Inhibitory activity of human urine on calcium oxalate crystal growth: effects of sodium urate and uric acid

B. FELLSTRÖM, U. BACKMAN, B. G. DANIELSON, K. HOLMGREN*, S. LJUNGHALL AND B. WIKSTRÖM

Department of Internal Medicine and *Department of Urology, University Hospital, Uppsala, Sweden

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Summary

1. Freshly voided urine from healthy subjects was pooled and ultrafiltered (10 000 daltons). The ultrafiltrable and the macromolecular portions were preincubated with calcium oxalate, uric acid and sodium urate crystals and the effects on the inhibitory activity of calcium oxalate crystal growth assessed by monitoring the disappearance of $^{14}$Coxalate from the solution.

2. The inhibitory activity of the ultrafiltrate was higher than that of the urinary macromolecular fraction. Both uninhibited and inhibited crystal growth processes followed second-order kinetics.

3. Calcium oxalate crystals adsorbed almost all the urinary macromolecular inhibitors whereas sodium urate or uric acid crystals adsorbed only 10–20%.

4. In the presence of a metastable solution of calcium oxalate, the incubation of the urinary macromolecular fraction with sodium urate crystals caused a pronounced reduction in the inhibitory activity. A similar effect was seen with uric acid crystals, but to a lesser degree.

5. We conclude that the effect of sodium urate or uric acid crystals alone on naturally occurring urinary macromolecular inhibitors of calcium oxalate crystal growth is weak, but that in the presence of metastable calcium oxalate this is greatly enhanced. A substantial adsorption of the inhibitors on to the crystals is suggested, possibly secondary to epitaxial growth of calcium oxalate on the surface of the urate crystals.

Key words: calcium oxalate, crystals, growth inhibitors, sodium urate, uric acid, urinary macromolecules, urinary ultrafiltrate.

Introduction

It has been claimed that hyperuricosuria promotes calcium oxalate stone formation. One of the suggested mechanisms is an interference by urate with naturally occurring macromolecular inhibitors of calcium oxalate crystal growth and aggregation in urine [1]. Microcrystals of sodium urate have been shown to adsorb heparin [2, 3], a glycosaminoglycan, which is a potent inhibitor of calcium oxalate crystal growth and aggregation in vitro [4]. The predominant types of glycosaminoglycans in the urine are, however, chondroitin sulphates, whereas heparin is excreted in human urine only in minute amounts [5–8]. Chondroitin sulphates could not, however, be shown to adsorb to any of the urate salts tested [9].

Accordingly we have studied the relation between urinary macromolecular and ultrafiltrable inhibitors of calcium oxalate crystal growth, and investigated to what extent the macromolecular fraction isolated from human urine would interact with sodium urate and uric acid crystals. This has been estimated from changes in the inhibition of calcium oxalate crystal growth.

Material and methods

The inhibition of calcium oxalate crystal growth was studied by following the disappearance of $^{14}$Coxalate from a metastable solution of calcium oxalate after adding calcium oxalate seed
crystals. The inhibitory activities of the ultrafiltrable and the macromolecular fractions of human urine were studied both before and after incubation with crystals and solutes, as shown in Fig. 1.

Metastable calcium oxalate solution

The supersaturated, metastable calcium oxalate solution was freshly prepared through a slow mixture of equal amounts of prefiltered (0.45 μm Millipore filter) CaCl₂ solution (3.4 mmol/l) and sodium oxalate solution (0.22 mmol/l) containing trace amounts of [14C]oxalate (The Radiochemical Centre, Amersham, U.K.). The original specific radioactivity was 75 mCi/mmol of oxalate. The final concentrations after addition of 1 ml of sodium chloride solution (150 mmol/l: saline) or urine fraction to be tested were calcium 1.55 mmol/l and oxalate 0.1 mmol/l, i.e. a relative supersaturation of calcium oxalate > 0.6. This solution was stable for several hours. All solutions were prepared from deionized water, buffered with sodium cacodylate, 10 mmol/l (pH 6.0), and pH was adjusted to 6.0 with either NaOH or HCl. The ionic strength was 0-15 [4].

Crystals

Calcium oxalate crystals were prepared basically by the method of Meyer & Smith [10], and sodium urate and uric acid crystals by the method of Pak & Arnold [11]. The calcium oxalate crystals had a mainly rounded shape in the light microscope. The linear size of the urate crystals was 10–40 μm.

