Characterization of glycosaminoglycans in tubular epithelial cells: Calcium oxalate and oxalate ions effects

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Background. The interaction between tubular epithelial cells and calcium oxalate crystals or oxalate ions is a very precarious event in the lithogenesis. Urine contains ions, glycoproteins and glycosaminoglycans that inhibit the crystallization process and may protect the kidney against lithogenesis. We examined the effect of oxalate ions and calcium oxalate crystals upon the synthesis of glycosaminoglycans in distal [Madin-Darby canine kidney (MDCK)] and proximal (LLC-PK1) tubular cell lines.

Methods. Glycosaminoglycan synthesis was analyzed by metabolic labeling with 35S-sulfate and enzymatic digestion with specific mucopolysaccharidases. Cell death was assessed by fluorescent dyes and crystal endocytosis was analyzed by flow cytometry.

Results. The main glycosaminoglycans synthesized by both cells were chondroitin sulfate and heparan sulfate most of them secreted to the culture medium or present at cellular surface. Exposition of MDCK cells to oxalate ions increased apoptosis rate and the incorporation of 35S-sulfate in chondroitin sulfate and heparan sulfate, while calcium oxalate crystals were endocyted by LLC-PK1, induced necrotic cell death, and increased 35S-sulfate incorporation in glycosaminoglycans. These effects seem to be specific and due to increased biosynthesis, since hydroxyapatite and other carboxylic acid did not induced cellular death or glycosaminoglycan synthesis and no changes in sulfation degree or molecular weight of glycosaminoglycans could be detected. Thapsigargin inhibited the glycosaminoglycan synthesis induced by calcium oxalate in LLC-PK1, suggesting that this effect was sensitive to the increase in cytosolic calcium.

Conclusion. Tubular cells may increase the synthesis of glycosaminoglycans to protect from the toxic insult of calcium oxalate crystals and oxalate ions, what could partially limit the lithogenesis.

Key words: calcium oxalate, potassium oxalate, glycosaminoglycan, proteoglycan, MDCK, LLC-PK1.

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Nephrolithiasis is a multifactorial disease. Many aspects of its pathogenesis have been widely investigated. One of them is the interaction between calcium oxalate, the crystal present in most of the human stones, and tubular epithelial cells. The effects of oxalate ion upon tubular cells have also been studied. High oxalate concentrations provide conditions for precipitation of calcium oxalate crystals in the urine, and both calcium oxalate crystal and oxalate ions induce renal injury [1, 2], which generates cellular debris and promotes crystal nucleation. The surviving tubular cells may adapt to the toxic insult by inducing cell proliferation, and synthesis of extracellular matrix (ECM) proteins and crystallization inhibitors [3, 4].

It has been previously shown that glycosaminoglycans inhibit the growth and aggregation of calcium oxalate crystals in vitro [5–7]. The logical extension of these observations was that the urinary glycosaminoglycans could function as inhibitors of calcium oxalate urolithiasis. If endogenous glycosaminoglycans are important factors in the inhibitors of urolithiasis, one might expect significant differences in the urinary glycosaminoglycan levels of stone formers in comparison to nonstone formers. In fact, our laboratory has shown a definite decrease in the urinary glycosaminoglycan concentration of stone forming subjects, both children and adults [8]. Furthermore, there are evidences suggesting that the adhesion of calcium oxalate crystals to Madin-Darby canine kidney (MDCK) cells can be inhibited by heparan sulfate, chondroitin 4-sulfate, and hyaluronic acid [9–11].

In humans, the main urinary glycosaminoglycans are chondroitin sulfate (~80% of total) and heparan sulfate (~20% of total), with small amounts of dermatan sulfate (1% to 2%) and trace amounts of hyaluronic acid and keratan sulfate. Similar glycosaminoglycan compositions have also been found in other mammalian species such as cat [12] and horse [13], but not rat, which excretes lower proportions of chondroitin sulfate [14, 15]. There are evidences suggesting that the urinary chondroitin sulfate is of systemic origin and filtered in the glomerulus.
[12, 16], while the urinary heparan sulfate and, possibly, dermatan sulfate could be of systemic origin or come from the kidney and/or urinary tract [12].

