Renal calcinosis and stone formation in mice lacking osteopontin, Tamm-Horsfall protein, or both

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Departments of 1Urology and 2Pathology, New York University School of Medicine, New York; 3Veterans Affairs Medical Center in Manhattan, New York, New York; 4Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough, Maine; 5Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, Indiana; 6Department of Chemistry and Biochemistry, Miami University, Oxford, Ohio; and 7Division of Nephrology and Hypertension, Mayo Clinic College of Medicine, Rochester, Minnesota

Submitted 12 August 2007; accepted in final form 24 September 2007

Renal calcinosis and stone formation in mice lacking osteopontin, Tamm-Horsfall protein, or both. Am J Physiol Renal Physiol 293: F1935–F1943, 2007. First published September 26, 2007; doi:10.1152/ajprenal.00383.2007.—Although often supersaturated with mineral salts such as calcium phosphate and calcium oxalate, normal urine possesses an innate ability to keep them from forming harmful crystals. This inhibitory activity has been attributed to the presence of urinary macromolecules, although controversies abound regarding their role, or lack thereof, in preventing renal mineralization. Here, we show that 10% of the mice lacking osteopontin (OPN) and 14.3% of the mice lacking Tamm-Horsfall protein (THP) spontaneously form interstitial deposits of calcium phosphate within the renal papillae, events never seen in wild-type mice. Lack of both proteins causes renal crystallization in 39.3% of the double-null mice. Urinalysis revealed elevated concentrations of urine phosphorus and brushite (calcium phosphate) supersaturation in THP-null and OPN/THP-double null mice, suggesting that impaired phosphorus handling may be linked to interstitial papillary calcinosis in THP- but not in OPN-null mice. In contrast, experimentally induced hyperoxaluria provokes widespread intratubular calcium oxide crystalization and stone formation in OPN/THP-double null mice, while completely sparing the wild-type controls. Whole urine from OPN-, THP-, or double-null mice all possessed a dramatically reduced ability to inhibit the adhesion of calcium oxalate monohydrate crystals to renal epithelial cells. These data establish OPN and THP as powerful and functionally synergistic inhibitors of calcium phosphate and calcium oxalate crystallization in vivo and suggest that defects in either molecule may contribute to renal calcinosis and stone formation, an exceedingly common condition that affects up to 12% males and 5% females.

kidney stone; urolithiasis; uromodulin

CLINICAL KIDNEY STONE DISEASE is classified into a number of forms, each requiring a specific plan for medical treatment. It has only been recently demonstrated through a series of biopsy studies of renal papillae from human stone formers that the different stones can develop through different pathways (7, 13). Whether the pathogenetic process leading to stone formation involves interstitial plaque or intratubular crystal deposits, a range of host factors appears to be involved, some acting as facilitators and others as inhibitors. Under normal conditions, there is a fine equilibrium between pro- and anticrostalization forces, which helps keep the kidneys stone-free so that they can carry out important physiological functions. However, when the equilibrium breaks down, urine constituents can precipitate out, allowing insoluble crystals to form. Although the mechanisms that mediate retention of urinary crystals into stones remain poorly defined, it is clear that the degree of urinary supersaturation predicts stone risk and type (24, 28, 38). Furthermore, the urine of most individuals is frequently supersaturated with calcium oxalate and calcium phosphate. These observations suggest a critical role for urinary supersaturation and inhibitors of crystallization. This paradigm of nephrolithiasis has compelling experimental support, as elevated urinary level of either calcium or oxalate alone, two major constituents of human kidney stones, can trigger nephrolithiasis (5, 18, 19). Much less is known, however, about whether deficient quantities or function of the anticrostalization factors can also fundamentally alter the normal equilibrium to the extent that unwanted mineralization occurs in the kidney.

Osteopontin (OPN) and Tamm-Horsfall protein (THP) are two major urinary macromolecules that exhibit various activities that can influence calcium crystallization in vitro (10, 24, 34). OPN is a ubiquitously expressed phosphoglycoprotein that regulates bone mineralization and ectopic calcification (9, 16, 23). Renal OPN expression is normally restricted to the thick ascending limb of Henle’s loop (TALH) and renal papillary epithelium. Found frequently as a component of human kidney stones, can trigger nephrolithiasis (5, 18, 19). Much less is known, however, about whether deficient quantities or function of the anticrostalization factors can also fundamentally alter the normal equilibrium to the extent that unwanted mineralization occurs in the kidney.

