Mechanisms involved in calcium oxalate endocytosis by Madin-Darby canine kidney cells

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Abstract

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Calcium oxalate (CaOx) crystals adhere to and are internalized by tubular renal cells and it seems that this interaction is related (positively or negatively) to the appearance of urinary calculi. The present study analyzes a series of mechanisms possibly involved in CaOx uptake by Madin-Darby canine kidney (MDCK) cells. CaOx crystals were added to MDCK cell cultures and endocytosis was evaluated by polarized light microscopy. This process was inhibited by an increase in intracellular calcium by means of ionomycin (100 nM; N = 6; 43.9% inhibition; P<0.001) or thapsigargin (1 µM; N = 6; 33.3% inhibition; P<0.005) administration, and via blockade of cytoskeleton assembly by the addition of colchicine (10 µM; N = 8; 46.1% inhibition; P<0.001) or cytochalasin B (10 µM; N = 8; 34.2% inhibition; P<0.001). Furthermore, CaOx uptake was reduced when the activity of protein kinase C was inhibited by staurosporine (10 nM; N = 6; 44% inhibition; P<0.01), or that of cyclo-oxygenase by indomethacin (3 µM; N = 12; 17.2% inhibition; P<0.05); however, the uptake was unaffected by modulation of potassium channel activity with glibenclamide (3 µM; N = 6), tetraethylammonium (1 mM; N = 6) or cromakalim (1 µM; N = 6). Taken together, these data indicate that the process of CaOx internalization by renal tubular cells is similar to the endocytosis reported for other systems. These findings may be relevant to cellular phenomena involved in early stages of the formation of renal stones.

Introduction

Calcium oxalate (CaOx) crystals, the main constituent of urinary calculi, adhere in a specific manner to the plasma membrane of renal tubular epithelial cells *in vitro* and *in vivo*, and this process is rapidly followed by endocytosis. The tubular cells then exhibit a proliferative response, rearranging the cytoskeleton, increasing expression of "early-immediate" transcription genes (c-myc, c-fos, nur-77), synthesizing fibrogenic substances such as platelet-derived growth factor and connective tissue growth factor, and increasing absolute cell counts and viability (for a review, see Ref. 1).

There are controversies about the elements required for the development of urinary calculi. Although the exact role of the above mentioned phenomena has not been defined, recent experimental and clinical evidence points to the interaction between CaOx crystals and renal tubular epithelium as a factor involved in the genesis of urolithiasis.

As observed in cell cultures, human kidney cells internalize crystals and proliferate when exposed to high concentrations of CaOx *in vivo* (e.g. primary hyperoxaluria, Crohn's disease-related malabsorption; 2,3). CaOx-induced excessive cell proliferation or damage (at higher concentrations) could lead to detachment of epithelial cells and basal membrane exposure. Epithelial denudation would permit a more intensive crystal adhesion (4,5). Additional anchoring of CaOx particles would follow, allowing an expressive crystal nucleation and calculus growth (6). The traffic of particles through the basement membrane or even through intact tubular epithelium could also explain the presence of CaOx crystals in tubular interstitium. Considering the CaOx stimulus of proliferation, interstitial scarring sometimes seen in urolithiasis could also be a consequence of CaOx internalization by tubular cells.

Different substances have been described as modulators of adhesion and/or endocytosis processes. Nevertheless, despite the possible relevance of the CaOx-tubular cell interaction, none of these drugs seems to be suitable for clinical use (1,7-12). The cellular pathways involved in endocytosis of CaOx crystals can constitute potential targets for drugs designed for the prophylaxis and/or treatment of urolithiasis. However, the mechanisms by which tubular cells take up CaOx need additional investigation.

The objective of the present study was to further clarify the cellular events related to this particular type of endocytosis employing Madin-Darby canine kidney (MDCK) cell culture.

