Normal Calcium Oxalate Crystal Growth Inhibition in Severe Calcium Oxalate Nephrolithiasis

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ABSTRACT

Urine from mammalian kidneys is regularly supersaturated with respect to calcium oxalate monohydrate, the most common solid phase in human nephrolithiasis, and also inhibits the nucleation, growth, and aggregation of calcium oxalate crystals. Nephrolithiasis is often associated with increased supersaturation, and it is assumed that this increase overbalances the inhibition effects, causing stones. However, some patients form stones in the absence of increased supersaturation, and in those patients, one might assume that reduced inhibition is the cause of their stones. This hypothesis was tested in 25 patients who formed at least ten stones each, yet lacked the usual metabolic abnormalities that increase supersaturation. Compared with 25 age- and sex-matched control subjects, urine supersaturation among the patients was not increased; this is an expected result of this study’s selection criteria. Compared with the same age- and sex-matched control subjects, urine from the patients showed no evidence for reduced inhibition of calcium oxalate crystal growth, so low inhibition of growth did not contribute to pathogenesis of stones in our highly selected study population, despite their otherwise unexplained and active stone formation. These results do not support the hypothesis that growth inhibition defects are a cause of stone disease.

Key Words: Inhibitors, nephrolithiasis, calcium oxalate

Much evidence suggests that inhibitors of calcium oxalate crystal nucleation, growth, and aggregation normally defend against nephrolithiasis (NL), and that defects of inhibitors can cause NL. Urine from healthy humans inhibits calcium oxalate monohydrate (COM) nucleation, growth, and aggregation (1–5), and most of the effects arise from nondialyzable molecules, primarily proteins (6,7). Some studies have (1,8–11), and others have not (3,12,13), found stone-former urine to be less efficient than normal urine in inhibiting COM crystallization. Nephrocalcin (NC) (14,15) and uropontin (UP) (16) both inhibit COM growth, to an equal extent; disorders of NC have been reported among stone-forming patients (11). Tamm-Horsfall protein (THP) inhibits aggregation (17), and abnormal forms of THP have been reported (18) among a few patients who formed more than ten calcium oxalate stones each.

Fifteen to 20% of patients with calcium oxalate NL lack usual stone risk factors such as systemic stone-forming diseases, idiopathic hypercalciuria, hyperuricosuria, hypocitraturia, or abnormally low urine volume (19–24); in them, abnormal inhibition as a cause of stones seems especially inviting. Despite its appeal, the inhibitor theory has not been tested as rigorously as one might like. Ideally, patients should be selected with the goal of minimizing heterogeneity and offering the highest probability of finding inhibition defects. Patients with NL have a great variety of metabolic disorders, and also vary in the number of stones they form; some forming only one and others as many as 100 stones (25). Also, control subjects should match patients in age and sex distributions. The assay should, as much as possible, avoid errors resulting from urine volume differences between patients and control subjects, which could variably dilute protein inhibitors. One critical study found that urine volume differences were responsible for apparent differences in crystallization inhibition (12). No studies to date have used stringent selection, matched controls, and an assay that avoids errors from urine volume effects.

We present a study of 25 patients with over ten separate calcium oxalate stones each; none of these patients have significant metabolic abnormalities presently recognized as causing stones; we compare them with 25 sex- and age-matched control subjects. Crystal growth inhibition was measured directly with urine proteins, using a well-established assay (26) modified to remove the effects of urine volume.

