}\)Ca\(^{2+}\) flux is shown across confluent monolayers of OK cells grown on semiporous filters and treated with vehicle (Control), ouabain (100 \(\mu\)M), or in the absence of cells (No Cells; A) or vehicle (Control), the transient receptor potential (TRP) inhibitor ruthenium red (10 \(\mu\)M), or the sodium/calcium exchanger (NCX) inhibitor KB-R7943 (10 \(\mu\)M; B). \(^{45}\)Ca\(^{2+}\) flux across confluent monolayers of OK cells in the presence of a transepithelial concentration gradient (directed apical to basolateral) of the magnitude denoted on the \(x\)-axis. D: \(^{45}\)Ca\(^{2+}\) flux across confluent monolayers of OK cells in the presence of a transepithelial osmotic gradient (directed apical to basolateral) of the magnitude denoted on the \(x\)-axis. Error bars correspond to means \(\pm\) SE. For all conditions, \(n = 6\) and \(*P < 0.05\).
resulting blots were probed with mouse anti-HA antibody (HA.11 Clone 16B12, Covance, San Diego, CA) and then goat anti-mouse antibody conjugated with horseradish peroxidase before visualization as above.

**Immunohistochemistry.** Visualization of NHE3 exposed at the cell surface of OK-NHE3 \(^\text{vec}\) cells was accomplished essentially as previously described (1). In brief, confluent live cells were incubated at 4°C with mouse anti-HA (HA.11 Clone 16B12, Covance) in a buffer containing: 10 mM HEPES, pH 7.4, 135 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 10 mM glucose. They were then incubated with a secondary Dylight 549-conjugated donkey anti-mouse IgG antibody (Jackson Immunoresearch, West Grove, PA) in the above buffer, which also contained 4,6-diamidino-2-phenylindole (Invitrogen). The cells were then mounted on the stage of a spinning-disk confocal microscope (WaveFx, Quorum Technologies, Guelph, ON) set up on an Olympus IX-81 inverted stand (Olympus, Markham, ON), employing a \(\times60\) objective. Images were obtained with an EMCCD camera (Hamamatsu, Japan) driven by velocity 5.0.3 software.

TRPV5 protein expression was quantified by immunofluorescence as previously described (2). Staining of kidney sections for TRPV5 was performed on 5-μm cryosections of paraffin-paraformaldehyde-fixed kidney samples. Antigen retrieval was achieved by boiling the samples in a buffer containing 0.01 M Na-citrate titrated to pH 6.0 with citric acid. Sections were blocked in a buffer containing 0.1 M Tris·HCl (pH 7.6), 0.15 M NaCl, and 0.5% Blocking Reagent (PerkinElmer Life and Analytical Science, Shelton, CT) for 1 h at room temperature. Sections were stained with rabbit anti-CaT-2/ECAC1 (Alpha Diagnostic, San Antonio, TX), 1:200 in the blocking buffer overnight at 4°C. After washing with 0.1 M Tris·HCl (pH 7.6), 0.15 M NaCl, and 0.05% Tween 20, the sections were incubated with a secondary anti-rabbit biotin-conjugated antibody (Santa Cruz Biotechnology) at 1:2,000 in the wash buffer. Amplification of the signal was then completed with a TSA PLUS fluorescence systems kit (PerkinElmer Life and Analytical Science) per the manufacturer’s instructions. Images were obtained with a Zeiss fluorescence microscope equipped with a digital photo camera (Infinity 3, from Lumerana, Ottawa, ON). For semiquantitative determination of protein levels, images were analyzed with Image-Pro Plus 4.1 image analysis software (MediaCybernetics, Silver Spring, MD), and then protein levels were quantified as the mean of integrated optical density.

**In vivo 45Ca\(^{2+}\) absorption assay.** Intestinal Ca\(^{2+}\) absorption from wild-type and NHE3 \(^{-/-}\) mice was determined by measuring serum 45Ca\(^{2+}\) levels at time intervals post-oral gavage of a 45Ca\(^{2+}\)-containing solution essentially as described previously (2). Animals were anesthetized with ketamine (37.5 mg/kg) and xylazine (7.5 mg/kg) administered intraperitoneally, and their body temperature was maintained with warming lamps throughout the procedure. The solution used to measure Ca\(^{2+}\) absorption contained 125 mM NaCl, 0.1 mM CaCl\(_2\), 17 mM Tris, and 1.8 g/l dextrose and was enriched with 20 μCi 45CaCl\(_2\)/ml (PerkinElmer); 15 μl/g body wt of this solution was administered by oral gavage after which blood samples were obtained at 0, 1, 2, and 4 min. Five microliters of serum per time point was analyzed by a LS6500 multipurpose scintillation counter (Beckman Coulter). The change in the plasma Ca\(^{2+}\) concentration was calculated by 10.22±0.33.1 on April 3, 2017 http://ajprenal.physiology.org/ Downloaded from