Material and Methods

Cell culture

MDCK cells obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS; 5%), 2 g/l $NaHCO_3$, 2.6 g/l HEPES, 10.000 IU/l penicillin, 50 mg/l streptomycin and 100 mg/l neomycin. Cells were placed in polystyrene bottles at 37ºC in a humidified gas mixture (95% air and 5% CO_2). Before each experiment cells were kept in DMEM without FBS for 24 h in order to obtain cells in the G_0 phase of the cell cycle. At maximal confluence, cell treatments were performed and cell suspensions were obtained by exposure to trypsin-EDTA solution (0.25/0.02%, w/v, 0.5 ml/bottle). Cell viability was evaluated by the lactate dehydrogenase (LDH) assay (13) and confirmed by Trypan blue dye (0.2%; 5 to 15 min) exclusion. Values higher than 90% in both methods were considered adequate.

Preparation of calcium oxalate (CaOx) crystals

Equimolar solutions (0.4 M, 100 ml) of calcium chloride and potassium oxalate were combined and the mixture was added to distilled and deionized water (DDW, 300 ml) by constant dripping for 2 h. This suspension was maintained under continuous stirring at 75ºC for 5 h and then washed with DDW to remove potassium chloride present in the supernatant. The remaining saturated solution was maintained at 37ºC for 15 days until CaOx crystallization. CaOx crystals were then sterilized in ethylene oxide and culture medium without FBS was added, yielding a new suspension. In order to uniformize particle size, the suspension was sonicated for 12 min. Qualitative analysis of CaOx was performed by X-ray diffractometry and crystal size was evaluated by laser chromatography with a CILAS 330 laser granulometer.

MDCK cell exposure to CaOx

Confluent cell cultures were washed twice with PBS and exposed to the CaOx suspen-

sion (200 µg/ml) for different periods of time (2, 6 and 24 h). Cultures were then washed again in PBS and trypsinized. These procedures yield only cells containing internalized or strongly adhered crystals. Cell suspensions were analyzed by polarized light microscopy and CaOx crystal endocytosis was scored arbitrarily on a 0 to 4 scale, according to a previous report (14), as follows: 0 for cells without intracellular CaOx crystals, 1 for cells containing a single small CaOx crystal, 2 for cells with less than 50% of the cytoplasm area occupied by CaOx crystals, 3 for cells with 50 to 75% of the cytoplasm area filled with CaOx crystals, and 4 for cells with more than 75% of the cytoplasm area occupied by CaOx crystals. At least 100 cells per sample were counted and the final score was calculated according to the equation: (score 0 cells x 0) + (score 1 cells x 1) + (score 2 cells x 2) + (score 3 cells x 3) + (score 4 cells x 4)/total number of cells. In another set of experiments, after determination of the best incubation period, cells were exposed to different concentrations of CaOx (10, 50, 100, 200 and 300 µg/ml) for 6 h and the CaOx cell uptake was evaluated. The intracellular position of CaOx crystals was confirmed in some representative cell cultures at different incubation times by laser scanning confocal microscopy (BioRad 1024-UV confocal system coupled to a microscope Zeiss Axiovert 100, BioRad, Hercules, CA, USA).

Effect of different drugs on CaOx crystal endocytosis

MDCK cells were exposed to CaOx (200 µg/ml, 6 h) in the absence (vehicle only) or in the presence of each of the following drugs, added to the medium at submaximal concentrations 30 min before CaOx administration: 10 nM staurosporine (a protein kinase C inhibitor), 3 µM indomethacin (a cyclo-oxygenase inhibitor), 3 µM glibenclamide (an ATP-sensitive potassium channel

blocker), 1 mM tetraethylammonium (a non-specific potassium channel blocker), 1 µM cromakalim (an ATP-sensitive potassium channel opener), 100 nM ionomycin (a calcium ionophore), 1 µM thapsigargin (an endoplasmic reticulum calcium ATPase inhibitor), 10 nM nifedipine (a voltage-dependent calcium channel blocker), 10 µM colchicine (a microtubule assembly inhibitor) or 10 µM cytochalasin B (an actin microfilament assembly inhibitor). CaOx crystal uptake was then evaluated as described above.