THP is the most abundant urinary protein in most mammals and is expressed exclusively by the TALH cells of the kidney (34). While THP is also found in some human kidney stones, its in vitro activity affecting calcium crystallization ranges from promotion to inhibition and to lack of any activity, depending on the pH, ionic strength, and THP’s aggregation status (24). Ablation of THP in mice allowed spontaneous formation of calcium microcrystals in the kidney, suggesting that the net effect of THP is protective. Although crystals were detected in only 16% of the THP-null mice (29), oxalate

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overload resulted in a significantly greater frequency (74%) and severity of renal calcium crystallization in the THP-null mice, but not in the wild-type controls. Interestingly, in the absence of THP, oxalate overload triggered a dramatic induction of OPN in all renal epithelial cells (29). This, plus the fact that the lack of either OPN or THP predisposed mice to kidney calcinosis under hyperoxaluric conditions, raises the interesting possibility that these two structurally unrelated proteins are functionally synergistic inhibitors of renal calcium crystallization and that a combined deficiency is sufficient to elicit renal calcinosis. To address these questions critically, we generated mice deficient for OPN and THP, either alone or in a combination, and examined whether they are prone to a specific type of renal calcification under natural as well as experimental conditions. We also studied the physiological defects resulting from the loss of these urinary macromolecules that might underlie renal mineral crystallization. Finally, we examined whether the deficiency of urinary macromolecules impairs the ability of urine to inhibit the attachment of calcium crystals to renal epithelial cells, a crucially important step in nephrolithiasis. Our results provide direct experimental evidence indicating that OPN and THP are potent inhibitors that can cooperate in vivo to prevent renal calcium crystallization, and strongly suggest that their defects can lead to renal stone formation.

METHODS

Generation of null mice lacking urinary macromolecules. Mice lacking OPN (OPN-null mice) and THP (THP-null mice) were both generated using a “replacement” strategy that substituted a portion of the target gene with a Neo-resistant gene in mouse embryonic stem cells. Consequently, the OPN-null mice lacked exons 4–7 of the OPN gene (25), whereas the THP-null mice lacked a 650 proximal promoter region, exons 1–4, introns 1–3, and partial intron 4 (30). Both null mouse strains and their wild-type controls had been maintained on a 129/SvEv background for at least six generations at the start of this study, thus allowing phenotypic comparison in a similar genetic background. Genotyping of the OPN-null mice was performed by PCR amplification of tail DNA using primers located in introns 3 and 4 (In3 forward: 5′-CCATACAGGAAAGAGAGACC-3′; In4 reverse: 5′-AACTGTTTTGCTTGCATGCG-3′) that amplified a 600-bp wild-type allele; and using an additional primer in the Neo′ gene (reverse: 5′-CGTCTGTAAGTCTGCAAG-3′) that amplified a 500-bp mutant allele. Genotyping of the THP-null mice was carried out by Southern blotting of EcoRI-digested tail DNA using a probe outside of the 5′-targeting region that detected a 7.0-kb wild-type allele and an 8.5-kb mutant allele.

For analysis of the potential synergism between OPN and THP, double-null mice were produced by intercrossing homozygous OPN-null mice with homozygous THP-null mice. Additional crossing of the double heterozygous offspring yielded double homozygous null mice for both OPN and THP, as well as single homozygous mice and wild-type mice, the latter of which were used as controls. Male mice aged 2–3 mo were used throughout the study. All animal experiments were carried out in accordance with federal guidelines and were approved by Institutional Animal Care and Use Committee.

Detection and analysis of renal calcium deposits and stones. For histochemical detection of spontaneous renal calcinosis in OPN-null, THP-null, and OPN/THP double-null mice, kidneys were freshly dissected, fixed in 10% buffered formalin, and paraffin-embedded. Five-micrometer-thick sagittal sections were cut, deparaffinized, and stained with a von Kossa procedure that specifically detects calcium deposits. After a brief counterstaining with 1% neutral red, the slides were viewed by light microscopy. Fourier transform infrared microspectroscopy (μ-FTIR) was performed to determine the chemical composition of the renal papillary crystals that were positively identified by von Kossa staining. Two paraffin blocks from each of the three experimental groups (OPN-null, THP-null, and double-null mice) and the wild-type controls were selected for this analysis. Crystals were analyzed in parallel by μ-FTIR to generate standard spectra to identify calcium oxalate or calcium phosphate. Seven-micrometer-thick paraffin sections mounted between two barium fluoride salt plates were analyzed in transmission mode with a 50 × 50-μm aperture. The best spectra were collected in the ATR mode. In this configuration, the germanium internal reflection element reduced the aperture size to 12.5 μm and thereby reduced the size of mineral area sampled to 12.5 μm or greater. Infrared spectra were gathered with Perkin-Elmer Autolmage infrared microscope interfaced to Perkin-Elmer Spectrum 2000 Fourier transform spectrometer (Perkin-Elmer, Shelton, CT).