**Fig. 2.** Paracellular Ca\(^{2+}\) flux across OK cells stably overexpressing epithelial sodium/proton exchanger (NHE3). A: immunoblot of whole cell lysate from OK cells stably expressing pcDNA 3.1(+) (vector) or NHE3 with an extracellular HA epitope (NHE3). B: representative images of the apical plane (XY) and from z-stacks (XZ) of OK cells stably expressing NHE3 with an extracellular epitope, immunolabeled live with anti-HA antibody (red). The nuclei are stained with 4,6-diamidino-2-phenylindole (DAPI; blue). C: 45Ca\(^{2+}\) flux across confluent monolayers of OK cells stably expressing pcDNA 3.1(+) (vector) or NHE3 (NHE3) grown on semipuraffin filters. D: 45Ca\(^{2+}\) flux across confluent monolayers of OK cells stably expressing pcDNA 3.1(+) (vector) or NHE3 (NHE3) in the presence or absence of Na\(^{+}\) (−Na\(^{+}\)). Error bars correspond to means ± SE. For all studies, \(n = 8\) and \(*P < 0.05\).
from the $^{45}$Ca$^{2+}$ content of the plasma samples and the specific activity of the $^{45}$Ca$^{2+}$ administered.

**Ussing chamber studies.** $^{45}$Ca$^{2+}$ flux across the duodenum of NHE3$^{+/+}$ and NHE3$^{-/-}$ mice was performed based on the method detailed elsewhere (15). Under pentobarbitone sodium anesthesia, the duodenum was dissected and the second centimeter was isolated, cut longitudinally, and then mounted in an Ussing chamber (EM-CSYS-2 system with P2300 chambers and P2303 sliders, all from Physiologic Instruments, San Diego, CA). The tissue was incubated with a solution that consisted of 118 mM NaCl, 3 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 23 mM NaHCO$_3$, 10 mM glucose, and 2 mM mannitol for 15 min before the experiments were performed. This solution and those used throughout the studies were continuously bubbled with 5% vol/vol CO$_2$/95% vol/vol O$_2$ and maintained at 37°C. Electrical recordings were performed with a pair of Ag/AgCl electrodes made with 3.0 M KCl in 1.5% wt/vol agar, and the potential difference was recorded via a preamplifier (model DVC-1000; World Precision Instruments, Sarasota, FL) connected to a PowerLab 8SP series (ADInstruments, NSW, Australia). Another pair of Ag/AgCl electrodes provides a short-circuit current ($I_{sc}$) that was measured by a PowerLab 8SP series, recorded with Chart 4.2.2 for Windows (AD-Instruments) and connected in series to a DVC-1000 current-generating unit. TEER was determined by solving for resistance in Ohm’s equation.

Unidirectional (mucosa-serosa) Ca$^{2+}$ fluxes were determined by exchanging the apical solution for a fresh solution of the same composition that had been spiked with 5 μCi/ml $^{45}$Ca$^{2+}$. Seven 50-μl samples were then collected from the basolateral compartment over time (at 0, 5, 10, 20, 30, 45, and 60 min) to ascertain the rate of Ca$^{2+}$ flux across the duodenum (the rate of $^{45}$Ca$^{2+}$ appearing in the basolateral compartment was linear over this time range). The calculations were performed employing the same equation as detailed above (see **Cell culture studies**). To ensure tissue integrity, at the end of each experiment TEER was again measured and compared with the initial tissue equilibrated value.

**Micro-computed tomography evaluation of tibial bone mass and mineral density.** The right tibial metaphysis from all animals were scanned using a Skyscan 1076 micro-computed tomography (CT) imager (Skyscan NV, Kontich, Belgium). Image projections were obtained at 18-μm resolution using an X-ray source voltage of 70 kVp and 139 mA, with beam filtration through a 1.0-mm Aluminum filter, with a 0.5° rotation step. Reconstruction was performed employing a modified Feldkamp back projection algorithm. The resulting raw image data were Gaussian filtered and globally thresholded at the fixed range of 0.0 – 0.0752 cross section-to-image conversion to extract the mineral phase. Using transverse image slices, trabecular bone was segmented from cortical bone using vendor-supplied analysis software (CT-Analyser, Skyscan NV) with semiautomated contouring.