Statistical analysis

Data related to CaOx endocytosis were reported as mean scores ± standard error of the mean (SEM). When different groups were compared, data were presented as percent of control (absolute values in the text). Laser granulometry results were expressed as non-cumulative frequency of distribution of crystal sizes in only one sample. After log transformation of raw data, experimental and control groups were compared by the unpaired Student t-test. P values of less than 0.05 were considered significant.

Drugs and reagents

PBS, DMEM, penicillin/streptomycin/neomycin solution, Triton X-100, NADH, sodium pyruvate, Tris-HCl, Trypan blue, ionomycin, indomethacin, colchicine, nifedipine, thapsigargin, tetraethylammonium, cromakalim, cytochalasin B, and glibenclamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin-EDTA solution and FBS were obtained from Sigma Chemical Co. or Cultilab (Campinas, SP, Brazil). Ethanol P.A., DMSO and all salts were purchased from Merck S.A. (Rio de Janeiro, RJ, Brazil). Staurosporine was supplied by Calbiochem-Novabiochem Co. (La Jolla, CA, USA). Glibenclamide, staurosporine and cytochalasin B were dissolved in DMSO. Ionomycin, indomethacin, nifedi-

pine and cromakalim were dissolved in ethanol. The remaining substances were dissolved in DDW or 0.9% NaCl. The final concentrations of ethanol or DMSO in the culture medium never exceeded 0.01%. We assured that these substances had no detectable effect on cell viability or CaOx endocytosis process.

Results

Physical characteristics of CaOx crystals

CaOx sample analysis by X-ray diffractometry yielded a purity grade higher than 95. The mean and median size of CaOx crystals in a single sample evaluated by laser granulometry was 3.94 and 4.99 μm, respectively (data not shown).

Time- and concentration-dependent CaOx endocytosis

MDCK cells internalized CaOx crystals in a time-dependent manner. As can be seen in Figure 1A, the score obtained after a 2-h exposure period was 0.67 ± 0.04, reaching 1.09 ± 0.08 at 6 h (N = 6; P<0.001). The magnitude of endocytosis did not change significantly after 24 h of incubation (N = 6; P = ns). At 6 h, MDCK endocytosis exhibited a concentration-dependent pattern from 10 to 300 μg/ml (0.11 ± 0.01 to 1.43 ± 0.11; N = 6-34) (Figure 1B). Although the concentration-response curve did not reach a plateau, higher CaOx concentrations were not employed due to cytotoxicity. Cell viability was not impaired by exposure to CaOx (200 μg/ml) for 6 h, with a mean intracellular LDH activity of $99.2 \pm 0.3\%$ and $98.7 \pm 0.4\%$ (N = 6; P = ns) for control and exposed cells, respectively (Figure 1C).

Effect of different drugs on CaOx endocytosis

As can be observed in Figure 2A, increasing $[Ca^{2+}]_i$ by means of endoplasmic reticulum Ca^{2+}-ATPase blockade with thapsigargin significantly inhibited the endocytotic response of MDCK cells (33.3% inhibition; N = 6; P<0.005). Similar results were obtained when $[Ca^{2+}]_i$ was enhanced by changing plasma membrane permeability to

