Effect of Brush Border Membrane Enrichment With Phospholipids on Nucleation of Calcium Oxalate

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ABSTRACT

Kidney Stone (renal calculi) is a solid mass composed of crystals, which form in the urine and accumulate in the kidney tubules. Crystal nucleation, growth, and retention are all involved with the development of kidney stones. We investigated the crystallization of calcium oxalate (CaOx) since it is the most common component of kidney stones. Cell membrane fragments derived from renal tubular epithelial cells are common in the human urine. They contain phospholipids such as Sphingomyelin (SM), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), Phosphatidylcholine (PC), and Phosphatidylinositol (PI) that can promote nucleation of CaOx crystals. Brush Border Membrane (BBM) can be enriched with specific phospholipids to determine which phospholipid is involved in crystal nucleation.

The BBM was obtained from normal male rats weighing at least 200-250 grams. A constant composition crystallization system was used to determine the crystallization potential of calcium oxalate solution with or without BBM. The experiment was carried out in Pipes buffer saline at two different relative supersaturations (RSS), RSS 6 and RSS 10. The buffer solution was composed of 0.01 M PIPES and 0.15 M NaCl. The pH was adjusted to 6.3 with either NaOH or HCl. The calcium and oxalate titrants were prepared separately by adding 0.05 M of calcium chloride for the calcium and 0.05 M of sodium oxalate for the oxalate to 100ml of buffer. Then the desired amount of calcium titrant was added into the reaction vessel and allowed to equilibrate for an hour. After one hour, the desired amount of oxalate titrant was added for RSS 6 or RSS 10. Next, the autotitrator endpoint is set and titration was started. Finally, the BBM enriched with different phospholipids was added, and the rate of oxalate addition for each phospholipid was compared to the rate for the normal BBM. Our study demonstrated that at an RSS of 10 the BBM enriched with PE and PC titrated at a faster rate when compared to the normal BBM. Membrane enriched with PI, SM, and PS titrated at a slower rate when compared to the normal BBM.

INTRODUCTION

Nephrolithiasis (kidney stone disease) is a common urological disease, accounting for approximately 200,000 hospitalization per year in the US alone. The ratio of men stone formers to women stone formers is 4:1 (1, 2). This disease usually peaks between the ages of 25 to 30 years, but it may continue through old age. For
the individuals who are affected by this disease, there is a 60 to 80% chance of reoccurrence within a lifetime and a 10% chance of reoccurrence within the first year following an episode (3). Furthermore, most cases of urinary stones within the United States are unknown. For that reason, we are researching the complexity of stone formation, which may help improve treatments as well as prevent this disease from reoccurring.

Calcium oxalate (CaOx) crystals make up 70 to 80% of those seen in stone forming patients. Many factors may contribute to the cause of renal calculi. These include urine supersaturation, crystal formation, and maturation of the crystal (4). CaOx crystals are known to damage the renal tubular membranes as they pass through the kidney. Current research has focused on the function these membranes may have when they are disrupted and their lipids are released (1,2,4). It is our hypothesis that membrane and their lipids, particularly the phospholipids, act as a substrate for the nucleation and growth of renal calculi (1). Membranes contain phospholipids such as sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylinositol (PI). We further hypothesize that these lipids differ from each other in their crystallization promotion capabilities. To determine the involvement of membranes and their lipids is the main aim of this investigation. Membranes were isolated from rat renal proximal tubular brush border and incubated in a metastable solution of CaOx using a constant composition crystallization system.

**MATERIALS AND METHODS**

The experiment was carried out in Pipes buffer saline at two relative supersaturations (RSS), RSS 6 and RSS 10. A constant composition system was set up in a small reaction vessel containing the sample into which is placed a calcium ion selective electrode. The electrode measures the calcium concentration with a pH meter that is connected to an autotitrator. The autotitrator controls two auto-burettes, one for the calcium and one for the oxalate titrant. Through a feed back loop the autotitrator maintains a set level of calcium concentration. Each titrant was composed of 100 mls of pipes buffer with 0.05M calcium chloride and sodium oxalate added respectively. The pH each solution was adjusted to 6.3 with either NaOH or HCl followed by filtration with 0.22 *m filter. To the reaction vessel the desired amount of calcium titrant was slowly added to yield a RSS 6 or RSS 10 and allowed to equilibrate for an hour. After one hour, the desired amount of oxalate titrant was added to yield a RSS 6 or RSS 10. Once the calcium ion electrode is stable, usually within 5 minutes, the endpoint is set and the titration experiment begins (5).