METHODS

Patients and Controls

From 2527 patients in our clinic, we selected 25 patients (18 men and seven women) who formed at least ten separate new stones and whose analyzed stones contained at least 50% calcium oxalate, and no struvite, uric acid, or cystine. Because of their large numbers of stones, we refer to them as having accelerated nephrolithiasis (ANL) (25). Also, pretreatment urine chemistry studies, performed on 24-h urine samples at time of entry into the clinic, met the following criteria (in mg/day): calcium <350 in men, <300 in women; oxalate <50; citrate >300 in men, >400 in women; urate <1000 in men, <900 in women. These criteria minimize
well-known metabolic risk factors in stone pathogenesis (27). We excluded patients who had systemic disorders of stone formation, including hyperparathyroidism, granulomatous disease, intestinal disease or prior intestinal resection, and renal tubular acidosis. We matched each patient with a same-sex control subject, matched to within 5 yr of age; no control subject had kidney stones, a history of stone formation in immediate family members, renal disease, or systemic disease known to involve the kidneys. Each patient and control subject was personally interviewed, and all patients examined by one of us; these examinations included screening for protein and glucose by using a dipstick.

**Urine Collections**

Each patient and control collected a 24-h urine sample, which was kept unrefrigerated, using thymol as preservative. Patients discontinued stone-prevention treatments such as thiazide diuretics and allopurinol for 6 half-lives before the urine collection, but did not discontinue potassium citrate salts. Of the 25 stone-forming patients studied, 15 were being treated with potassium citrate at the time the 24-h urine sample was collected. The protocol was approved by our institutional human research committee.

**Sample Preparation and Chemistries**

Upon receipt, urine volume was measured, and an aliquot of each 24-h urine sample was taken for measurement of chemistries. Creatinine, calcium, uric acid, magnesium, phosphorous, sodium, potassium, and chloride concentrations were measured by standard laboratory techniques on a Beckman Synchron CX5 (Beckman Instruments, Brea, CA). Sulfate was determined by barium precipitation, oxalate was measured enzymatically with oxalate oxidase (Sigma Diagnostics, St. Louis, MO), and citrate was measured enzymatically with citrate lyase (Boehringer Mannheim, Mannheim, Germany). By using these measurements, we calculated an empirical estimate of COM supersaturation—the activity product (AP) (28). AP for patients was calculated using the urine chemistries at the time of entry into the kidney stone clinic, when the patients were actively forming stones. Another aliquot (40 mL) was dialyzed for 72 h against water, at 4°C, with three changes of water using 3.5-kd mol wt cutoff membranes. The protein concentration was then measured, and the volume reduced by lyophilization.

**Protein Measurements**

Total protein was measured using ninhydrin, as prior studies have shown that many common reagents fail to recognize all of the urine proteins (11). A 25- to 75-μL aliquot was hydrolyzed in 0.5 mL of 1 N NaOH for 8 h. Thereafter, pH was adjusted to 5.7 with 0.5 mL of 7.5% acetic acid, and 25 μL of ninhydrin added. The resulting color development during 15 min of incubation in a boiling water bath was measured at 570 nm. A standard curve was constructed for each run by using BSA. Urine samples were diluted so that their optical density values fell within the range of the albumin standard curve. Lysozyme standards were also run, as “unknowns” with each set of urine samples, as a second test of the system.

In addition to total protein, we measured albumin by using a commercial double-antibody immunoassay (Vectastain; Vector Laboratorities, Burlingame, CA), and Tamm-Horsfall protein by ELISA using a commercial polyclonal antiserum (Behring, La Jolla, CA) and a monoclonal antibody produced in our laboratory (29).

**Crystal Growth Inhibition**

To a 2-mL solution of calcium chloride and sodium oxalate (1.0 mM each) containing 90 mM NaCl, buffered with 10 mM Tris–HCl buffer, pH 7.2, stirred at 1200 rpm, and contained in a quartz cuvette heated to 37°C and continuously monitored at 214 nm, we added 24 to 64 μg of COM crystals, verified before use by x-ray crystallography. The amount of seed crystal added is varied whenever a new crystal slurry was used, to maintain a steady kinetic rate constant for the assay, defined as a control initial velocity of 1.6 to 2.1 × 10^{-4} mmol/min ligand consumption. Consumption of oxalate begins immediately after seed addition, and is monitored for 400 s by disappearance of absorbance at 214 nm. Data was collected using a Lambda 7 spectrophotometer (Perkin Elmer Corp., Norwalk, CT) interfaced to an Apple IIE computer (Apple Computers, Cupertino, CA) by an Adalab data acquisition system (Interactive Microwave Inc., State College, PA).