Urine preparation

Freshly voided urine from five healthy subjects was pooled and centrifuged to eliminate solid particles. The supernatant was separated by ultrafiltration with a filter having a cut-off at 10,000 daltons (YM-10, Amicon). The non-ultrafiltrable, macromolecular fraction of the urine was restored to the initial volume with saline buffered with sodium cacodylate, 10 mmol/l (pH 6.0).

Preincubation with crystals

The ultrafiltrate and non-ultrafiltrable macromolecular fractions of the urine were incubated with crystals or solutes for 2 h at 37°C in Pyrex glass tubes with Teflon-coated magnetic stirrers. The incubations were performed with calcium oxalate crystals in concentrations of 45 or 90 mmol/l and with sodium urate and uric acid crystals in concentrations of 37 or 75 mmol/l. Sodium urate and uric acid crystals (37 mmol/l) were also incubated with the macromolecular fraction in a metastable calcium oxalate solution (relative supersaturation > 0.6).

Preincubations were further made in saturated solutions of calcium oxalate and uric acid, and in metastable calcium oxalate solution (relative supersaturation > 0.6). Control incubations were performed in buffered saline. After incubation for 2 h the moieties were centrifuged, filtered (Millipore, 0.45 μm) and the filtrates tested for inhibitory activity against calcium oxalate crystal growth.

Assay of inhibitory activity

Crystal growth. The crystal growth experiments were executed by adding 1 ml of the urinary fraction to be tested to 10 ml of the metastable solution containing [14C]oxalate for monitoring the disappearance of oxalate from solution. The addition of 250 μl of calcium oxalate seed crystals from a slurry (6 mg/ml = 200 mmol/l) started the crystal growth process. Then aliquots were drawn, filtered (Millipore 0.45 μm), added to a scintillant mixture (Picofluor-30, Packard) and radioactivity was counted (Beckman LS-250). Each experiment was made in triplicate and the mean values were presented as percentages of the initial radioactivity.
Calculations. In order to estimate the fit the second-order kinetics of crystals growth, i.e.

\[-\frac{d(Ox^*_t)}{dt} = k(Ox^*_t - Ox^*_0)^2\]

the data were transformed according to the asymptotic solution of the differential equation

\[(Ox^*_t - Ox^*_0)^{-1} = k \cdot t + A\]

where \(k\) is the rate constant, \(Ox^*_t\) the radioactivity at time \(t\), \(Ox^*_0\) that at time \(= \infty\) and \(A\) the integration constant [10, 12]. The infinity value was approximated by the 3 h value.

Inhibition. Inhibitory activity index could be estimated as

\[I_R = 100 \times \left(1 - \frac{k_i}{k_c}\right)\]

where \(k_i\) is the rate constant of the inhibited system and \(k_c\) that of the control system with 1 ml of saline. The estimate could be simplified by calculating the quotient of radioactivities at a specific time, e.g. 60 min, as:

\[I_Q = 100 \times \frac{(Ox^*_t - Ox^*_c)}{(Ox^*_0 - Ox^*_c)}\]

where the indices are: s, non-seeded + saline, c, seeded + saline and i, seeded + inhibitor.

Results

The addition of 1 ml of centrifuged urine inhibited the crystal growth more than either the ultrafiltrate or the macromolecular fraction of the urine alone. The ultrafiltrate retarded the initial growth more than the macromolecular fraction (Fig. 2). The standard error of the mean (SEM) values were <2% units on all occasions. The concentration of oxalate after 3 h was estimated to be 0.03 mmol/l (31% of the initial 0.1 mmol/l) and assuming a 1:1 stoichiometry [10] the final calcium concentration would be 1.48 mmol/l. This corresponded to a concentration product of \(4.49 \times 10^{-8}\) (mol/l)^2.

Both the uninhibited and all three inhibited crystal growth systems followed second-order kinetics, as shown by the transformed values, which could well be fitted to a straight line (\(r = 0.98\)–0.99) (Fig. 3). The rate constants and inhibitory indices are shown in Table 1.