Figure 1 - A, Time-response internalization of calcium oxalate (CaOx) crystals (200 μg/ml) by MDCK cells. *P<0.001 compared to 6 and 24 h (Student t-test). B, Concentration-response curve for CaOx crystal internalization by MDCK cells after a 6-h exposure period. C, Viability of MDCK cells with (closed column) or without (open column) CaOx exposure for 6 h expressed as percentage of intra- and extracellular lactate dehydrogenase (LDH) activity. The columns represent the mean and the bars the SEM of at least 6 experiments.
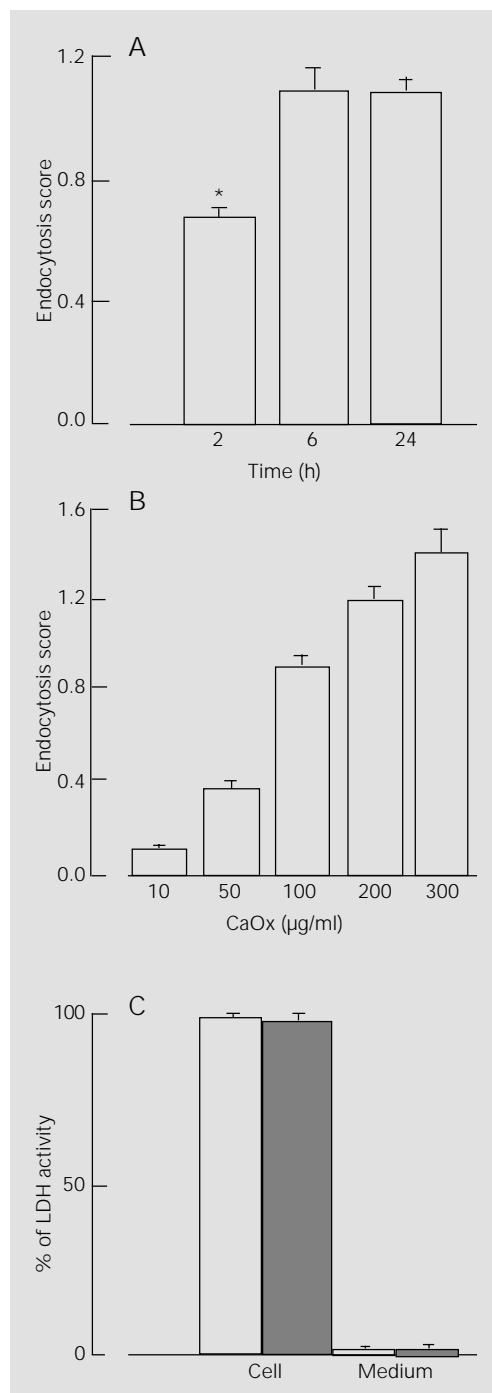
extracellular calcium with ionomycin (43.9% inhibition; N = 6; P<0.001). On the other hand, the inhibition of voltage-dependent calcium channels by nifedipine did not promote alterations in cellular CaOx uptake (N = 6; P = ns) (Figure 2A). The absolute scores of the control groups compared to the thapsigargin, ionomycin and nifedipine groups were 1.32 ± 0.10, 1.27 ± 0.06 and 1.22 ± 0.06 (N = 6), respectively.

Staurosporine markedly reduced (44% of control; N = 6; P<0.01) the uptake of CaOx crystals after an exposure time of 6 h (Figure 2B). On the other hand, indomethacin had only a discrete, although statistically significant, inhibitory effect on CaOx endocytosis (17.2% of control; N = 12; P<0.05) (Figure 2B). The scores obtained for the

control groups were 1.32 ± 0.10 (N = 6) and 1.22 ± 0.03 (N = 12) for staurosporine and indomethacin, respectively.

According to the data in Figure 2C, the inhibition of actin microfilament assembly by cytochalasin B significantly decreased the endocytotic response of MDCK cells (34.2% of control; N = 8; P<0.001). Similarly, colchicine, a microtubule formation inhibitor, significantly reduced CaOx uptake by tubular cells (46.1% of control; N = 8; P<0.001). The control absolute score for both groups was 1.17 ± 0.05 (N = 6).