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**Table 2. Blood-gas analysis**

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<th>pH</th>
<th>$P_{O_2}$, mmHg</th>
<th>$P_{CO_2}$, mmHg</th>
<th>HCO$_3^-$, mM</th>
<th>Hct, %</th>
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<td>20.0 ± 0.6</td>
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<td>KO (n = 7)</td>
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<td>59 ± 3</td>
<td>20.5 ± 1.0</td>
<td>40 ± 3</td>
<td>134 ± 12</td>
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</table>

Values are means ± SE. WT, wild-type; KO, knockout.
ing. Bridging of the metaplasmyal growth plate was used as the anatomic landmark for the proximal origin of trabecular bone. The selected region of interest spanned ~50 slices and was analyzed using morphometric software to determine trabecular bone volume ratio [bone volume/tissue volume (BV/TV)] and volumetric cortical bone mineral density (g/cm³), after calibration with known hydroxyapatite "phantoms." Serum osteocalcin concentration and urine C-terminal telopeptide of type I collagen (CTX-1) concentration were determined with kits following the manufacturer’s directions (both from Immunodiagnostic Systems).

Statistical analysis. Data are presented as means ± SE. ANOVA and Student’s t-test were carried out using Excel software (Microsoft, Santa Monica, CA). A P value of <0.05 was considered statistically significant.

RESULTS

An osmotic gradient, approximating that observed across the proximal tubule, is sufficient to drive paracellular Ca²⁺ flux. We employed the OK cell line to study paracellular Ca²⁺ flux in an in vitro model system resembling the proximal tubule. We choose this model, as it is known to have low-resistance tight junctions, which approximate those of the proximal tubule in vivo (31, 34). Consistent with this, TEER measurements demonstrated that by 5 days after plating OK cells on semipermeable filters they formed a tight junction with a resistance of 10.2 ± 1.5 Ω-cm². We proceeded to assess Ca²⁺ flux across confluent monolayers with the radiotracer ⁴⁵Ca²⁺. Flux across the filter itself was greatly reduced by the presence of OK cells (Fig. 1A). Neither 100 μM ouabain, 10 μM ruthenium red, nor 10 μM KB-R7943 altered this process (Fig. 1A, and B), confirming that the Na⁺/K⁺-ATPase, TRPV5/6, or NCX1, respectively, are not required for transepithelial Ca²⁺ flux. Thus in this model system the majority of Ca²⁺ flux occurs paracellularly.

Micropuncture experiments have identified a small but significant difference in Ca²⁺ concentration between ultrafiltrate and tubular fluid from late proximal tubular puncture sites (19, 48). At its maximum, this difference was measured to be 0.2 mM (48). We therefore imposed this concentration gradient across confluent monolayers of OK cells grown on semipermeable filters to assess whether this gradient could induce Ca²⁺ flux in our model system. We found that a Ca²⁺ concentration gradient of 0.2 mM, equivalent to that measured in vivo, was not sufficient to induce significant Ca²⁺ flux (Fig. 1C).

An osmotic gradient of ~12 mosmol/l has been observed across the proximal tubule of wild-type mice in vivo (47). Given that NHE3⁻/⁻ mice have significantly reduced proximal tubular water reabsorption, due to reduced Na⁺ flux, it follows that they also have a reduced osmotic gradient across the proximal tubule of ~40 mosmol/l (33, 47). Therefore, we measured Ca²⁺ flux after imposing altered osmotic gradients across confluent monolayers of OK cells. These experiments revealed that an osmotic gradient of only 12 mosmol/l (i.e., that which NHE3 contributes to the generation of in vivo) was sufficient to more than double paracellular Ca²⁺ flux compared with control conditions lacking an osmotic gradient (Fig. 1D). Overexpression of NHE3 increases Ca²⁺ flux. To explore the role of NHE3 in this proximal tubular cell culture model, we overexpressed NHE3 containing a triple HA tag in the first extracellular loop, NHE3-₃SH₃A₃, in OK cells (1). We were able to detect a single band of the appropriate molecular weight (~95 kDa) in whole cell lysate from the stable transfectants but not from a cell line overexpressing the empty vector (Fig. 2A). Immunostaining of the exofacial HA tag confirmed apical localization of NHE3 in this model system (Fig. 2B). Next, we performed ⁴⁵Ca²⁺ flux studies in the wild-type cells and observed a near doubling of Ca²⁺ flux across confluent monolayers of cells overexpressing NHE3 compared with cells expressing the vector only (Fig. 2C). This increased flux was due to NHE3 activity as confirmed by repeating the studies in the absence of Na⁺. The removal of Na⁺ from the Ca²⁺ flux medium prevented the increase in Ca²⁺ flux induced by the overexpression of NHE3 but had no effect on baseline Ca²⁺ flux in the vector-transfected control (Fig. 2D).