The rate of consumption follows second-order kinetics, and from the integrated rate equation (26), we derive the velocity of the reaction.

The assay was run with or without addition of urine protein (16 μg/mL) in an alternating protocol, such that each urine protein sample was sandwiched between two control measurements, using only buffer. The amount of crystal added to the assay was constant during this comparison of control measurements to urine protein inhibition. Each protein sample was run in duplicate, and duplicates that failed to match within 10% were remeasured. Growth rate velocity was expressed as 100× (v/vo), where vo is the average velocity of the two controls for the protein sample, and v is the velocity of the protein sample. Thus, when inhibition is absent, v does not differ from vo, and the growth-rate ratio approaches 100%.

**Supersaturation Estimation**

Calcium oxalate supersaturation was estimated using an empirical AP(CaOx)-Index (28) that approximates the true ion activity product of calcium oxalate multiplied by 10^{4}:

$AP(CaOx)\text{-Index} = 1.9(Ca^{2+})(Ox)(Mg^{2+})(Cl^{-2})(V^{-1.03})$

where calcium, oxalate, magnesium, and citrate are measured in mmol and V is the volume of the urine collection in L. Given that the equilibrium activity product, experimentally determined in vitro, for CaOx is 2.25 × 10^{-9} (30), a value for the AP of 22 approximates equilibrium. A value of 2.8 was found to approximate the solubility limit (28), which corresponds to a supersaturation of about 12.7.

**Statistical Methods**

Significance of mean value differences between groups were tested using t tests that assumed unequal variances between groups. Distributions of data were compared using quantile plots (Systat, Evanston, IL).

**RESULTS**

Patient urine inhibited crystal growth as well as normal urine (Figure 1, Table 1). Values from patients overlapped those of matched control subjects. Because three patients showed very low growth rates (below 45%), meaning high inhibition, we postulated that these three outliers may have hidden an inhibitor deficiency in the rest of the patients when the group was treated as a whole. However, if these three outli-
 ers are deleted, mean growth rate of patients (63% ± 2) remained undifferentiated from their corresponding control subjects (P = .083). Also, patients had a distribution of AP values and a mean value that were no different than normal (Figure 2, Table 1). Because calcium, oxalate, and citrate concentrations exert major effects on calcium oxalate supersaturation, and in the AP equation we calculated the simple ratio of the calcium oxalate molar product to the citrate molarity, the ratios in patients and control subjects did not differ (Table 1). As expected, AP and this ratio were highly correlated (r = .822, P < .001).

The urine excretion rate of THP by ANL patients exceeded that of normal (Figure 3), but excretion of total protein and albumin did not, whether expressed as 24-h excretion rate or as the ratio of protein to creatinine (Table 1). The THP excretion rate did not correlate with crystal growth inhibition, or with AP

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**TABLE 1. Principal laboratory findings in patients and control subjects**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>ANL Patients</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Rate (%)</td>
<td>61 ± 2</td>
<td>59 ± 1</td>
</tr>
<tr>
<td>AP</td>
<td>.74 ± .1</td>
<td>.72 ± .1</td>
</tr>
<tr>
<td>(Ca x Ox)/Ct (mM)</td>
<td>.30 ± .05</td>
<td>.26 ± .02</td>
</tr>
<tr>
<td>TP/Cr (mg/gm)</td>
<td>159 ± 13</td>
<td>150 ± 13</td>
</tr>
<tr>
<td>THP/Cr (mg/gm)</td>
<td>24 ± 3</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>ALB/Cr (mg/gm)</td>
<td>5 ± 1</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

*ANL patients with accelerated nephrolithiasis; AP, estimate of supersaturation; Ca, Ox, Ct: calcium, oxalate, and citrate concentrations; TP/Cr, ratio of total protein to creatinine concentration; THP/Cr, ratio of Tamm-Horsfall protein to creatinine concentration. ALB/Cr, ratio of total albumin to creatinine concentration. 

