Kidney International, Vol. 28 (1985), pp. 636–639

Bladder secretion of inhibitors of calcium oxalate crystal growth

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Bladder secretion of inhibitors of calcium oxalate crystal growth. Differences in calcium oxalate crystal growth inhibition were studied in normally voided urine (bladder urine) and in urine collected directly from the kidney (kidney urine) in nine dogs. Urine samples were collected before and 10 days after bilateral ureterostomies. Calcium oxalate crystal growth inhibition was measured in a standard seeded crystal growth system. The alcian blue-precipitable material of the urine samples was determined. Significantly lower values were observed in kidney urine than in bladder urine for calcium oxalate crystal inhibition (mean difference, 0.07 ± 0.02 inhibitor units/mg creatinine; $P < 0.01$) and for the alcian blue-precipitable material (mean difference, 0.07 ± 0.02 mg/mg creatinine; $P < 0.01$). We conclude that the bladder adds calcium oxalate crystal growth inhibition to urine. Glycosaminoglycans from the bladder mucosa may be responsible; however, other acidic polymers such as RNA fragments or glycopeptides have been shown to be a constituent of the alcian blue-precipitable material. These are potent inhibitors of calcium oxalate crystal growth, and their participation in the increase of inhibition observed in bladder urine cannot be excluded. Total calcium oxalate crystal growth inhibition present in normally voided urine may be an overestimation of the actual inhibition present at the level of the kidney, where calculi usually form.

Sécrétion vésicale d'inhibiteurs de la croissance des cristaux d'oxalate de calcium. Les différences dans l'inhibition de la croissance des cristaux d'oxalate de calcium ont été étudiées dans des urines émises normalement (urines vésicales) et dans des urines directement collectées dans le rein (urines rénales) chez neuf chiens. Les échantillons urinaires ont été collectés avant et 10 jours après urétérostomie bilatérale. L'inhibition de la croissance des cristaux d'oxalate de calcium a été mesurée dans un système de croissance standard autour d'un cristal. Le matériel précipitable au bleu alcian dans les échantillons urinaires a été déterminé. Des valeurs significativement moindres ont été observées dans les urines rénales par rapport aux urines vésicales pour l'inhibition des cristaux d'oxalate de calcium (différence moyenne, 0,07 ± 0,02 unités inhibitrices/mg créatinine; $P < 0,01$) et pour le matériel précipitable au bleu alcian (différence moyenne 0,07 ± 0,02 mg/mg créatinine; $P < 0,01$). Nous concluons que la vessie ajoute un inhibiteur de la croissance des cristaux d'oxalate aux urines. Les glycosaminoglycans de la muqueuse vésicale pourraient être responsables; cependant, d'autres polymères acides, comme des fragments de RNA ou des glycopeptides pourraient être des constituants du matériel précipitable au bleu alcian. Ce sont des inhibiteurs puissants de la croissance des cristaux d'oxalate, et leur participation à l'augmentation de l'inhibition observée dans les urines vésicale ne peut être exclue. L'inhibition totale de la croissance des cristaux d'oxalate de calcium présente dans les urines normalement émises pourrait être une surestimation de la véritable inhibition présente au niveau rénal, où les calculs se forment d'habitude.

Received for publication January 30, 1984,
and in revised form April 1, 1985

The presence of inhibitors of calcium oxalate crystal growth and aggregation in urine has been considered important for the prevention of calcium oxalate stone formation because urine usually is supersaturated with calcium oxalate [1]. A decrease of inhibitory activity in the urine of patients who form calcium oxalate calculi within the urinary tract has been suggested as a risk factor for calcium stone formation [2]. Yet, measurements of the total inhibitory activity in 24-hr urine specimens from normal subjects and from patients with idiopathic formation of calcium oxalate stones have led to conflicting results. Robertson et al [2] reported a decrease in the urinary excretion of calcium oxalate crystal growth inhibitors in patients with idiopathic calcium urolithiasis. Their data showed a wide overlap between the individual values of inhibitory activity for patients in whom stones formed and for normal subjects. Conversely, others [3, 4] found no differences in the level of calcium oxalate crystal growth inhibition in 24-hr urine specimens from patients with calcium oxalate stones and from normal subjects.