Heparan sulfate is the main kidney glycosaminoglycan, occurring both in cell surface proteoglycans of the syndecan and glypican families, and in perlecans, the main basement membrane proteoglycan [17]. Other basement membrane proteoglycan is bamacan. It is a chondroitin sulfate proteoglycan expressed transiently during nephrogenesis that disappears from the glomerular basement membrane (GBM) upon maturation [18]. Furthermore, the expression of the core protein of four small proteoglycans, decorin, biglycan, fibromodulin, and lumican, has been observed in the renal interstitium, with accumulation around tubules. Low expression of these core proteins was also found in mesangial matrix [19]. But their glycosylation was not assessed.

Decorin core protein was also detected in the urine and glomerulus of patients with membranous nephropathy [19] and IgA nephropathy [20], but not in normal subjects. Moreover, we have previously shown that mesangial cells isolated from diabetic rats synthesized in vitro increased amounts of dermatan sulfate proteoglycans of the small leucine-rich proteoglycan family (SLRPG), and this increase was proportional to the duration of diabetes [21]. Increased expression of dermatan sulfate proteoglycans was also shown in experimental models of chronic renal failure [15] and diabetes mellitus [14] in rats.

On the other hand, increased expression of syndecan-1, a cell surface heparan sulfate proteoglycan, has been demonstrated in rat kidney during calcium oxalate nephrolithiasis [22]. The increased expression of syndecan-1 and heparan sulfate in a cell line derived from MDCK cells led to a decrease in calcium oxalate attachment to these cells [23], suggesting a preventive role for this proteoglycan in calcium oxalate nephrolithiasis.

The goal of the present study was to investigate the effects of calcium oxalate crystals and oxalate ions upon the synthesis of glycosaminoglycans by distal (MDCK) and proximal (LLCP-K1) tubular epithelial cell lines, and upon cell death. The effects of other crystal (hydroxyapatite) also commonly found in renal stones, and other carboxylic anion (formate) was also investigated, as well as the effects of thapsigargin, a calcium ionophore.

METHODS

Materials

Potassium oxalate (K2-oxalate), NaHCO3, N(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (Hepes), NH4OH, and ethanol were purchased from Merck EGA (Darmstadt, Germany). Chondroitinase AC from Flavobacterium heparinum was prepared as described by Aguiar et al [24], and heparitinase II, also from F. heparinum, was prepared as described by Nader et al [25]. Chondroitin 4-sulfate (from whale cartilage) and chondroitin 6-sulfate (from shark cartilage) were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Heparan sulfate (from bovine pancreas) was obtained from Opocrin Laboratories (Modena, Italy). Hyaluronic acid from bovine tracheal cartilage and umbilical cord, Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin, phosphate-buffered saline (PBS), papain, Triton X-100, Trizma base (Tris[hydroxymethyl]aminomethane), x-ray film (Kodak SSB), toluidine blue, thapsigargin, acrydine orange, ethidium bromide, and HOE 33342 [(bisbenzimide Hoe 33342 (2′-(etoxifenil)-5-(4-metil-1-piperazinil)-2,5′-bi-benzimidazole and HCl)] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Agarose were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Cetavlon (cetyl, trimethyl ammonium bromide), N,N-methylenbisacrylamide, 1,3-diaminopropane (PDA) and ethylenediamine were from Aldrich Chemical Co. (Milwaukee, WI, USA). Fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA, USA). Trypsin-ethylenediaminetetraacetic acid (EDTA) solution was purchased from Cultilab (Sao Paulo, SP, Brazil). H235SO4 was purchased from Instituto de Energia Nuclear (Sao Paulo, SP, Brazil) and Whatman Paper number 1 was from Whatman International, Ltd. (W & R Balston Ltd., Maidstone, England).