NHE3⁻/⁻ mice have normal serum Ca²⁺ but increased serum 1,25(OH)₂D₃ levels. To assess the role of NHE3 in vivo, we measured serum Ca²⁺ and calcitropic hormone levels in NHE3⁻/⁻ mice. To avoid the potentially confounding effect of metabolic acidosis, we performed these measurements in young animals between 7 and 8 wk of age (30). At this age, although the mice are smaller than wild-type animals (Fig. 3A), their blood gases are not significantly different (Table 2). Serum electrolytes were not different between wild-type and NHE3⁻/⁻ animals, including total and ionized Ca²⁺ levels (Fig. 3B and Table 3). Analysis of PTH levels revealed no statistically significant difference between groups; however, serum 1,25(OH)₂D₃ levels in the NHE3⁻/⁻ mice were signif-

Table 3. Serum electrolytes

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<th>Na⁺, mM</th>
<th>K⁺, mM</th>
<th>Cl⁻, mM</th>
<th>Intracellular Ca²⁺, mM</th>
<th>Glucose, mM</th>
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<td>WT (n = 12)</td>
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<td>5.1 ± 0.1</td>
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<td>1.26 ± 0.01</td>
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<td>5.2 ± 0.5</td>
<td>109 ± 0.9</td>
<td>1.25 ± 0.001</td>
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<td>3.1 ± 0.2</td>
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Values are means ± SE.

Table 4. Metabolic cage data

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<th>Chow Eaten, mg g⁻¹·24 h⁻¹</th>
<th>Urine Volume, ml/24 h</th>
<th>Urine Volume, μl g⁻¹·24 h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (n = 13)</td>
<td>22.5 ± 0.8</td>
<td>1,400 ± 290</td>
<td>55 ± 11</td>
<td>1.9 ± 0.1</td>
<td>77 ± 6</td>
<td>0.48 ± 0.07</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>KO (n = 8)</td>
<td>16.4 ± 1.0*</td>
<td>1,410 ± 250</td>
<td>73 ± 14</td>
<td>2.0 ± 0.1</td>
<td>105 ± 7*</td>
<td>0.41 ± 0.08</td>
<td>21 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 compared with WT.
Table 5. Urine analysis

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Osmolarity, mosmol/l</th>
<th>Ca2+, μmol/24 h</th>
<th>Ca2+ flux, nmol·g⁻¹·24 h⁻¹</th>
<th>Creatinine, μmol/24 h</th>
<th>Creatinine flux, nmol·g⁻¹·24 h⁻¹</th>
<th>Ca2+ /Creatinine ratio</th>
<th>FECa2++, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (n = 12)</td>
<td>5.2 ± 0.3</td>
<td>3990 ± 220</td>
<td>1.3 ± 0.2</td>
<td>48 ± 6</td>
<td>6.7 ± 0.6</td>
<td>273 ± 27</td>
<td>0.18 ± 0.01</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>KO (n = 9)</td>
<td>5.5 ± 0.5</td>
<td>3170 ± 250*</td>
<td>1.0 ± 0.2</td>
<td>75 ± 14*</td>
<td>3.2 ± 0.6*</td>
<td>216 ± 32*</td>
<td>0.35 ± 0.04*</td>
<td>19 ± 5*</td>
</tr>
</tbody>
</table>

Values are means ± SE. FECa2++, fractional excretion of Ca2+. *P < 0.05 compared with WT.

significantly increased by more than four times that of wild-type mice (Fig. 3, C and D).

NHE3⁻/⁻ mice have increased fractional excretion of Ca²⁺ and less concentrated urine. To assess whether Ca²⁺ filtered by the glomerulus was being reabsorbed along the nephron, we collected 24-h urine and measured serum and urine Ca²⁺ and creatinine (Tables 3, 4, 5, and 6). This enabled us to calculate the fractional excretion of calcium, which was significantly increased in the NHE3⁻/⁻ mice (Fig. 4A), consistent with decreased tubular reabsorption. An increased luminal collecting duct concentration of Ca²⁺ has been reported in rodents to acidify and dilute the urine (40–42). A failure to absorb Ca²⁺ from the proximal tubule, as we predict is happening in NHE3⁻/⁻ mice, was being reabsorbed along the nephron, we consequently stone formation in rodents (42). This occurs via decreased expression in NHE3⁻/⁻ mice (Fig. 5, A–C). Decreased luminal collecting duct Ca²⁺ concentration has been hypothesized to increase urine volume so as to prevent calcium phosphate supersaturation and consequently stone formation in rodents (42). This occurs via decreased aquaporin-2 expression (13, 18, 42). We therefore assessed the expression of aquaporin-2 in wild-type and NHE3⁻/⁻ mice and found decreased aquaporin-2 expression in the NHE3⁻/⁻ animals (Fig. 4, D and E).