The activation or the inhibition of ATP-sensitive potassium channels by cromakalim and glibenclamide, respectively, did not alter significantly CaOx endocytosis (N = 6; P = ns). Similarly, the tetraethylammonium-
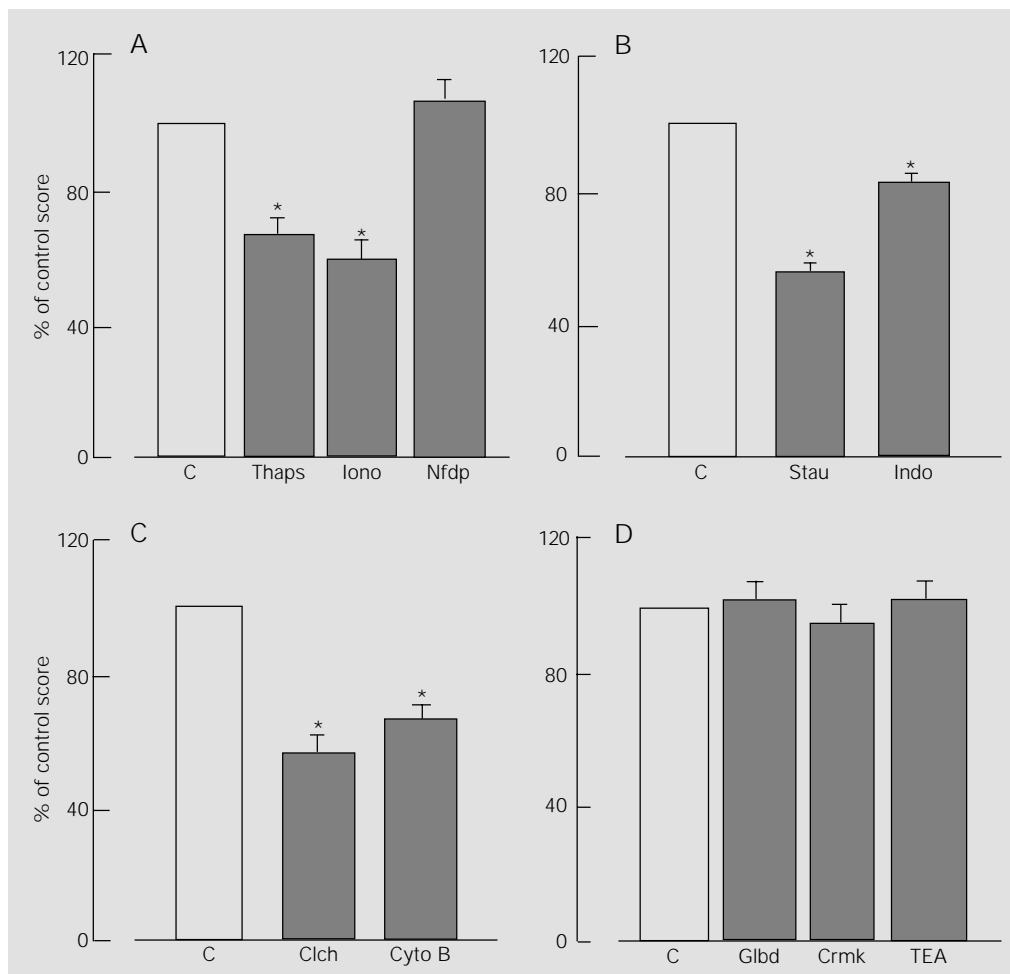

Figure 2 - Effect of different drugs on CaOx crystal endocytosis (200 µg/ml, 6 h exposure time) by MDCK cells. Data are reported as percentage of control mean endocytosis scores. The columns represent the mean and the bars the SEM of at least 6 experiments. A, Thapsigargin (Thaps, 1 µM), ionomycin (Iono, 100 nM) and nifedipine (Nfdp, 10 nM). *P<0.005 compared to control (C) (Student t-test). B, Staurosporine (Stau, 10 nM) and indomethacin (Indo, 3 µM). *P<0.05 compared to control (C) (Student t-test). C, Colchicine (Clch, 10 µM) and cytochalasin B (Cyto B, 10 µM). *P<0.001 compared to control (C) (Student t-test). D, Glibenclamide (Glbd, 3 µM), cromakalim (Crmk, 1 µM) and tetraethylammonium (TEA, 1 mM).

induced nonspecific blockade of potassium channels did not change the cytosolic CaOx content of MDCK cells exposed to relatively high crystal concentrations (N = 6; P = ns) (Figure 2D).