Tubular cell culture

Distal (MDCK) and proximal (LLC-PK1) tubular epithelial cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM supplemented with 5% FBS, glucose and antibiotics at 37°C, under 5% CO2 humidified atmosphere. At confluence, the cells were detached with trypsin, collected by centrifugation, resuspended in DMEM, and subcultured in 25 cm2 culture plastic flasks.

Preparation of calcium oxalate crystals

Solutions of CaCl2 (0.4 mol/L, 100 mL) and K2-oxalate (0.4 mol/L, 100 mL) were added to distilled and deionized water (300 mL) by constant dripping for 2 hours (1 mL/min). This suspension was maintained under continuous stirring at 75°C for 5 hours, and then washed with deionized water to remove the KCl present. The remaining saturated solution was maintained at 37°C for 15 days for calcium oxalate crystallization. Calcium oxalate crystals were sterilized in ethylene oxide or steam autoclave and culture medium before FBS was added. No difference was observed between the both procedures. The suspension was sonicated for 15 minutes to obtain particles of uniform size.
Exposure of tubular epithelial cells to calcium oxalate or K$_2$-oxalate

Prior to the experiments, cells were transferred to a 6-well plate (1 × 10$^5$ cells/well) and maintained under the culture conditions for 3 days. Previously, we noticed that tubular cells were significantly affected by exposure to deprived culture medium (DMEM-BASE), so we chose to expose tubular cells to oxalate and calcium oxalate crystals diluted in DMEM culture medium to control experimental conditions. At confluence, MDCK and LLC-PK1 were exposed for 24 hours to either DMEM (without FBS, control) or DMEM containing calcium oxalate crystals (100, 200, 400, or 500 μg/mL, corresponding to 0.78, 1.56, 3.12, or 3.9 mmol/L, respectively), K$_2$-oxalate (0.5, 1.0, 2.0, and 4.0 mmol/L), sodium formate (4.0 mmol/L), hydroxyapatite (calcium phosphate) crystals (500 μg/mL), or thapsigargin (2.0 μmol/L).

At the same time, H$_2^{35}$SO$_4$ (100 μCi/mL) was also added to the culture medium for the metabolic labeling of the glycosaminoglycans. The addition of H$_2^{35}$SO$_4$ did not modify the pH of culture medium. After 24-hour incubation, the medium, cell surface, intracellular fraction, and ECM fraction were obtained as previously described [26, 27]. In brief, 1 mL of the culture medium was collected in a centrifuge tube and labeled “medium fraction.” The cells were incubated with Triton X-100 (0.5%) solution in 10 mmol/L Tris-HCl, pH 7.4, buffers. This buffer allowed removing the cellular surfaces without lysing the cell. After 5 minutes’ incubation in an ice bath, under agitation, the supernatant solution was collected and labeled as “cell surface.” Subsequently, NH$_4$OH (0.02 mol/L) solution in the same buffer was added to the phase. After 5 minutes' incubation in an ice bath under agitation, the solution was collected and labeled as “cell fraction.” A sodium dodecyl sulfate (SDS) (2%) solution in the same buffer was added and the residue was suspended with a cell scraper. The solution was collected and labeled as “ECM.” All steps were followed by observation under a phase contrast inverted microscope.

In some experiments, only medium and cell fractions were extracted. The culture medium (1 mL) was collected and labeled “medium fraction.” The cells were treated with 3.5 mol/L urea in 50 mmol/L Tris-HCl, pH 7.4, and scraped from the flask surface with a cell scraper. The solution and transferred to a centrifuge tube and labeled “cell extract” [27].

In both experimental situations, chondroitin sulfate and heparan sulfate (100 μg each) were added, as carriers,
to each fraction. After addition of 3 volumes of ethanol, the mixtures were maintained at −20°C for 24 hours. The precipitates formed were collected by centrifugation and vacuum dried.

For the glycosaminoglycan extraction, the precipitates formed in each fraction were resuspended in 100 μL of phosphate-cysteine buffer (pH 6.5) containing EDTA and 100 μg of papain.