NHE3⁻/⁻ mice have decreased intestinal Ca²⁺ absorption. We next evaluated intestinal Ca²⁺ handling. To this end, the expression of vitamin D-regulated genes, implicated in the transepithelial absorption of Ca²⁺, were measured. Employing quantitative real-time PCR (qPCR) we found no difference in the expression of TRPV5 and PMCA1b; however, calbindin-D₉K expression was significantly decreased in NHE3⁻/⁻ mice (Fig. 5, A–C). Decreased expression of calbindin-D₉K was confirmed at the protein level by semi quantitative Western blotting (Fig. 5, G and H). We also evaluated the expression of genes recently implicated in paracellular intestinal Ca²⁺ flux by qPCR (20). This revealed that jejunal claudin-2 and -15 expression was decreased, while claudin-12 expression was unaltered (Fig. 5, D–F). To determine the functional consequence of these findings, we measured serum ¹⁴CßCa²⁺ uptake, after oral gavage, from wild-type and NHE3⁻/⁻ mice. The knockout animals displayed significantly decreased Ca²⁺ absorption at 1 min after gavage, a difference that was not detectable at 2 and 4 min (Fig. 6A). To confirm the significance of these findings, we measured Ca²⁺ flux across isolated duodenum under conditions of voltage clamp in Ussing chambers. The potential difference and TEER across the duodenum of wild-type and NHE3⁻/⁻ mice were not different; however, NHE3⁻/⁻ mice display decreased duodenal Ca²⁺ flux (Fig. 6, B and C).

Renal expression of Ca²⁺-transporting genes is decreased in NHE3⁻/⁻ mice. The expression of 1,25(OH)₂D₃-sensitive Ca²⁺-transporting genes was also examined in the kidney. First, by qPCR we measured the expression of the transepithelial Ca²⁺-transporting genes TRPV5, cabindin-D₂₈K, NCX1, and PMCA1b. TRPV5 expression was decreased; however, there was no difference in the expression of the other genes between wild-type and NHE3⁻/⁻ mice (Fig. 7, A–D). We then analyzed TRPV5 protein expression by semiquantitative immunofluorescence microscopy and calbindin-D₂₈K expression by semiquantitative immunoblotting. Both proteins demonstrated decreased expression in NHE3⁻/⁻ mice (Fig. 7, G–J). Claudin-16 and -19 have been implicated in the paracellular reabsorption of Ca²⁺ from the thick ascending limb of Henle (26). Therefore, we assessed their expression by qPCR and found that claudin-16 expression was unaltered, while claudin-19 expression was decreased (Fig. 7, E and F).

NHE3⁻/⁻ mice are osteopenic. Finally, the effects of both decreased intestinal and renal tubular Ca²⁺ (re)absorption on bone health were ascertained by micro-CT analysis of right hindlimb bones from wild-type and NHE3⁻/⁻ mice. Figure 8 demonstrates that NHE3⁻/⁻ mice have thinner and smaller bones. NHE3⁻/⁻ mice were measured to have significantly reduced volumetric cortical bone mineral density (0.82 ± 0.07 g/cm³) compared with wild-type animals (0.93 ± 0.02 g/cm³) (Fig. 8, A and B). In terms of trabecular bone formation, NHE3⁻/⁻ mice also display a significantly reduced trabecular bone volume ratio (1.2 ± 0.6%) compared with wild-type mice (5.1 ± 1.9%) (Fig. 8, C and D). Additional measurements of trabecular bone morphometry by micro-CT confirmed that NHE3⁻/⁻ mice had significantly reduced trabecular thickness (mm) and trabecular number (1/mm), with a corresponding increase in trabecular spacing (mm) compared with wild-type mice. The trabecular structural model index indicated a transition from the plate-like architecture in the wild-type mice to a rod-like architecture for the NHE3⁻/⁻ mice (Table 7). Given the hypomineralized bones observed in the NHE3⁻/⁻ animals we sought to assess the mechanism leading to this. We there-