Inhibition of crystal adhesion to cultured renal epithelial cells. Spot urine samples were collected from five mice representing each of the four genotypes under study by gently massaging lower abdominal area and stored at −80°C until use. Madin-Darby canine kidney (MDCK) cells were cultured in DMEM containing 10% calf serum and 1.6 μmol/l biotin to reach confluence. Before adhesion measurement, the media were aspirated and the cells were rinsed once with PBS (pH 7.4). [14C]Calcium oxalate monohydrate (COM) crystals at a final concentration of 200 g/ml (41.6 g/cm² of cells) were precoated with urine samples in an Eppendorf tube subjected to end-over-end rotation for 15 min (26, 27). Crystals were then washed twice, and adhesion of the precoated crystals to MDCK cells was assessed in a 2-min binding assay. After being washed in PBS, the cells along with attached crystals were then dissolved in 10% NaOH and the radioactivity was measured in a scintillation counter. Crystal adhesion in the presence of wild-type mouse urine was set at 100%, and those from the null mice were expressed relative to the measurement from the wild-type mice.

Experimental hyperoxaluria. For induction of experimental hyperoxaluria, wild-type and OPN-, THP-, and double-null mice (2-mo-old mice) were exposed to 1% ethylene glycol and 4 IU/ml vitamin D₃ (Twin Laboratories, Hauppauge, NY), the latter as a water suspension, in the drinking water continuously for 1 mo. Urine samples from these mice were collected and viewed using a dark-field microscope equipped with polarized light to visualize birefringent calcium crystals. The chemically treated mice were then killed and their kidneys were removed and processed for von Kossa staining and in selected cases for μ-FTIR as described above.

Urinalysis. Spot urine samples from individual mice of each of the four genotypes under study were assayed before and after hyperoxaluric treatment. Urine samples were collected directly into Eppendorf tubes, centrifuged at 5,000 g for 5 min to dispose of cell debris, and stored at −80°C until use. Urinary concentrations of oxalate, calcium, and other determinants of supersaturation were measured in the Mayo Clinic Renal Function Laboratory, or kindly performed by Dr. Y. Nakagawa, University of Chicago Kidney Stone Program (Chicago, IL) as previously described (6). Supersaturations were calculated using the EQUIL2 program (36).

Statistical analysis. Pair-wise Student’s t-test was performed to assess the statistical difference of urine physiological parameters and cell-crystal adhesion measurements among wild-type, OPN-null, THP-null, and OPN/THP-double-null mice, using Web-based SPSS software. Pair-wise X² tests were used to assess the frequency of renal calcinosis among the four experimental groups under normal and hyperoxaluric conditions. P values <0.05 were considered statistically significant. To correct for multiple comparisons in Table 2, P values <0.017 (α < 0.05) were accepted as significant.
RESULTS

Generation of single- and double-null mice lacking urinary macromolecules. To examine the in vivo roles of urinary macromolecules in renal homeostasis, and to explore the mechanism(s) whereby the deficiency of urinary macromolecules leads to renal calcinosis and stone formation, we generated null mice lacking OPN, THP, or both. Intercross breeding was carried out initially between OPN-null mice and THP-null mice, yielding offspring that were heterozygous for both OPN- and THP-null alleles. Additional crosses among these heterozygous siblings gave rise to a number of genotypes, of which four (wild-type, OPN-null only, THP-null only, and OPN/THP-double null mice; Fig. 1A) were most relevant to the current study and therefore chosen for further investigation. Northern blot, RT-PCR, and immunohistochemical analyses clearly established that OPN- and THP-null mice lacked the corresponding mRNA and protein in the kidneys and that OPN/THP-double null mice lacked renal expression of both proteins (Fig. 1, B and D; data not shown). Urine samples of the null mice also lacked the corresponding protein(s), as evidenced by Western blot analysis using the polyclonal antibodies (Fig. 1C). It is important to note that, despite their identical location in the TALH, the lack of OPN did not affect the expression level or the subcellular localization of THP, and vice versa.