The BBM was obtained from male rats, each weighing between 200-250 grams using standard techniques (2). The BBM was enriched with various phospholipids, sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) obtained from Avanti Polar Lipids. Liposomes were made by suspending 5 mg of individual oil based lipid in an aqueous solution with the aid of sonication. The liposome mixture is then added to the BBM in a ratio of 1:10 BBM to Liposomes and incubated one at least 1 hour at room temperature, with gentle mixing. After one hour the liposome BBM mixture is centrifuged at 18,000 rpms for 20 minutes to pellet the membranes. The BBM enriched membranes are then washed twice with 10 mls of Tris buffer to remove any excess liposomes. The membranes are
finally reconstituted in 1-2 mls of Tris buffer and analyzed for their lipid content (4).

A small aliquot is taken for protein analysis before the enriched membranes are checked for lipids. The lipids were extracted from the membrane isolates using Chloroform:Methanol (CM) (2:1) with the aid of sonication for 15 mins followed by overnight extraction at 4C. The next day two volumes of normal saline is added to the CM mixture and allowed to phase separate into three layers, organic, interface and aqueous with the aid of centrifugation. The organic is Folch-washed 3 times using theoretical upper phase and filtered using Whatmann #1 phase separating paper to remove contaminating debris. All phases are dried under nitrogen stream and lyophilized to complete dryness. Samples are weighed, re-suspended in a known volume of chloroform with 0.1% BHT added to prevent oxidation, placed in a brown bottle, blanketed with nitrogen, and stored at -70*C until further analysis. The organic is separated into various lipid classes by using a Bio Sil A column. The phospholipids were isolated with 250 mls of methanol, dried under nitrogen, weighed, reconstituted in a known volume of CM (2:1), and stored at -70*C. The phospholipids are then analyzed for acidic and neutral phospholipids and for phosphorus content. Further separation of the phospholipids and identification of individual lipids was accomplished by thin layer chromatography with known standards run in tandem. Individual phospholipids were quantitated by densitometry and the Bartlet method.

RESULTS

The addition of BBM to calcium oxalate solutions of RSS 10 resulted in consumption of calcium and oxalate and crystallization of CaOx. Most runs were terminated after 165 minutes after the onset of the titration or when the calcium consumption became constant (5). No crystallization occurred in a solution with RSS 6. BBM enriched with PE and PC titrated at a faster rate when compared to the normal BBM (Figure 1). Membrane enriched with PI, SM, and PS titrated at a slightly slower rate when compared to the normal BBM (Figure 1).

DISCUSSION AND CONCLUSIONS
The formation of kidney stones involves nucleation, growth, and aggregation of stone forming crystals and their retention in the kidneys (3). First step is crystal nucleation, which requires urine to be supersaturated with respect to crystallizing salts such as CaOx. Nucleation can be with or without the presence of a substrate. The former requires extremely high CaOx supersaturation, approximately 70 while the highest CaOx supersaturation found in the urine of idiopathic stone formers is 30. Thus, human urine requires a substrate for crystal nucleation. Since high oxalate can be injurious to renal epithelial cells resulting in their death and breaking apart into membrane vesicles, we hypothesized that cell membranes of these vesicles can act as nucleators of CaOx.

The results of this study show that cell membranes derived from the renal epithelial cells can promote crystallization of CaOx in a solution with calcium and oxalate concentrations and CaOx supersaturation which can exist in the urine of human stone formers, thus supporting our hypothesis. Results also demonstrate that enrichment of BBM with PC and PE increased the rate of CaOx crystallization while enrichment with PS, PI, and SM decreased the rate of CaOx crystallization. Thus, the results also support the hypothesis that membrane phospholipids differ from each other in their capabilities to promote nucleation of CaOx crystals.

Previous studies have suggested that the damage and release of these lipids from the membranes can support crystal nucleation and adhesion within the renal tubules. Renal tubular brush border membrane, the largest membrane pool in the kidney, is rich in phospholipids and very susceptible to injury resulting in exposure of the phospholipids (4). Thus, one can assume that the BBM would be an excellent promoter of CaOx crystallization, although there was a decrease in the time it took to form the crystals, causing nucleation to occur sooner than it would normally occur (3). This increase in the rate of crystallization may suggest that more crystals are being nucleated, and more growth occurs than would occur without BBM (3).

Human urine is a complex solution containing more ions than calcium and oxalate and a variety of proteins and other organic substances excreted in the urine. Our experiments were carried out in simple solution and so this does not exactly simulate the conditions in the kidneys. Results, however, suggest that an insult to the kidneys causing cell death and destruction can promote crystallization of CaOx and put kidneys to the risk of stone formation.

REFERENCES