Table 6. Urine electrolytes

<table>
<thead>
<tr>
<th></th>
<th>Na⁺, μmol/24 h</th>
<th>K⁺, μmol/24 h</th>
<th>Cl⁻, μmol/24 h</th>
<th>Cl⁻ flux, nmol·g⁻¹·24 h⁻¹</th>
<th>PO₄²⁻, μmol/24 h</th>
<th>PO₄²⁻ flux, nmol·g⁻¹·24 h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (n = 10)</td>
<td>182 ± 33</td>
<td>7.9 ± 1.31</td>
<td>353 ± 66</td>
<td>15.2 ± 2.6</td>
<td>283 ± 47</td>
<td>12.3 ± 1.9</td>
</tr>
<tr>
<td>KO (n = 7)</td>
<td>21.7 ± 5.9*</td>
<td>1.1 ± 0.7*</td>
<td>116 ± 33*</td>
<td>6.9 ± 2.0*</td>
<td>161 ± 47</td>
<td>9.8 ± 3.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 compared with WT.
fore measured serum osteocalcin, a marker of bone formation, and urine CTX-1 concentration, a marker of bone resorption. Consistent with decreased bone formation and increased bone resorption, we observed increased serum osteocalcin and decreased urinary CTX-1 in the NHE3−/− mice relative to wild-type animals (Fig. 8, E and F).

DISCUSSION

We have provided evidence that NHE3 plays a critical role in Ca\(^{2+}\) homeostasis. These studies demonstrate that an osmotic gradient, of a similar magnitude to that observed across the proximal tubule and largely generated by NHE3 activity, is sufficient to drive paracellular Ca\(^{2+}\) flux. Furthermore, overexpression of NHE3 in a proximal tubular cell culture model doubles transepithelial Ca\(^{2+}\) flux. NHE3−/− mice were found to have normal serum Ca\(^{2+}\) and increased 1,25(OH)\(_2\)D\(_3\) levels. They also display reduced tubular Ca\(^{2+}\) reabsorption as evidenced by increased fractional excretion of Ca\(^{2+}\). Given the known expression of NHE3, this is likely due to decreased proximal tubular reabsorption. Despite increased 1,25(OH)\(_2\)D\(_3\) levels, NHE3−/− mice have unaltered intestinal TRPV6 and decreased calbindin-D\(_{9K}\) expression. qPCR analysis revealed that jejunal claudin-2 and -15 expression was reduced, while claudin-12 expression was unaltered. This likely contributes to decreased intestinal Ca\(^{2+}\) absorption and reduced duodenal Ca\(^{2+}\) flux in NHE3−/− mice. Surprisingly, in the presence of increased 1,25(OH)\(_2\)D\(_3\), renal mRNA expression of TRPV5 was decreased while expression of calbindin-D\(_{28K}\), NCX1, and PMCA1b was unaltered. The protein expression of TRPV5 and calbindin-D\(_{28K}\) was decreased. The renal expression of claudin-16 was unchanged, and claudin-19 expression was reduced. Hence, decreased distal tubular Ca\(^{2+}\) reabsorption likely also contributes to the increased fractional excretion of Ca\(^{2+}\) observed in the NHE3−/− mice. Ultimately, decreased renal tubular and intestinal Ca\(^{2+}\) absorption contributed to decreased cortical bone mineral density and trabecular bone volume in NHE3−/− animals as measured by micro-CT.

These findings provide a molecular link between Na\(^{+}\) and Ca\(^{2+}\) homeostasis. It has been appreciated that a high-salt diet induces hypercalciuria (16, 24). Similarly, restriction of salt intake is a first-line therapy for the treatment of kidney stones, in particular those that are the result of hypercalciuria. There is also a clinical association between salt intake, volume expansion, hypertension, and hypercalciuria (14, 45). However, to date, the molecular mechanism(s) underlying these findings has been incompletely appreciated. Our studies demonstrate that in the absence of NHE3, Ca\(^{2+}\) absorption from the small intestine and the renal tubule is greatly diminished.
Therefore, we have identified the epithelial sodium/proton exchanger as a potential molecular link between Na\(^+\)/H\(^+\) and Ca\(^{2+}\)/H\(^+\) homeostasis. Although decreased NHE3 activity may account for the hypercalciuria associated with volume expansion, it is important to note that NHE3\(^{-/-}\) animals are volume contracted and relatively hypotensive (43). Thus whether altered NHE3 activity accounts for hypercalciuria in volume-expanded and subsequently hypertensive individuals remains to be determined.