Physiological alterations of the null mice. Compared with the wild-type controls, all three types of null mice were completely fertile and had expected litter sizes, and exhibited no aberration in growth, daily activity, feeding pattern, or behavior. Histological examination of the kidneys from all null mice showed normal glomeruli and tubular segments with no evidence of interstitial fibrosis or cell injury (data not shown). However, urinalysis revealed several major alterations in physiological parameters in the null mice (Table 1). In particular, both sodium and chloride concentrations were significantly lower in the OPN-null mice than in the wild-type controls (P = 0.02 for sodium and P = 0.04 for chloride). In stark contrast, concentrations of these two ions were significantly elevated in the THP-null mice (P = 0.01 vs. wild-type mice for both ions). Since the body size and the concentrations of urine creatinine as well as other ions, such as calcium, magnesium, and citrate, were not significantly different between the null mice and the wild-type controls, the altered NaCl concentrations in OPN- and THP-null mice do not appear to result from renal dilution and concentration effects, respectively. Therefore, these changes in urinary ion levels appear to be a direct result of the physiological defects in NaCl reabsorption due to the depletion of OPN or THP in the TALH. It is worth emphasizing that the lack of OPN and THP in our current study had exactly the opposite consequences, one (OPN-null) significantly lowering the NaCl concentration while the other (THP-null) significantly raising it (Table 1). This suggests that the effects of macromolecule ablation on NaCl handling within the TALH segment are protein-specific. Interestingly, our double-null mice lacking both OPN and THP exhibited normal urinary levels of Na and Cl (Table 1), possibly reflecting a “neutralization” of the NaCl-lowering and -raising effects from the two proteins.

Another significant physiological alteration was the elevated level of urine phosphorus and calcium phosphate (brushite) supersaturation in the THP-null and OPN/THP-null mice. It is important to note that, despite their identical location in the TALH, the lack of OPN did not affect the expression level or the subcellular localization of THP, and vice versa.

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Because these changes were not detected in the OPN-null mice, they appeared to be related to the loss of functional THP. It is possible that the renal NaCl wasting in THP-null mice resulted in a compensatory increase of food intake, hence increased phosphorus absorption and excretion. This is consistent with the fact that other correlates of oral intake (sodium, potassium oxalate, uric acid) all increased in this group, while OPN-null mice did not demonstrate evidence of renal salt wasting or hyperphosphoruria (Table 1). Together, these data indicate that, although the deficiency of OPN, THP, or both did not produce any morphological abnormality, they significantly reduced the ability of the renal epithelial cells to properly handle electrolytes and minerals.