*Differs from corresponding controls (P < .02).
(not shown). The difference in THP excretion rate (mg/gm creatinine) was significant among men (19 ± 2 versus 10 ± 2 for patients and control subjects, \( P < .002 \)) but not women (38 ± 9 versus 19 ± 3, \( P = .074 \)). However, there was a small number of women in the study (seven patients, eight control subjects). Urine chemistry values of patients differed from those of their matched control subjects in a few particulars (Table 2). Among men, uric acid excretion rates and weight were higher in patients than in control subjects, and among women, urine oxalate and weight measurements were lower in patients than in control subjects. Given that the selection criteria for patients avoided the majority of known stone-forming metabolic disorders, the correspondence of laboratory values is expected. No differences between patients and same-sex control subjects were found in urine creatinine, sodium, potassium, magnesium, sulfate, phosphate, or chloride values (not shown).

Because 60% of our patients remained on citrate supplements during the urine collection, we compared the growth inhibition and THP excretion rate of patients who did and did not take citrate. Growth-rate ratio was 62.5 ± 2.6 versus 58.3 ± 3.9 \( (P = 0.39) \), and THP excretion (mg/day) was 39.6 ± 5.6 versus 40.8 ± 7.1 \( (P = 0.89) \) (mean ± SE), for the patients treated with citrate and not treated with citrate, respectively. These findings support our initial assumption that citrate would not interfere with our assays once it was dialyzed out of the urine samples.

**DISCUSSION**

We found no evidence for abnormal COM growth inhibition in the urine of ANL patients. Control subjects were selectively matched to patients by sex and age. Instead of adding urine either in fixed volumes or fixed fractions of total volume, we added fixed amounts of dialyzed protein. Our patients were selected for the highest \( a \) \( \text{prior} \) probability of having inhibition defects; all developed at least ten separate stones, and none had impressive urinary biochemistry abnormalities of the kind usually thought to promote stones. The fact that we could find no reduction of growth inhibition in patient urine is a strong argument against the inhibition pathogenesis hypothesis, at least for the non-dialyzable protein inhibitors of urine. However, we cannot exclude the possibility that aggregation or nucleation inhibition could be abnormal.

Possibly, depressed COM growth inhibition may arise from some features of NL patients that we excluded or minimized in the ANL group. One such feature could be high calcium oxalate supersaturation or hypercalciuria. Pak and Holt (10) observed reduced COM growth inhibition in urine from patients with hypercalciuria and primary hyperparathyroidism, but not those with normocalcicuric nephrolithiasis. In previous studies (9), Coe et al. found exactly the same result; inhibition was low in urine from hypercalciuric patients, not those with normocalcicuric, hyperuricosuric, calcium oxalate NL. Pak and Holt and Coe et al. found that supersaturation did not predict inhibition abnormalities, in that the normocalcicuric patients in Pak and Holt’s study, those with normal inhibition, had elevated supersaturations (10), and in the study by Coe et al. (9), no correlation was found between supersaturation and inhibition. In the study presented here, we also found no correlation between the AP CaOx Index of supersaturation and inhibition, or between urine calcium, oxalate, or citrate, or our ratio of calcium oxalate product to citrate concentration and inhibition. If hypercalciuria itself somehow reduces COM crystal growth inhibition, then hypercalciuria may act through some direct effect of calcium on protein inhibitors, not merely through increased supersaturation, crystallization, and adsorption of inhibitors on crystals (31). On the other hand, absence of any correlation between urine calcium and inhibition raises the possibility that hypercalciuria, an inherited trait (32), may somehow be linked to inherited inhibition defects. Possibly, clinical expression of stone disease in hypercalciuric people may depend upon reduced inhibition, so that non-stone-forming stones will not form.