The majority, >90%, of ingested Ca\(^{2+}\) is reportedly absorbed from the small intestine (28). Three distinct processes have been described that mediate transepithelial Ca\(^{2+}\) flux. The best described occurs via an active transcellular process in the duodenum. It predominates in the presence of a low-Ca\(^{2+}\) diet. Luminal Ca\(^{2+}\) entry occurs, at least in part, via TRPV6 (6). Cytosolic Ca\(^{2+}\) is then buffered and transported to the basolateral membrane by calbindin-D\(_{9K}\), where efflux into the circulation occurs through PMCA1b. Under conditions of normal to high Ca\(^{2+}\) intake, absorption is via the paracellular pathway. The mechanism mediating this is either via the simple diffusion of Ca\(^{2+}\) down its electrochemical gradient or by a process known as solvent drag. This latter phenomenon involves the movement of water between epithelial cells, which “drags” Ca\(^{2+}\) with it.

We found that intestinal Ca\(^{2+}\) absorption and more specifically Ca\(^{2+}\) flux across the duodenum of NHE3\(^{-/-}\) mice was reduced (Fig. 6, A and B). Ussing chambers with equimolar Ca\(^{2+}\) on both sides of the epithelium under conditions of voltage clamping were used to make this latter determination. Thus reduced Ca\(^{2+}\) flux across the duodenum of the null mice is not a function of simply decreased passive diffusion. In the absence of NHE3, there is significantly reduced water and Na\(^+\) absorption from the intestine, which results in diarrhea (21). This absorptive defect likely accounts for the decreased body weight and consequently diminished creatinine production by the null mice (Tables 4 and 5). The cause of reduced intestinal Ca\(^{2+}\) absorption is therefore likely due to decreased solvent drag-mediated flux, although we cannot exclude a decreased concentration gradient for Ca\(^{2+}\) mediating part of this affect. We found decreased expression of calbindin-D\(_{9K}\) and therefore cannot exclude the transcellular pathway from contributing to the phenotype either. However, as the paracellular pathway predominates under conditions of normal to high Ca\(^{2+}\) intake, and the mice were fed a diet replete with Ca\(^{2+}\) (0.81%), we

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**Fig. 5.** Characterization of intestinal Ca\(^{2+}\) handling. Quantitative (q) PCR analysis of duodenal TRPV6 (A), calbindin-D\(_{9K}\) (B), and PMCA1b (C) and jejunal claudin-2 (CLDN2; D), claudin-12 (CLDN12; E), and claudin-15 (CLDN15; F) expression. The results are expressed as a percentage of WT and are normalized to the expression of GAPDH; \(n = \geq 7\) group. G and H: representative immunoblot (G) and quantification (H) of calbindin-D\(_{9K}\) protein expression from WT and NHE3\(^{-/-}\) (KO) mice duodenum; \(n = 6\) group. Note that \(\beta\)-actin was blotted (bottom) as a loading control. Error bars correspond to means ± SE. *\(P < 0.05\).
favor decreased paracellular transport as the mechanism mediating reduced calcium flux.

Renal tubular Ca\(^{2+}\) absorption is less well characterized. Ca\(^{2+}\) reabsorption from the distal convoluted tubule and connecting tubule occurs in a paracellular fashion. The molecular mechanism mediating this is analogous to the duodenum. Apical entry is mediated by TRPV5, buffering and shuttling of Ca\(^{2+}\) to the basolateral membrane by calbindin-D28K, and efflux by NCX1 and PMCA1b (25). Ca\(^{2+}\) reabsorption from the loop of Henle occurs in a passive paracellular fashion largely driven by the luminal positive potential generated through Na\(^{+}\) backflux into the lumen (27). Claudin-16 and -19 are essential for this (27). The majority of Ca\(^{2+}\) reabsorption (>60%) occurs from the proximal tubule via the paracellular pathway. This process is intimately dependent on Na\(^{+}\) absorption. NHE3\(^{-/-}\) mice have significantly reduced proximal tubular Na\(^{+}\) and water reabsorption (33, 43), and we observed greatly reduced renal tubular Ca\(^{2+}\) reabsorption. Given that NHE3 is predominantly expressed in the proximal tubule (8), decreased tubular Ca\(^{2+}\) reabsorption is likely the result of failed paracellular proximal tubular reabsorption. Consistent with an increased luminal collecting duct Ca\(^{2+}\) concentration, we observed decreased aquaporin-2 expression. As previously described, decreased aquaporin-2 membrane expression in the NHE3\(^{-/-}\) mice occurs in the presence of elevated vasopressin levels (3). The etiology of this paradoxical downregulation of aquaporin-2 has not been completely explained (3, 12). However, based on our results, it likely occurs via activation of the collecting duct calcium-sensing receptor, which is a consequence of increased Ca\(^{2+}\) delivery from the proximal nephron.