### Table 1. Urine chemistry of single- and double-null mice

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<tr>
<th></th>
<th>WT</th>
<th>OPN Null</th>
<th>THP Null</th>
<th>O/T Null</th>
<th>Chemically Treated Mice</th>
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<td>pH</td>
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<td>pH</td>
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<td></td>
<td>Na, meq/l</td>
<td>6.3 ± 0.3</td>
<td>7.4 ± 1.5</td>
<td>6.6 ± 0.8</td>
<td>6.8 ± 1.0</td>
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<td>Cl, meq/l</td>
<td>111.2 ± 25.3</td>
<td>77.8 ± 31.8*</td>
<td>144.0 ± 22.4*</td>
<td>125.8 ± 25.8</td>
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<td></td>
<td>Ca, mg/dl</td>
<td>63 ± 4.2</td>
<td>48 ± 1.4</td>
<td>6.6 ± 2.4</td>
<td>5.5 ± 1.5</td>
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<td>Oxalate, mg/dl</td>
<td>6.6 ± 2.8</td>
<td>7.9 ± 3.5</td>
<td>9.8 ± 4.7</td>
<td>6.4 ± 1.9</td>
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<td>P, mg/dl</td>
<td>176.0 ± 92.3</td>
<td>172.8 ± 57.5</td>
<td>206.6 ± 66.8*</td>
<td>254.1 ± 80*</td>
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<td>Mg, mg/dl</td>
<td>71.3 ± 45.6</td>
<td>54.5 ± 15.5</td>
<td>58.1 ± 8.8</td>
<td>61.4 ± 10.0</td>
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<td>K, meq/l</td>
<td>330.5 ± 126.1</td>
<td>301.2 ± 81.8</td>
<td>418.8 ± 76.8*</td>
<td>370.8 ± 73.6</td>
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<td>Citrate, mg/dl</td>
<td>163.5 ± 61.5</td>
<td>193.0 ± 61.5</td>
<td>153.9 ± 64.0</td>
<td>167.2 ± 56.3</td>
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<td>UA, mg/dl</td>
<td>12.4 ± 6.7</td>
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<td>15.0 ± 3.2</td>
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<td>CaOx SS (DG)</td>
<td>0.7 ± 1.0</td>
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<td>BR SS (DG)</td>
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<td>0.4 ± 0.7</td>
<td>0.7 ± 0.5*</td>
<td>0.6 ± 0.4*</td>
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<td>UA SS (DG)</td>
<td>4.6 ± 3.7</td>
<td>10.9 ± 8.5</td>
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<td>Creatinine, mg/dl</td>
<td>56.3 ± 22.2</td>
<td>47.7 ± 16.6</td>
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<td>Ca/Cr, mg/g</td>
<td>112.2 ± 62.2</td>
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<td>Ox/Cr (mg/g)</td>
<td>110.1 ± 44.7</td>
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<td>6.3 ± 0.1</td>
<td>6.0 ± 0.5</td>
<td>5.7 ± 0.0</td>
<td>5.8 ± 0.1</td>
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<td>75.6 ± 42.1</td>
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<td>81.6 ± 20.3</td>
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<td>100.1 ± 37.0</td>
<td>126.7 ± 43.8</td>
<td>104.6 ± 41.6</td>
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Values are means ± SD; n = 10 for untreated groups, n = 5–10 for chemically treated groups. Differences between null mice and wild-type (WT) mice showing statistical significances (P < 0.05) were marked with an *; UA, uric acid; BR, brushite; SS, supersaturation; O/T null, osteopontin (OPN)/Tamm-Horsfall protein (THP) double-null mice.

Fig. 2. Renal calcinosis in mice lacking urinary macromolecules. Representative cross-sections of renal papillary regions of WT (A and E), OPN-null (B and F), THP-null (C and G), and OPN/THP-double null mice (D and H) were stained with von Kossa method that specifically detects calcium deposits in the tissue (8). While wild-type mice were without exception devoid of any calcium deposits (Fig. 2, A and E), certain OPN-null mice (10%) showed calcium deposits in the renal papilla (Fig. 2B). On high magnifications the crystals were located in the interstitial space near both thin loops of Henle and medullary collecting ducts (Fig. 2F). The interstitial crystals varied in shape from small granular objects to rods ranging from 30 to 200 μm in length (Fig. 2F). Similar calcium crystals were not noted in a previous study analyzing the OPN-null mice using a similar staining method (37), possibly due to the fact that the crystals in these null mice are confined to the renal papillae that are small in mice and not readily captured in all kidney sections. A slightly higher percentage...
(14.3%) of the THP-null mice exhibited similarly sized and shaped interstitial calcium crystals, located exclusively in the renal papillae (Table 2; Fig. 2, C and G). An even greater percentage (39.3%) of the OPN/THP-double null mice spontaneously developed renal papillary calcium deposits ($P = 0.0011$ vs. wild-type; $P = 0.025$ vs. OPN-null mice; $P = 0.05$ vs. THP-null mice; Table 2; Fig. 2, D and H). This result strongly suggests that OPN and THP act synergistically to inhibit renal crystal formation, and their combined deficiency is sufficient to allow spontaneous renal calcium crystallization.

Notably, the naturally occurring renal papillary calcium crystals observed in the null mice were not accompanied by tubular cell damage, interstitial fibrosis, or inflammatory cell infiltration, as evidenced by the lack of staining by macrophage-specific antibodies (data not shown). It appears, therefore, that renal crystallization in the null mice is not a direct result of renal epithelial damage, but of abnormalities in mineral supersaturation and/or other mechanisms.