**TABLE 2. Laboratory values for patients and control subjects**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>ANL Men</th>
<th>Control Men</th>
<th>ANL Women</th>
<th>Control Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml/day)</td>
<td>1772 ± 158</td>
<td>1463 ± 137</td>
<td>2184 ± 286</td>
<td>2105 ± 225</td>
</tr>
<tr>
<td>Calcium (mg/day)</td>
<td>196 ± 14</td>
<td>171 ± 25</td>
<td>152 ± 21</td>
<td>129 ± 21</td>
</tr>
<tr>
<td>Oxalate (mg/day)</td>
<td>34 ± 2</td>
<td>35 ± 3</td>
<td>26 ± 3(^a)</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>Citrate (mg/day)</td>
<td>549 ± 36</td>
<td>710 ± 97</td>
<td>606 ± 49</td>
<td>525 ± 74</td>
</tr>
<tr>
<td>pH</td>
<td>6.07 ± .08</td>
<td>6.21 ± .12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urate (mg/day)</td>
<td>713 ± 24(^b)</td>
<td>583 ± 42</td>
<td>445 ± 53</td>
<td>484 ± 39</td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>96 ± 4(^c)</td>
<td>79 ± 3</td>
<td>61 ± 2(^c)</td>
<td>72 ± 4</td>
</tr>
<tr>
<td>Number</td>
<td>18</td>
<td>18</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

\(^a\) All values are mean ± SE.

\(^b\) \( P < .05 \) from same-sex control.

\(^c\) \( P < .01 \) from same-sex control.
hypercalciuric people might have normal urine crystal growth inhibition levels. This latter question is not as yet explored.

In addition, we must consider whether our experimental design might have prevented us from finding a growth inhibition defect. We allowed our patients to continue using citrate during the collection of the 24-h urine samples. We assumed that citrate would not affect the growth assay because it would be dialyzed out of the urine before study. Also, the only urinary protein that citrate is known to affect is THP (33), which inhibits crystal aggregation but has no inhibitory activity against crystal growth (31). In analyzing our results, we found no difference between the patients treated with citrate and those not treated with citrate, suggesting that our results were not affected by the citrate supplementation. Our patients and control subjects were studied while they were on a random diet, and it is possible that excretion rates of lithogenic substances and AP could have been affected by dietary avoidance of specific foodstuffs. However, the AP was calculated from the urine chemistry studies of the patients at the time of their entry into the stone clinic, when the patients were actively making stones and before they received any dietary counseling. We feel that studying patients in this manner provides a more accurate reflection of their stone-forming risk than would studying the patients while they were on a prescribed diet. Finally, calcium phosphate salts may act as a nucleation site for calcium oxalate stones (34). A defect in the inhibition of calcium phosphate crystal nucleation is not addressed in this study and could be an explanation for the stone formation in ANL.

The fact that the THP excretion rates of ANL patients exceeded normal rates is another surprising result, and one that was not specifically a part of our hypothesis. We have no explanation for it, or any reason why such a difference could explain stone forming. The fact that albumin and total protein excretion rates did not differ significantly argues against some general disorder of renal protein handling. THP does not inhibit COM growth (31), but does inhibit aggregation (17,18). THP could also promote nucleation, because it self-aggregates into protein particles in high-calcium, high-sodium, low-pH solutions (18), and when light chains bind to it (35). Although the risk for self-aggregation could increase from hypercalciuria, this could not explain the results in our study, because calcium excretion rates from patients and control subjects were similar.

Overall, our critical test of the growth inhibition hypothesis provides a negative answer. Among ANL patients, defective crystal growth inhibition is not present; however, other aspects of crystal inhibition, such as nucleation, and aggregation, may be abnormal and may account for ANL. Increased THP excretion, observed incidently, is unexplained.

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