In the absence of detailed micropuncture measurements, we are unable to determine the exact amount and concentration of Ca\(^{2+}\) delivered to distal sites in the NHE3\(^{-/-}\) mice and therefore are unable to definitively prove this theory. Although interesting, this mechanism does not appear to play a significant role in preventing stone formation in hypercalciuric patients (5, 29).

Additionally, we observed decreased expression of Ca\(^{2+}\)-transporting proteins in the distal convoluted tubule and loop of Henle. Thus a reduction in these transporters may contribute to the Ca\(^{2+}\)-wasting phenotype. However, given the location of NHE3 expression, it is more likely that decreased Na\(^{+}\) and water flux from the proximal tubule results in decreased tubular Ca\(^{2+}\) absorption. Whether the mechanism mediating this is simply one of increasing luminal Ca\(^{2+}\) concentration (via Na\(^{+}\) and consequently water removal) or via solvent drag is not clearly differentiated with these studies. However, in our cell culture model, the imposition of a small concentration gradient similar in magnitude to the one measured across the proximal tubular epithelium in vivo was not sufficient to drive Ca\(^{2+}\) flux. In contrast, an osmotic gradient approximating that observed in vivo doubled paracellular Ca\(^{2+}\) flux. More specific studies will be needed to clearly determine which mechanism is more important in vivo.

Perhaps the most striking finding is the observation that, despite significantly increased 1,25(OH)\(_2\)D\(_3\) levels, NHE3\(^{-/-}\) mice have greatly reduced intestinal Ca\(^{2+}\) absorption. Moreover, multiple genes known to be upregulated by 1,25(OH)\(_2\)D\(_3\), including TRPV5, TRPV6, calbindin-D9K, and calbindin-D28K, have either no alteration in expression or decreased expression in NHE3\(^{-/-}\) mice (Figs. 5 and 7). The simplest explanation would be that NHE3\(^{-/-}\) mice have altered vitamin D receptor (VDR) signaling and are therefore tissue resistant. A more complicated and intriguing possibility however, invokes the relationship between 1,25(OH)\(_2\)D\(_3\) and renin. There is clear evidence that activation of the VDR by 1,25(OH)\(_2\)D\(_3\) suppresses activation of the renin-angiotensin-aldosterone system (38). However, whether components of the renin-angiotensin-aldosterone system affect VDR signaling is not known.

Additional evidence that activation of the VDR via 1,25(OH)\(_2\)D\(_3\) supersedes is not only because of changes in the expression of VDR (VDR) signaling and are therefore tissue resistant. A more complicated and intriguing possibility however, invokes the relationship between 1,25(OH)\(_2\)D\(_3\) and renin. There is clear evidence that activation of the VDR by 1,25(OH)\(_2\)D\(_3\) suppresses activation of the renin-angiotensin-aldosterone system (38). However, whether components of the renin-angiotensin-aldosterone system affect VDR signaling is not known.

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aldosterone system on VDR activation. Further study is required to test this hypothesis.

In conclusion, we provide evidence that NHE3 is a molecular link between Na\(^+\) and Ca\(^{2+}\) homeostasis. Not only does the overexpression of NHE3 in a proximal tubule cell culture model double transepithelial Ca\(^{2+}\) flux, NHE3\(^{-/-}\) mice also display profound defects in Ca\(^{2+}\) handling. Decreased proximal tubule/intestinal Na\(^+\)/H\(^+\) transport, likely inhibits Ca\(^{2+}\) absorption via the paracellular pathway, as observed in the NHE3\(^{-/-}\) mice. These animals have increased circulating 1,25(OH)_2D_3 levels, yet surprisingly have decreased intestinal and renal Ca\(^{2+}\) (re)absorption. Consistent with the functional
data is decreased expression of a number of renal (TRPV5, calbindin-D_{28K}, and claudin-19) and intestinal (calbindin-D_{9K}, claudin-2, and -15) Ca^{2+}/H^{+}-transporting genes. This raises the possibility of a potential contribution of these pathways to the Ca^{2+}-absorptive and -reabsorptive abnormalities observed. Ultimately, decreased renal and intestinal Ca^{2+} absorption leads to hypomineralized bones in NHE3^{-/-} mice. We propose that a predominant mechanism mediating this observation is reduced intestinal and proximal tubular paracellular Ca^{2+} flux, in the absence of a driving force generated by NHE3.

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