Discussion

Our technique for CaOx crystal production provided particles with dimensions and purity grade suitable for performing endocytosis assays. Moreover, the analysis of CaOx endocytosis kinetics confirmed previous data (15), showing that MDCK cells take up CaOx crystals in a time- and concentration-dependent manner, without detection of cell toxicity at the several concentrations tested, further validating our methods.

Alterations in $[Ca^{2+}]_i$ play an important role in endocytosis and intracellular transport (16,17). Enhancements in $[Ca^{2+}]_i$ from extra- or intracellular calcium sources induced by ionomycin and thapsigargin, respectively, significantly inhibited the MDCK cell CaOx internalization. On the other hand, in our study, the blockade of calcium influx via voltage-dependent channels by nifedipine did not promote relevant changes in crystal internalization. Indeed, a direct causative relationship between massive increments in intracellular calcium and endocytotic process inhibition has not been determined, as shown by Wu and Betz (18). The use of different calcium channel blockers and the measurement of $[Ca^{2+}]_i$ in MDCK cells after crystal internalization could help us solve this question.

Different investigators have reported considerable decreases of endocytosis in different models using protein kinase C inhibitors, with special emphasis on plasma membrane receptor recycling (19,20). This phenomenon does not only apply to protein endocytosis and was well documented in MDCK cells when activators (such as derivatives of phorbol esters) and/or inhibitors of protein kinase C were employed (21,22). This fact

was evident also in the present study, with marked inhibition of CaOx internalization after exposure to staurosporine.

Another enzyme involved in the mechanisms of endocytosis is cyclo-oxygenase. Singhal et al. (23), for instance, have demonstrated the production of prostaglandin E_2 by rat mesangial cells following colloidal gold particle internalization. In our model, indomethacin induced a slight inhibitory response, indicating a minor role for cyclo-oxygenase in CaOx endocytosis. Curiously, Lieske et al. (24) reported an inhibitory effect on CaOx endocytosis after administration of arachidonic acid (AA) and some of its metabolites (PGE_1 and PGE_2) to MDCK cells. Thus, the involvement of AA cascade metabolites in these events deserves further investigation.

Cytochalasin B or colchicine also significantly reduced cellular CaOx crystal uptake. Several studies have demonstrated the participation of the cytoskeleton in endocytotic processes by inhibition of microtubules and/or actin microfilament assembly (25-27). This especially occurs in epithelial cells where actin filaments and tubulin are relevant not only for cell polarity establishment and maintenance, but also for the intra- and transcellular process of particle distribution. The earlier intracellular events triggered by CaOx are related to the cytoskeleton. As already observed elsewhere, tubular cell crystal endocytosis is accompanied by F-actin concentration and cytokeratin net reorganization (28). Our data showed that alterations in the tubulin net are also present in this phenomenon, although data from Lieske et al. (28) pointed to an opposite direction. Furthermore, the inhibitory effect of cytochalasin B suggests that actin polymerization may be a more precocious event than CaOx crystal internalization, in contrast to the results of the above mentioned authors. However, although contradictory data exist, cytoskeleton mobilization is indispensable for the development of the CaOx internalization

process by tubular epithelial cells.

Reductions in culture medium potassium concentration enhance CaOx internalization by tubular cells (1). In our study, potassium channel inhibitors (glibenclamide and tetraethylammonium) did not modify the magnitude of CaOx endocytosis by MDCK cells. The same occurred when the ATP-sensitive potassium channel activator, cromakalim, was employed, excluding the participation of some potassium channels in such phenomenon.

In conclusion, our study confirms and extends previous data showing that the process of CaOx uptake by tubular cells is inhibited by $[Ca^{2+}]_i$ increases, cytoskeleton assembly blockade, protein kinase C and, to a lesser extent, cyclo-oxygenase inhibition, but not by potassium channel blockade or activation. Thus, MDCK cells employ mechanisms for CaOx internalization quite similar to those observed in several types of endocytosis. These results may contribute to a better understanding of a phenomenon probably related to the pathophysiology of urolithiasis.

Acknowledgments

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