The preincubation of the macromolecular fraction with calcium oxalate crystals caused an almost complete disappearance of the inhibitory activity in this fraction of the urine. This effect was slightly less when incubation was performed with the lower concentration of calcium oxalate

![Fig. 2. Crystal growth with saline (——), urinary macromolecular fraction (■——■), ultrafiltered urine (□——□) and centrifuged urine (▲——▲) along with the stable, unseeded solution (○——○). The ordinate shows [14C]oxalate radioactivity in solution as % of initial radioactivity. Each point represents the mean of three experiments. SEM values were <2% units in all, and are not marked on the Figure.](image1)

![Fig. 3. [14C]Oxalate (as % of initial radioactivity) at various times (Ox^*_t), transformed according to the solution of the kinetics equation. Ox^*_0 is approximated by the 3 h value. The additives were saline (●——●), urinary macromolecular fraction (■——■) and ultrafiltered (□——□) or centrifuged urine (▲——▲). Means ± SEM of three experiments are shown.](image2)
crystals. Preincubation of the macromolecular fraction with uric acid crystals caused less than 10% decrease in the inhibitory activity compared with results of incubation with saline. Preincubation with sodium urate crystals caused a slightly greater decrease in the inhibitory activity, but still less than 18%.

When the low concentration (37 mmol/l) of sodium urate crystals was incubated with the macromolecular fraction in the presence of a metastable calcium oxalate solution 80% of the inhibitory activity disappeared, compared with 13% without calcium oxalate. The corresponding reduction with uric acid crystals (37 mmol/l) was 55% (Fig. 4).

**TABLE 1.** Rate constants (means ± SEM) and inhibition indices for the crystal growth systems with various additives

<table>
<thead>
<tr>
<th>Additive</th>
<th>Rate constant [(mmol/l min)-1]</th>
<th>Inhibition index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>8.0 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>Centrifuged urine</td>
<td>1.3 ± 0.1</td>
<td>84 ± 37</td>
</tr>
<tr>
<td>Ultrafiltered urine</td>
<td>1.7 ± 0.1</td>
<td>79 ± 23</td>
</tr>
<tr>
<td>Macromolecular fraction</td>
<td>2.8 ± 0.2</td>
<td>65 ± 18</td>
</tr>
</tbody>
</table>

Preincubation of the macromolecular fraction with the saturated solutions of calcium oxalate or uric acid did not change the inhibitory activity, but on incubation with a metastable calcium oxalate solution a 25% decrease was observed.

Preincubation of the ultrafiltrate with saturated solutions did not cause any substantial impairment of the inhibitory activity of the ultrafiltrate compared with incubation with saline, but a slight decrease was seen on incubation with calcium oxalate, uric acid or sodium urate crystals (Table 2).

**Discussion**

The inhibition of calcium oxalate crystal growth can be assessed by studying the effects of an added test substance to a seeded crystal growth system [4, 13–15]. Monitoring of the process by studying the disappearance of a radioactive tracer from the solution has been shown to be useful in the case of calcium oxalate [14, 15]. An exchange of [14C]oxalate in solution and oxalate on the crystals does take place, but is negligible compared with the incorporation of [14C]oxalate corresponding to crystal growth in the oxalate concentration used here (results not shown). The present study revealed that the crystal growth process, as investigated by [14C]oxalate disappearance from solution, followed very closely second-order kinetics of crystal growth (Fig. 3). This could be predicted from the degree of supersaturation of the solutions used, indicating a mainly surface-regulated crystal growth [10, 12, 16]. Furthermore, the final concentration of oxalate when estimated at 3 h from the fractional [14C]oxalate in solution, the corresponding final calcium concentration, and assuming a 1:1 stoichiometry of calcium oxalate crystal growth yielded a concentration product of 4.49 ± 0.75 × 10⁻⁸ (mol/l)². This is only slightly above the solubility product (3.44 ± 0.55 × 10⁻⁸ (mol/l)²), indicating that the process was almost finished after 3 h and corresponded to a 91% reduction of the initial supersaturation, which had a concentration product of 1.55 ± 0.75 × 10⁻⁸(mol/l)².

In our study the ultrafiltrate was a more potent...
inhibitor than the macromolecular fraction, and urine which had only been centrifuged retarded the crystal growth even more. In contrast the major inhibitory activity has been claimed to be contained in the non-dialysable fraction of urine [6] but similar inhibition by the ultrafiltrable and the macromolecular fractions of urine has also been reported [17]. A possible explanation may be that in our study relatively large volumes (9%) of the substances to be tested were added. The endogenous calcium oxalate content in the ultrafiltrate may have disturbed the preset, standardized supersaturation but the significance of this is uncertain. Furthermore, the present method measures crystal growth but the macromolecular fraction of the urine is probably more important as an inhibitor of crystal aggregation (W. G. Robertson, personal communication).