To establish the chemical composition of the renal crystals detected with the von Kossa or Yasue staining, paraffin blocks (2–3/ genotypetype) that contained renal papillary crystals were subject to microinfrared spectroscopy. Papillary interstitial crystals from OPN-null mice (Fig. 2f), THP-null mice (Fig. 2f), and OPN/THP-double null mice (data not shown) all produced spectra that were identical to that of hydroxyapatite (calcium phosphate).

**In vitro effects of macromolecule deficiency on crystal-epithelial adherence.** Crystal attachment to the renal epithelial cells or sites of exposed interstitial plaque (Randall’s plaque) is a critical step in nephrolithiasis, because unattached intratubular or renal pelvic crystals can be readily expelled along with the urine flow without causing any harm to the kidneys (21, 24). On the other hand, urinary macromolecules are believed to play a key role by coating intratubular crystals, thereby preventing their adhesion to the renal epithelial cells. To examine whether the loss of OPN and THP leads to reduced ability of mouse urines to inhibit the crystal-epithelial interaction, we incubated COM crystals with cultured MDCK cells in the presence of urine samples from the wild-type, OPN-null, THP-null, and double-null mice. We found a 3-fold increase of crystal adhesion to epithelial cells with OPN-null urine, 2-fold increase with THP-null urine, and a 3.5-fold increase with double-null urine (Fig. 3). These data provide direct experimental evidence implicating OPN and THP as effective in vivo inhibitors of crystal:epithelial adhesion and suggest that defects in this defense mechanism contribute significantly to renal calcinosis.

**Susceptibility of macromolecule-null mice to renal crystallization under hyperoxaluric conditions.** If OPN and THP indeed prevent urine mineral supersaturation and crystal adhesion in vivo, we reasoned that under hypercalcuric and hyperoxaluric conditions the combined deficiency of these two molecules would dramatically exacerbate renal calcium crystallization or even stone formation. We therefore treated the null and control mice with vitamin D3, which was intended to increase the intestinal absorption of calcium, and ethylene glycol, a precursor of oxalate (20). Despite increased vitamin D intake, urine calcium excretion did not significantly increase when the animals were fed with normal calcium diet (Table 1). In contrast, hyperoxaluria was present to the same extent in all mice, including the wild-type mice, and calcium oxalate supersaturation increased similarly in all four groups. Remarkably, however, calcium crystalluria (Fig. 4, A–D) and renal calcium crystal formation (Fig. 4, E–H) were observed only in null but not in wild-type mice (Table 2). OPN-null mice developed the least frequent (65%) and smallest amounts (+) of renal crystals, while THP-null mice more frequently (71.3%) developed moderate amounts (++) of crystals, and OPN/THP-null mice almost universally (95.4%) developed large amounts (+++ to +++++) of crystals (Table 2 and data not shown). Unlike the spontaneously occurring calcification which was almost exclusively in the papillary interstitium, the crystals in chemically treated mice were invariably intratubular with most of them concentrated at the outer medullary region (Fig. 4, F–H). Many crystals were closely attached to the apical surfaces of renal epithelial cells, consistent with our in vitro adhesion study, where the loss of OPN and/or THP dramatically increased crystal-epithelial interaction (Fig. 3). After 1 mo on treatment, ~15% of the THP-null mice and 18% of the OPN/THP-double null mice developed large crystalline deposits at the tip of renal papillae (Fig. 4, I–J). Some of these deposits blocked the distal portions of ducts of Bellini, resulting in severe dilation of the proximal portions of the collecting ducts (Fig. 4, I and J). Stones were observed in the renal pelvic cavity (Fig. 4, K and L). A series of degenerative and atrophic changes of renal epithelial cells along with interstitial fibrosis was evident in chemically treated null mice as well as wild-type controls, although calcium crystallization and stone formation occurred only in the null mice (data not shown). This result suggests that renal epithelial injury alone does not lead to renal crystallization as long as urinary macromolecules are functionally intact and that oxaluria can promote tubular injury in the absence of crystals. However, the degree of fibrosis and tubular damage increased in null mice, suggesting that crystals