Aliquots (5 μL) of each fraction and a standard aliquot containing chondroitin sulfate and heparan sulfate were submitted to agarose gel electrophoresis in 0.05 mol/L 1,3-diaminopropane-actate buffer (pH 9) [28, 29]. The 35S-labeled compounds were visualized by exposure of the agarose gel slabs (after fixation, drying, and staining with toluidine blue) to Kodak x-ray film for 1 day.

For identification the gel bands were compared to chondroitin sulfate and heparan sulfate aliquots or degraded with specific mucopolysaccharidases (chondroitinase AC and heparitinase II).

For quantification, they were scraped of the gels and counted in Ultima Gold in a Beckman 6800 liquid
Fig. 3. Relative amounts of $^{35}$S-chondroitin sulfate and $^{35}$S-heparan sulfate synthesis by Madin-Darby canine kidney (MDCK) and LLC-PK1 in presence of calcium oxalate (CaOx) (0 to 500 µg/mL) or K$_2$Ox (0 to 4.0 mmol/L). The experiment was performed as described in Fig. 2. Quantitative results are expressed as counts per minute (cpm). Data are mean ± standard error of five experiments. *$P < 0.05$ was considered significant.
Fig. 4. Quantitative data of $^{35}$S-chondroitin sulfate and $^{35}$S-heparan sulfate from culture medium and cell extracted from Madin-Darby canine kidney (MDCK) (A) and LLC-PK1 (B) exposed to hydroxyapatite (HA) (500 µg/mL) and sodium formate (FOR) (4.0 mmol/L) for metabolic labeling of proteoglycans. Cells were incubated with H$_2^{35}$SO$_4$. After 24 hours, the culture medium and cell extract fractions were collected and the $^{35}$S-glycosaminoglycans were isolated as described in the text. Aliquots (5 µL) of each fraction thus obtained were submitted to agarose gel electrophoresis in 0.05 mol/L 1,3-diaminopropane-acetate (PDA), pH 9. The radioactive compounds were visualized by radioautography in the Cyclone Phosphor Image System of the gel slabs and quantified in the liquid cyntillation counter. Quantitative results are expressed as counts per minute (cpm). Data are mean ± standard error of five experiments. *$P < 0.05$ was considered significant.

Fig. 5. Effects of K$_2$Ox and calcium oxalate (CaOx) upon hyaluronic acid synthesis by Madin-Darby canine kidney (MDCK) and LLC-PK1. The experiment was performed as described in Fig. 2, except that hyaluronic acid was measured in cell and medium fractions of MDCK and LLC-PK1 cells exposed to K$_2$-oxalate (4.0 mmol/L) and calcium oxalate (500 µg/mL). Results were expressed as concentration of hyaluronic acid (ng/mL). Data are mean ± standard error of five experiments. *$P < 0.05$ was considered significant.
Fig. 6. Effect of K$_2$Ox and calcium oxalate (CaOx) treatment upon the cellular viability (necrotic and apoptotic cell death) on Madin-Darby canine kidney (MDCK) and LLC-PK1. MDCK exposed to K$_2$-oxalate (0.5 to 4.0 mmol/L) and LLC-PK1 exposed to calcium oxalate (100 to 500 µg/mL) suspensions were centrifuged and incubated with acrydine orange/ethidium bromide solutions or HOE 33342 [(bisbenz-imide Hoe 33342 (2′-(etoxifenil)-5-(4-metil-1-piperazinil)-2,5′-bi-benzimidazole and HCl)] dyes. Ethidium bromide (green) stains all cells while acrydine orange (orange) is excluded from viable cells. Green cells were consid- ered viable while orange cells were considered unviable. HOE 33342 stains the chromatin. Blue cells were considered nonapoptotic and condensed chromatin cells were considered apoptotic. At least 100 cells were counted and results expressed as percentage. Data are mean ± standard error of five experiments.

scintillation spectrometer. The results were expressed as counts per minute (cpm).

Molecular weight determinations were performed by polyacrylamide gel electrophoresis (PAGE) as previously described [30, 31].

Hyaluronic acid was measured as described by Martins et al [32].