The fact that the uroepithelium of the bladder is covered with the glycocalyx-containing glycosaminoglycans [5, 6] and that glycosaminoglycans have been shown to be potent inhibitors of both calcium oxalate crystal growth and aggregation [7, 8] suggests that the bladder may not behave as an inert reservoir in terms of calcium oxalate crystal formation. In a preliminary report, Edyvane, Ryall, and Marshall [9] noted a significant amount of inhibitory activity in saline washes of the bladder. This observation suggested that some of the inhibition of calcium oxalate crystal formation observed in voided urine specimens may come from the bladder. The possibility that urine contains significant amounts of calcium oxalate crystal growth inhibitors that are of bladder origin might have two major consequences. First, measurements of calcium oxalate crystal growth inhibition in voided urine would overestimate the inhibition present at the level of the kidney, where calcium oxalate stones usually form. Second, this possibility could explain the apparent lack of difference in the amount of calcium oxalate crystal growth inhibition between normal subjects and

patients with idiopathic calcium oxalate urolithiasis, as reported in some studies [3, 4].

The purpose of this study was to compare the amount and chemical nature of the inhibition of calcium oxalate crystal growth present in kidney urine with that present in voided (bladder) urine.

Methods

Nine female mongrel dogs weighing 13.8 ± 2.0 kg were used in these experiments. They were fed the same standard diet 4 days before the experiment and for its duration.

Technique of tubeless ureterostomies. Operation was performed with the dogs under phenobarbital anesthesia. The ureters were approached through a midline incision and were dissected away from the retroperitoneal space, and care was taken to preserve most of the vascular supply. After each ureter had been mobilized from the bladder junction to the lower pole of the kidney, it was divided at the ureterovesical junction. A tunnel was formed in the abdominal muscle and skin 5 cm below the costal margin on each side. The muscular tunnel easily accommodated the size of a finger. The skin orifice was 1.5 by 0.5 cm and rectangular. The ureter was then pulled through this musculocutaneous tunnel, and any twisting or tension was avoided. The distal 1 cm of the ureter was split longitudinally into two parts [10]. Each ureter was then anchored to the underlying tissues and sutured to the skin with 6/0 nylon sutures in order to fill the rectangular skin defect that was formed previously.

The dogs were given a 3-day course of antibiotics (penicillin-Colistimethate, 1 million units/day) and were kept in metabolic cages to provide good drainage of urine and to prevent skin irritation. Excretory urograms were performed with the intravenous injection of 30 ml of contrast medium (Hypaque-M, 90%) 18 days after operation. If ureteral dilatation was present, the dog was excluded from the study. Blood samples were withdrawn pre-operatively and 3 weeks post-operatively (at sacrifice) for the determination of serum creatinine.

*Technique of urine collection.*The periods of urine collection started 1 hr after the morning meal and lasted 6 hr. The dogs had free access to water during all collection periods. Two collections of bladder urine and kidney urine were available in each dog. The urine was kept frozen until processed for the measurement of calcium oxalate crystal growth inhibition, alcian blue-precipitable material, calcium, and creatinine.

Bladder (pre-operative) collections were performed in two ways. The first collection was made in a metabolic cage. When a dog did not micturate during the period of observation (6 hrs), the collection period was extended until urine was found in the collecting device. Although this method did not allow accurate 6-hr collections of urine, it was preferable to bladder catheterization, which would have required anesthesia and could have produced irritation of the bladder mucosa. A second collection was performed at the time of operation by direct suprapubic puncture and evacuation of the bladder. Operation was scheduled during the afternoon so that urine collected at that time corresponded to a 6-hr postprandial collection.

Kidney urine was collected 10 days after operation on 2 consecutive days. A collecting device, including a belt that supported two receptacles, was tightened on the dog's abdomen. The orifices of the two receptacles were applied to the ureterostomies. Because of the fragility of this device in association with the dog's movements, semi-immobilization in a harness was necessary. This collection device was satisfactory because it did not allow leakage of the urine. After collection, urine from the two kidneys was pooled before freezing.

Measurements of calcium oxalate crystal growth inhibitors. A seeded crystal growth system [11] was used to measure the inhibition present in the urine samples. In this system, one inhibitor unit corresponds to that concentration of any inhibitor of crystal growth necessary to produce a 50% reduction in the rate of crystal growth [12]. Results were expressed as inhibitor units per liter.