### Table 2. Renal calcinosis in single- and double-null mice

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<th>Spontaneous</th>
<th>Chemically Induced</th>
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<tr>
<td></td>
<td>WT (n = 21)</td>
<td>OPN Null (n = 20)</td>
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<tr>
<td></td>
<td>0 (0%)</td>
<td>2 (10%)</td>
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<tr>
<td>$P_{1}$ = 0.15</td>
<td></td>
<td>$P_{2}$ = 0.07</td>
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<tr>
<td>$P_{5} = 0.70$</td>
<td>$P_{6} = 0.05$</td>
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Numbers shown are mice exhibiting renal papillary calcinosis by von Kossa staining. Percentages of crystal-positive mice are shown in the parentheses. $P$ values are pair-wise comparison of OPN-null vs. wild-type ($P_{1}$), THP-null vs. wild-type ($P_{2}$), THP-null vs. OPN-null ($P_{3}$), OPN/THP double null vs. wild-type ($P_{4}$), OPN/THP double null vs. OPN-null ($P_{5}$), and OPN/THP double null vs. THP null ($P_{6}$). *$P$ values <0.017 ($\alpha < 0.05$) in multiple comparisons.
are an important promoter of renal injury. By microinfrared spectroscopy, the renal crystals and stones were exclusively of calcium oxalate (Fig. 4M). The fact that the wild-type mice did not develop any renal crystals at all despite severe hyperoxaluria strongly supports the idea that under normal circumstances, OPN and THP are powerful inhibitors of calcium crystallization and that acting together these two proteins are sufficient to suppress renal crystallization even under severely hyperoxaluric conditions. Our data also suggest that the deficiency of these macromolecules can render the host extremely vulnerable to hyperoxaluric conditions.

DISCUSSION

The data we presented here are significant on several fronts. First, they provide compelling experimental evidence indicating that deficiency in urinary macromolecules per se can disrupt the physiological balance between pro- and antilithogenic forces in favor of stone formation. An increased supersaturation of calcium salts as a result of macromolecule deficiency appears particularly relevant in this process, although it seems protein-dependent. In the case of THP, its deficiency led to a significant elevation of urine phosphorus and brushite (calcium phosphate) supersaturation. This effect appears to be THP-specific as it was reproducible in the THP/OPN-double null mice but was not observed in OPN-null mice. These data also suggest that renal calcium crystallization in OPN-null mice might be mediated by mechanisms other than an increased supersaturation of the calcium salts. Formal analyses using 24-h urine samples from the null mice are clearly required to establish a link between specific physiological defects and our observed phenotypes.

Second, our results reveal a novel concept of the functional redundancy of urinary macromolecules that are capable of acting synergistically in preventing renal calcification. Because of this, the loss of one inhibitor (e.g., OPN alone or THP alone) only permits occasional crystallization, when the other macromolecule is functionally intact, and under certain conditions, could even be upregulated in the absence of the other. As we previously demonstrated, OPN expression is dramatically induced under hyperoxaluric stress when THP is absent, possibly reflecting a compensatory response to the loss of a functionally synergistic partner (29). Such a functional redundancy is highly desirable for the kidneys to remain in a crystal-free environment to perform vital functions, and could therefore be a result of evolutionary pressure. Given that urine contains other putative macromolecule inhibitors, such as bikunin, prothrombin fragment 1, inter-alpha trypsin (3, 7, 22, 24), this type of redundancy may not be limited to OPN and THP, but may be applicable to other macromolecules. It will be of considerable interest to determine how other molecules respond to the loss of OPN and/or THP under normal and hyperoxaluric conditions, and whether loss of additional molecules could lead to more frequent and severe renal calcinosis and stones.