Calcium oxalate endocytosis experiments

Calcium oxalate endocytosis was determinate by flow cytometry using 488 nm excitation. In brief, the flow cytom-eter detects how the cell interacts with a focused laser beam in terms of how the cell scatter the incident light and emit fluorescence. In forward scatter light is diffracted at low angles, generally proportional to cell size and the side scatter light is reflected at high angles, proportional to cell granularity and complexity and detected at 90° to incident light axis.

Cultures of LLC-PK1 and MDCK cells were exposed, respectively, to calcium oxalate (100 to 500 µg/mL) and K$_2$-oxalate (0.5 to 4.0 mmol/L), as described above. After 24 hours' incubation, the cells were washed with PBS and incubated with the trypsin-EDTA solution. These proce-dures yield only cells containing internalized or strongly adherent crystals. Cell suspensions (1 × 10^6 cells/mL) were transferred to conical fluorescence-activated cell sorter (FACS) tubes and immediately analyzed on a FACScan flow cytometer (FacsCalibur analyzer; Becton-Dickinson Immunocytometry Systems, Fullerton, CA, USA). Data were acquired for up to 5 × 10^3 cells from each sample.

Histograms of the light intensity were constructed for each experimental situation and geometric mean of light intensity of the cellular population was determinate. Calci-um oxalate crystals were excluded from analysis using a gated analysis method based on forward light scatter and side scatter. MDCK and LLC-PK1 complexity (granular-ity) were considered as control parameters and compared with the cellular complexity after calcium oxalate or K$_2$-oxalate exposition.

Cellular viability experiments

Necrotic and apoptotic cells were evaluated respectively by acrydine orange/ethidium bromide and HOE 33342 dyes. MDCK and LLC-PK1 suspensions were centrifuged, resuspended in PBS, and incubated with acrydine orange/ethidium bromide solution for 5 to 15 minutes. Cells were then observed under light micro-scopy. Ethidium bromide stains all cells in green, while acrydine orange is excluded from viable cells. HOE 33342 stains the chromatin. Blue cells were considered nonapoptotic, and cell with condensed chromatin were consid-ered apoptotic. At least 100 cells per culture were counted, and the results were expressed as percentage of necrotic or apoptotic cells.
**Statistical analysis**

Results are given as mean ± SEM. Experimental and control groups were compared by the *t* test of Student. *P* values of less than 0.05 were considered significant.

**RESULTS**

Figure 1 shows that both MDCK and LLC-PK1 cell lines synthesized a mixture of chondroitin sulfate and heparan sulfate in different proportions. LLC-PK1 synthesized 23.2% of chondroitin sulfate and 76.8% of heparan sulfate, while MDCK synthesized 48.5% of chondroitin sulfate and 51.5% of heparan sulfate. In both cell lines, most of the glycosaminoglycans were present in the culture medium and cell surface fractions (40% to 70%), while smaller portions were observed in intracellular compartment (1% to 10%) and ECM (1% to 15%), especially for LLC-PK1 cells.

Both cell lines were exposed to increasing concentrations of K$_2$-oxalate (0.5 to 4.0 mmol/L) and calcium oxalate (100 to 500 µg/mL) during 24 hours. The different cell lines responded differentially to these treatments. High concentrations of K$_2$-oxalate increased the $^{35}$S-sulfate incorporation in glycosaminoglycans by MDCK cells, but had no significantly effects upon LLC-PK1 cells (Fig. 2). Both chondroitin sulfate and heparan sulfate were affected by K$_2$-oxalate, but not by calcium oxalate exposition (Fig. 3). In contrast calcium oxalate increased the $^{35}$S-sulfate incorporation in LLC-PK1, but there was no significantly effect on MDCK cells (Fig. 2). Again both chondroitin sulfate and heparan sulfate were increased in culture medium and surface fractions (Fig. 3).