Determination of the concentration of alcian blue-precipitable material. The concentrations of alcian blue-precipitable material in the urine samples were determined by the method of Whiteman [13]. Fifty microliters of urine was mixed with 1 ml of reagent that contained 0.05% alcian blue 8GN (MCB Chemicals, subsidiary of EM Science, Cincinnati, Ohio, USA), 50 mM magnesium chloride, and 50 mM sodium acetate buffer adjusted to pH 5.8 with acetic acid. After 2 hrs, the aliquots were centrifuged and the precipitate was washed with 2 ml ethanol and dissociated with 1 ml of 40% Manoxol 1B (BDH Chemicals, England). The absorbence of the resulting blue solution was measured on a spectrophotometer (DMS 90, Varian Associates, Inc., Sunnyvale, California, USA) at 620 nm. The content of alcian blue-precipitable material in the samples was determined by a reference curve, and chondroitin 4 sulfate (Grade III, Sigma Chemical Co., St. Louis, Missouri, USA), was used as a standard. All samples were analyzed in duplicate, and concentrations were expressed in milligrams per liter.

Analysis of data

The amounts of calcium oxalate crystal growth inhibition and alcian blue-precipitable material were expressed as inhibitor units per liter and milligrams per liter, respectively, divided by the creatinine concentration in milligrams per liter of the sample. The two values obtained for kidney and bladder urine were averaged. Analysis of the difference between kidney and bladder urine was done by the sign test.

Results

Normal renal function and absence of dilatation of the urinary tract. Plasma creatinine values were 1.05 ± 0.15 mg/dl pre-operatively and 0.97 ± 0.16 mg/dl post-operatively. The hourly creatinine excretion was 28 ± 4 mg, a value that is not different from the value of 26 ± 5 mg observed in normal dogs. Excretory urograms showed normal upper urinary tracts and ureters without obstruction.

Calcium oxalate crystal growth inhibition. The amount of calcium oxalate crystal growth inhibition per milligram of creatinine showed an increase in each animal from kidney urine (0.07 ± 0.01 inhibitor units/mg creatinine) to bladder urine (0.14 ± 0.03 inhibitor units/mg creatinine). Changes between kidney and bladder urine were statistically significant (mean change, 0.07 ± 0.02; $P < 0.01$). The increase in calcium oxalate crystal growth inhibition observed in bladder urine was 104 ± 30% of the value found in kidney urine (Fig. 1, left).

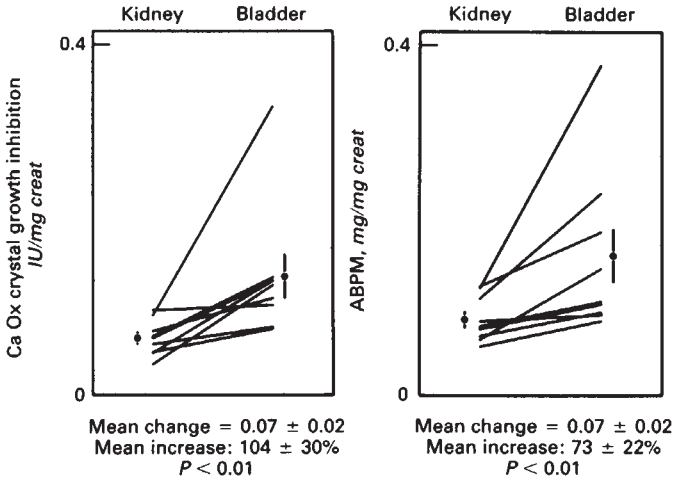

Fig. 1. Left Calcium oxalate (Ca Ox) crystal growth inhibition in kidney and bladder urine. **Right** Alcian blue-precipitable material (ABPM) in kidney and bladder urine. Abbreviations are: creat, creatinine; IU, inhibitor units.

Concentration of alcian blue-precipitable material. The amount of alcian blue-precipitable material similarly showed an increase from kidney urine (0.087 ± 0.009 mg/mg creatinine) to bladder urine (0.16 ± 0.03 mg/mg creatinine). The mean change (0.07 ± 0.02 mg/mg creatinine) was statistically significant ($P <$ 0.01). The increase in the content of alcian blue-precipitable material observed in bladder urine was 73 ± 22% of the value found in kidney urine (Fig. 1, right).

Discussion

An increase in calcium oxalate crystal growth inhibition as urine moved from the kidney to the bladder and a parallel increase in the alcian blue-precipitable material were observed in this study. The contribution of the bladder is substantial; the mean increases in inhibition of calcium oxalate crystal growth and in alcian blue-precipitable material in bladder urine were 104 ± 30% and 73 ± 22%, respectively.