Third, we found that the naturally occurring crystals in the renal papillae in our current study were primarily interstitial. It should be noted that we previously reported spontaneously formed intratubular crystals in the THP-null mice. This difference is mainly technical as we previously did not pay sufficient attention to the renal papillae, which in mice are extremely small and do not appear in all sections. We therefore cannot rule out the existence of intratubular crystals in any of our null mice. The intratubular crystals might have existed during the early stages of renal calcification, and when they are not firmly attached to the tubular epithelial cells, they might have been expelled, thus escaping detection. Further studies that include a larger number of the knockout mice at different ages and transmission electron microscopy that can better localize the crystals are clearly required to address this important question. Nevertheless, the actual existence of spontaneous interstitial crystals in our null mice suggests that the crystallization process in these mice bears resemblance to those observed in human patients with idiopathic kidney stones. The kidney biopsies from the patients often contain conspicuous interstitial deposits that are of calcium phosphate and are located in the renal papillae (14). These deposits are believed to be the precursors of Randall’s plaques, which appear to anchor calcium oxalate stones (12). Because the calcium phosphate deposits and Randall’s plaques are interstitial, they cannot be expelled from the kidney and may therefore play a key role to serve as a nidus for stone genesis. While the renal crystals we observed in our null mice are smaller than those found in humans, they are a significant size in the context of the small size of mouse renal papillae. We believe, therefore, that intrarenal crystals observed in these null mice may represent the earliest stages of renal stone formation as the unique consequence of the loss of urinary macromolecules. Thus far, only ~40% of our double-null mice develop spontaneous renal calcification. It will be important for us to find out whether renal calcification is age-dependent, occurring more frequently in older null mice. It will also be interesting to know whether placing mice on a high-calcium diet would increase the rate of calcium crystallization. The null mice will be useful for studying the potential alteration in interstitial ion composition as a result of OPN and/or THP deficiency. Studies of these null
Fig. 4. Susceptibility of macromolecule-deficient mice to renal crystallization and stone formation under hyperoxaluric conditions. A–D: urine samples examined with dark-field microscope equipped with polarized light showing no crystal in WT mice (A), small amounts of crystals in OPN-null mice (B), moderate amounts of crystals in THP-null mice (C), and large amounts of crystals in OPN/THP-null mice (D). E–H: von Kossa staining of outer medullary regions of the kidneys showing the absence of any calcium crystals in a WT mouse (E), and increasing amounts of crystals in OPN-null mouse (F), THP-null mouse (G), and OPN/THP-double null mouse (H). I–L: von Kossa staining showing renal stones at the tips of severely dilated ducts of Bellini of 2 THP-null mice (I and J) and in the renal pelvis of an OPN-null mouse (K) and an OPN/THP-null mouse (L). Magnification ×100. M. μ-FTIR spectroscopy of deep medullary crystals stained positive with von Kossa method. Top: 2 spectra were standard spectra from calcium oxalate and calcium phosphate (hydroxyapatite). Bottom: spectrum was from the deep medullary tissue of a WT mouse. Middle: 2 spectra were obtained from an OPN-null mouse and a THP-null mouse that had been treated with vitamin D3 and ethylene glycol, showing identical profiles to that of calcium oxalate.
models may offer mechanistic insights into how interstitial calcium crystals are formed. Since differences in gene expression could play a role in determining the susceptibility to renal crystallization, it will be of interest to determine whether such differences exist between the null mice and the wild-type controls.

Fourth, we found that the chemically treated null mice developed only intratubular calcium oxalate crystals, but not the interstitial calcium phosphate crystals found in untreated null mice. This difference might be due to different supersaturation states under different conditions. In chemically treated mice, urine is supersaturated with calcium oxalate (Table 1), thus driving calcium oxalate crystal formation. Supersaturation of brushite was also lower in chemically treated mice, perhaps due to lower pH. These could explain, at least in part, why chemically treated mice do not develop interstitial calcium phosphate crystals.

Finally, our findings suggest that deficiency of urinary macromolecules, either quantitative or qualitative, is a potentially important cause of nephrolithiasis. Results in this study also suggest that inhibitors could be important preventing the earliest interstitial lesions typical of idiopathic calcium oxalate stones (i.e., Randall’s plaques), as well as the tubular plugging observed under conditions of marked hyperoxaluria (1, 12). Evidence is mounting that renal stone is a polygenic disease (31, 35). Nearly half the human stone formers exhibit no underlying metabolic abnormalities such as hypercalciuria and hyperoxaluria. It is these patients that alternative causes including a genetic one should be thoroughly investigated. The fact that the majority of renal stones recur and a significant subset of patients have family history further points to the possibility of a common physiological defect in nephrolithiasis (17). There is a range of pathophysiological conditions that can affect the expression or structure of OPN and THP in humans (4, 11, 15, 16, 33, 34). It will be of particular interest and importance to define whether some of these molecular alterations, either quantitatively or qualitatively, contribute to the pathogenesis of human renal stones.

ACKNOWLEDGMENTS

The authors thank Y. Nakagawa for urinary biochemical determinations and G. Farell-Baril for technical assistance.

GRANTS

This work was supported in part by National Institutes of Health Grants DK-56903 and DK-69688 (to X.-R. Wu), DK-53399 and DK-60707 (to J. C. Lieske), and by a Merit Review Award from the Veterans Administration.

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AJP-Renal Physiol • VOL 293 • DECEMBER 2007 • www.ajprenal.org