In order to investigate if the effects of calcium oxalate and K$_2$-oxalate observed upon glycosaminoglycan synthesis are specific for these salts, the effects of other crystals also present in urinary stones (calcium phosphate or hydroxyapatite) and formate, which is a carboxylic acid (as oxalate) and shares oxalate transporter in LLC-PK1 cells [33] were also analyzed. Figure 4 shows that neither formate nor hydroxyapatite increased glycosaminoglycans synthesis in the cell lines here studied. This finding suggests that the effects of calcium oxalate and K$_2$-oxalate are specific.
To investigate if the increase in $^{35}$S-sulfate incorporation is due to increased sulfation degrees of the glycosaminoglycans, these compounds were submitted to enzymatic degradations with \textit{F. heparinum} mucopolysaccharidases. The products formed are shown in Table 1. There were no significant changes in the relative proportions of disaccharides that compose chondroitin sulfate or heparan sulfate. Also, the modal molecular weights of the polymers were unaltered, indicating that the changes in $^{35}$S-sulfate incorporation in glycosaminoglycans are not due to changes in sulfation degrees or chain length.

Furthermore, we observed that both MDCK and LLC-PK1 cells synthesized and secreted to the culture medium small amounts of hyaluronic acid (Fig. 5). Calcium oxalate caused discrete increase in hyaluronic acid concentrations upon LLC-PK1 cells and K$_2$-oxalate upon MDCK cells.

Figure 6 show that the treatment of MDCK cells with K$_2$-oxalate–induced apoptosis (34%) and necrosis (13%) while calcium oxalate had no effect upon cellular viability (data not shown). In opposition, calcium oxalate increased, in a concentration-dependent manner, necrotic cell death in LLC-PK1 cells and there was no difference in the cellular viability after the K$_2$-oxalate exposition in these cells.

Figures 7 and 8 show that calcium oxalate crystals were endocyted by both cells observed by the increase of the side scatter. Calcium oxalate crystals concentrated at the region R1 of the dot plot graphic, while MDCK or LLC-PK1 cell concentrate at region R2. When calcium oxalate crystals were exposed to both cells the concentration of crystals decrease in the region R1 and the cell populations migrated through side scatter axis of the dot plot graphic. LLC-PK1 cells endocyted more calcium oxalate crystals than MDCK, but no significant difference in cellular complexity was observed after K$_2$-oxalate exposition in both cells. Also in LLC-PK1 significant effects were observed upon glycosaminoglycan synthesis and cell necrosis. When the cells were exposed to K$_2$-oxalate, some calcium oxalate crystallizes in the culture medium. Nevertheless, very discrete changes in the cell complexity were observed under these conditions, indicating a much lower degree of calcium oxalate endocytosis (if any). So, the effects of K$_2$-oxalate upon MDCK cells are possibly due to the soluble ions.

To analyze the possible mechanism through which K$_2$-oxalate and calcium oxalate increased glycosaminoglycans synthesis, the effect of thapsigargin, an endoplasmic reticulum calcium adenosine triphosphatase (ATPase) inhibitor was studied (Fig. 9). Thapsigargin had no effect upon the glycosaminoglycan synthesis by both cell
lines. It had also no effect upon the increase in $^{35}$S-glycosaminoglycan synthesis induced by K$_2$-oxalate in MDCK cells, but significantly inhibited the increase in $^{35}$S-chondroitin sulfate and $^{35}$S-heparan sulfate synthesis and secretion to the culture medium induced by calcium oxalate (500 µg/mL) in LLC-PK1 cells.

**DISCUSSION**

The results here presented show that tubular epithelial cell lines (MDCK and LLC-PK1) synthesize a mixture of chondroitin sulfate and heparan sulfate proteoglycans, predominantly secreted to the culture medium and present in cellular surface. Hyaluronic acid was also detected both in cell and medium fraction of both cell lines.

MDCK and LLC-PK1 are affected differently by calcium oxalate and oxalate ions. Upon MDCK cells, K$_2$-oxalate increased the incorporation of $^{35}$S-sulfate in chondroitin and heparan sulfate found both in culture medium, but calcium oxalate had no effect; on the contrary, calcium oxalate increased the $^{35}$S-sulfate incorporation in glycosaminoglycans by LLC-PK1, while K$_2$-oxalate had no significant effect.