In urine, substances that precipitate with alcian blue include glycosaminoglycans, Tamm-Horsfall protein, RNA fragments [8], and possibly other acidic polyanions. After removal of Tamm-Horsfall protein, which is not a calcium oxalate crystal growth inhibitor in our seeded crystal growth system (unpublished results), glycosaminoglycans account for the main part of the alcian blue-precipitable material in urine by weight [13]. Other polyanions, such as RNA that precipitates with alcian blue, are believed to be present in urine in small quantities (from 5 to 20 mg/liter) and would account for approximately 2% of the alcian blue-precipitable material [8, 14]. The alcian blue-precipitable substance added to urine by the bladder was not characterized in our study.

If we assume that the increase in alcian blue-precipitable material found in bladder urine (mean change, 0.07 ± 0.02 mg/mg creatinine) was responsible for the increase in inhibition (mean change, 0.07 ± 0.02 inhibitor units/mg creatinine), it is possible to estimate the concentration at which the alcian blue-precipitable substance would give 1 inhibitor unit. If we assume that the molecular weight of this substance is between 20,000 and 150,000, the molar concentration that would give 1 inhibitor unit would be between 10^{-8} and 10^{-9}. In comparison, 4.6×10^{-6} M chondroitin sulfate from shark cartilage, 6.3×10^{-9} M heparin, and 3.2×10^{-9} M RNA from yeast will produce 1 inhibitor unit [15]. This would suggest that something other than chondroitin sulfate is responsible for the added inhibition in bladder urine observed in our study. However, it is unlikely that urinary chondroitin sulfate is identical to that obtained from shark cartilage. Glycosaminoglycans isolated from urine have been shown to be more potent inhibitors of calcium oxalate crystal growth than those obtained commercially [16]. Participation of other polyanions, such as the acidic urinary glycoprotein described by Ito and Coe [17], also a potent inhibitor of calcium oxalate crystal growth, cannot be excluded. The presence of glycosaminoglycans on the bladder mucosa [6] makes them an available source of the increased inhibition observed in bladder urine, but other more potent inhibitors may be involved.

The fact that urine contains less calcium oxalate inhibition in the kidney than in the bladder increases the relative importance of the inhibitors that are known to be present in kidney urine in patients with calcium oxalate stone formation. Low molecular weight inhibitors such as citrate [12] and pyrophosphate [18] are excreted at the level of the renal tubule and, therefore, are present in kidney urine. These two substances contribute 10 to 15% of the total calcium oxalate inhibition observed in voided (bladder) urine [8]. Because kidney urine contains about 50% less inhibition than voided urine, pyrophosphate and citrate could contribute 20 to 30% of the total inhibition present in kidney urine. Therefore, citrate and pyrophosphate would seem to be more important in calcium oxalate inhibition and stone formation than thought previously, and medical treatment that increases the urinary excretion of citrate and pyrophosphate should benefit patients with calcium oxalate stones. Some high molecular weight inhibitors, such as glycopeptide isolated from human kidney tissue culture, may also be present at the level of the kidney [19]. The contribution of this glycopeptide to calcium oxalate crystal growth inhibition, estimated to be 18 to 50% in voided urine [17], would be increased in urine at the level of the kidney.

The increase in calcium oxalate crystal growth inhibition in bladder urine compared with kidney urine observed in this study leads to three observations and speculations that may be of clinical importance: *1)* the measurement of calcium oxalate crystal growth inhibition in voided urine may be an inaccurate estimation of the inhibition present at the level of the kidney; *2)* citrate and pyrophosphate, as well as other inhibitor substances of renal origin, may be more important inhibitors than thought previously; and *3)* the study of the characterization and separation of the inhibitors of calcium oxalate crystal formation may be more fruitful when it is performed with kidney urine.

Acknowledgments

The authors wish to express their thanks to Mr. Jan H. Bergert for technical assistance and Mrs. Sherry L. Linander and Mrs. Patty Winkels for secretarial assistance.

An abstract of this report was presented at the meeting of the American Society of Nephrology, Washington, D.C., USA, December 4–6, 1983.

This investigation was supported in part by Research Grants AM-20605 and HL-16496 from the National Institutes of Health, Public

Health Service, and by a grant from the Fondation Pour la Recherche Medicale, Paris, France.

Dr. Martin was a visiting scientist from the Service D'Urologie et de Chirurgie de la Transplantation Hôpital E. Herriot (Pr Dubernard), INSERM U80 (Pr Traeger)-Lyon, France.

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