Another plausible explanation for the variable results is that there is no simple linear relation between the concentration and the activity of the inhibitors [18, 19]. High concentrations of the urinary fractions as used here do not necessarily mean equal increases in inhibitory activity. For the same reason one cannot expect a simple additive effect of inhibitors [18, 19]. In our study, the sum of the inhibitory activities in the ultrafiltrate and the macromolecular fractions exceeded that in whole urine, irrespective of how the degree of inhibition was deduced (Table 1).

The ultrafiltrate seems to behave differently from the macromolecular fraction in relation to calcium oxalate crystals. The inhibitory activity of the macromolecular fraction disappeared almost completely after incubation with a multitude of crystals, whereas that of the ultrafiltrate was only slightly affected (Fig. 4, Table 2). This indicates that the macromolecules remained adsorbed to the crystals when these were separated through centrifugation and Millipore filtration. It has been shown that glycosaminoglycans can be recovered through hydrochlorine dissolution from calcium oxalate crystals [6]. Most of the urinary glycosaminoglycans were probably contained in the non-ultrafiltrable fraction, and for steric reasons even some of the minor fraction of those with a molecular weight below 10,000 daltons [20]. Constituents of the ultrafiltrate (magnesium, citrate etc.) are probably not incorporated into the crystal lattice as permanently as are the macromolecules [21]. The slight impairment of the inhibitory activity seen in the ultrafiltrate after incubation with calcium oxalate crystals may be an effect of ultrafiltered pyrophosphate, which has been shown to poison the active growth sites of the crystals in a more permanent way than, e.g., magnesium [21]. The role of ultrafiltered, middle-molecular sized fragments of the glycosaminoglycans or acidic peptides [22] is a matter of speculation.

Preincubation of the macromolecular fraction with the large amount of sodium urate and uric acid crystals used here had only a slight effect on the inhibitory activity seen in the supernatant after centrifugation, which indicates that the macromolecules in the urine did not adsorb to these crystals per se as they did to calcium oxalate crystals. It has been shown that heparin adsorbs to sodium urate crystals [2, 3] and that the increase in formation product ratio of calcium oxalate seen by addition of heparin was completely attenuated after preincubation of heparin with sodium urate crystals [3]. Heparin was not adsorbed on to other urate salts tested, including potassium urate, ammonium urate and uric acid. Although heparin has been used as a model for urinary glycosaminoglycans [9], it is probably excreted in human urine only in minute amounts [5, 7, 8], if it exists extracellularly in man at all [20]. Chondroitin sulphates also caused an increase in the formation product ratio but did not adsorb to any of the tested urate salts [9]. Chondroitin sulphates and non-sulphated chondroitin are the major glycosaminoglycans in the urine [5, 7] and those adsorbed to calcium oxalate crystals were mainly chondroitin sulphates (C-4-S, C-6-S) [6].

It has been shown that the adsorption of heparin on to sodium urate crystals was greatly enhanced in the presence of calcium [2]. The polyanionic glycosaminoglycans are known to bind bivalent cations (Ca$^{2+}$) about 100 times better than univalent cations (Na$^{+}$) [20]. When sodium urate crystals were preincubated with the macromolecular fraction of urine in the presence of a metastable solution of calcium oxalate, 80% of the inhibitory activity was lost compared with 13% in the absence of calcium and oxalate. A similar, but less pronounced, reduction was seen with uric acid crystals. We suggest therefore that even large amounts of sodium urate or uric acid crystals do not adsorb into the macromolecular inhibitors of calcium oxalate crystal growth, but in the presence of calcium and oxalate in solution the potency for adsorption may be significant.

The mechanism in vivo whereby the inhibition of calcium oxalate aggregation by urinary glycosaminoglycans may be counteracted by the presence of urate [1] is not completely clear. The issue has been questioned by other investigators, using a similar technique for measuring crystal aggregation [23]. Nevertheless, a reduction of urinary urate seems
to increase the formation product ratio of calcium oxalate [24] and the inhibition of calcium oxalate crystal growth [25]. The amount of urate crystals used here was larger than could be expected in vitro. The results still suggest a mechanism whereby calcium oxalate adheres to the sodium urate crystal surface because of structural similarities [26, 27] and creates an adsorptive site for glycosaminoglycans. Whether or not such a mechanism is true in this situation has not yet been demonstrated. The possibility of calcium oxalate having precipitated and absorbed the inhibitors seems unlikely as calcium oxalate in this degree of supersaturation was shown to be stable for 3 h. A reduction in urinary urate perhaps diminishes the potential for heterogeneous growth of calcium oxalate on sodium urate microcrystals [11, 28], capable of adsorbing glycosaminoglycans in the urine.

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References