There was a small increase in hyaluronic acid concentrations, compared with chondroitin and heparan sulfate, when MDCK were exposed to oxalate. Hyaluronic acid has been implicated as a binding molecule for calcium oxalate crystals [34]. It is possible that tubular cells limit the synthesis of hyaluronic acid to avoid the increase in adhesion of calcium oxalate crystals.

In both cell lines, 4-sulfated and 6-sulfated disaccharide units compose chondroitin sulfate. Heparan sulfate is composed by monosulfated (DiNS and DiNAc 6S) and disulfated (DiNS 6S) disaccharides. The relative amounts of these disaccharides were unaltered by the treatment here used. Also, the glycosaminoglycan molecular weight was not increased, indicating that the increase in $^{35}$S-sulfate incorporation induced by K$_2$-oxalate or calcium oxalate are not due to increased sulfation or polymerization degree.

Several papers have shown polarized synthesis of proteoglycans by MDCK cells. Apical secretion of chondroitin sulfate proteoglycans [35] and expression of glypican, the main heparan sulfate proteoglycans at the MDCK cell surface [36] have been reported. Most of the proteoglycans are expressed in the apical surface of MDCK cells that resemble distal tubular epithelial cells, with only 17% at the basolateral side [37]. Furthermore, Erickson and Couchman [38] reported the synthesis of many ECM proteoglycans by these cells: perlecan, agrin, and type XVIII collagen (heparan sulfate proteoglycans) and biglycan, bamacan, and versican (chondroitin sulfate proteoglycans). These proteoglycans are also present in mammalian kidney tubules.

In contrast, there are only a few papers on the proteoglycans synthesized by LLC-PK1 cells. Although they are commonly used as a model of proximal tubular epithelial cells, the proteoglycans synthesized by these cells were poorly characterized and only heparan sulfate proteoglycans have been described [39, 40]. To our knowledge, this is the first paper that describes the synthesis of chondroitin sulfate proteoglycans by LLC-PK1 cells.

The effects of calcium oxalate crystals, as well as oxalate ions, upon MDCK and LLC-PK1 cells have been widely investigated concerning their binding, internalization, and induction of cell injury. Some results were similar to the present study [1, 41], and others did not [2, 42], possibly because of the methodologies or conditions employed. Nevertheless, their effects upon proteoglycan synthesis have not been fully analyzed.

Up-regulation of heparan sulfate proteoglycan in rat kidney [22] and increased expression of heparan sulfate core protein mRNA in both proximal and distal tubules [43] has been reported in nephrolithiasis. Although this is the first paper to describe that calcium oxalate crystals and oxalate ions have different effects upon the glycosaminoglycan synthesis in different cell lines and that keep resemblance to proximal (LLC-PK1) and distal (MDCK) tubular epithelial cells.

K$_2$-oxalate and calcium oxalate effects seem to be specific to, respectively, distal and proximal tubular cells. Neither exposition to hydroxyapatite nor formate increased glycosaminoglycan synthesis in theses cells. Studies report that MDCK cells bind 10 times more calcium oxalate compared to brushite crystals (calcium phosphate) [44]. It is possible that more calcium oxalate than hydroxapatite crystals bound to MDCK and LLC-PK1 and stimulated glycosaminoglycan synthesis in LLC-PK1.

Several methodologies were used to study calcium oxalate endocytosis, like light microscopy [41, 45] or radioactive labeled crystals [42, 44]. Some of them used different cell lines and observed different results in their experimental conditions. Through flow cytometry, we found that there was an increase in cellular complexity when both cells were exposed to crystals, but LLC-PK1 cells seemed to endocyt more crystals than MDCK.

K$_2$-oxalate induced necrotic and apoptotic cell death in MDCK, these alterations are attributed mainly to direct effects of oxalate ions and not by calcium oxalate crystallization from culture medium, since K$_2$-oxalate did not change cellular complexity. Calcium oxalate increased cellular complexity and induced necrotic cell death in LLC-PK1, indicating that calcium oxalate effects are attributed to crystal endocytosis.

These salt concentrations strongly correlate with the concentration that induces $^{35}$S-chondroitin sulfate and $^{35}$S-heparan sulfate synthesis. Calcium oxalate crystals and oxalate ions effects were observed in high concentrations, probably nonphysiologic, but experimental conditions were improved to do not affect the normal
Fig. 9. Effect of thapsigargin (Tap) (2.0 μmol/L) on the synthesis of 35S-chondroitin sulfate and 35S-heparan sulfate induced by K₂Ox (4.0 mmol/L) in Madin-Darby canine kidney (MDCK) and calcium oxalate (CaOx) (500 μg/mL, 3.9 mmol/L) in LL-PK1. Distal tubular cells exposed to either Dulbecco's modified Eagle's medium (DMEM) without fetal calf serum (FCS) or K₂-oxalate and K₂-oxalate plus thapsigargin (2.0 μmol/L) and proximal tubular cells exposed to DMEM without FCS or calcium oxalate and calcium oxalate plus thapsigargin (2.0 μmol/L) were incubated with H₂35SO₄. After 24 hours' culture medium and cellular extract fractions were collected and the 35S-glycosaminoglycans were isolated. Aliquots of culture medium and cell extract of each experimental situation were submitted to agarose gel electrophoresis. The 35S-glycosaminoglycans were identified and quantified. Results were expressed as mean ± standard error of percentage between 35S-glycosaminoglycans obtained after each treatment and, respectively, control situation. Control (CTL), MDCK or LLC-PK1 culture medium and cell extract control; K₂-oxalate, culture medium, and cellular extract from MDCK exposed to K₂-oxalate; K₂-oxalate + thapsigargin, culture medium and cellular extract from MDCK exposed to K₂-oxalate plus thapsigargin. Calcium oxalate, culture medium, and cellular extract from LLC-PK1 exposed to calcium oxalate; calcium oxalate + thapsigargin, culture medium, and cellular extract from LLC-PK1 exposed to calcium oxalate plus thapsigargin. Data are mean ± standard error of five experiments. *P < 0.05 was considered significant different than control situation; **P < 0.05 was considered significant different than calcium oxalate–treated situation.

pattern of glycosaminoglycan synthesis observed in these cells. Nevertheless, these results could be important since LLC-PK1 can physiologically handle oxalate ions [33] but once exposed to calcium oxalate crystals, a rare situation, shows a concentration-related increase in necrosis and glycosaminoglycan synthesis. Also, MDCK cells are frequently confronted to calcium oxalate crystals and could control their endocytosis, but once injured by oxalate, induce an up-regulation of glycosaminoglycans. These findings suggest that in our conditions, tubular cells show specificities to oxalate and calcium oxalate crystals treatment and the surviving cells increase glycosaminoglycan synthesis to protect against their deleterious effects.

Increase the cytosolic Ca^{2+} levels inhibit 35S-glycosaminoglycan synthesis induced by calcium oxalate crystals in proximal tubular cells. Campos and Schor [46] showed that thapsigargin inhibit calcium oxalate crystals endocytosis in MDCK. If it is also happening in our conditions, it is possible that 35S-glycosaminoglycan synthesis is inhibited because the endocytosis was prevented.

Another mechanism is related to K₂-oxalate effect since tapsigargin did not modify the 35S-glycosaminoglycans synthesis induced by K₂-oxalate in MDCK. Iida et al [47] observed that oxalate exposure increased intracellular calcium, as a sign of injury, and it was prevented by heparan sulfate. It is possible that oxalate induces up-regulation of glycosaminoglycan to revert the damage.

CONCLUSION

High oxalate concentrations propitiate calcium oxalate crystallization and both calcium oxalate crystal and oxalate ions induce tubular injury that could promote crystals nucleation. Tubular cells may protect from the toxic insult by inducing the synthesis of crystallization inhibitors, like glycosaminoglycans. Our work shows that oxalate ions and calcium oxalate crystals increase glycosaminoglycan synthesis in tubular epithelial cells and could partially limit the stone formation and nephrolithiasis development.

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