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Calcium oxalate monohydrate crystals internalized into renal tubular cells are degraded and dissolved by endolysosomes

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ABSTRACT

Interaction between calcium oxalate crystals and renal tubular cells has been recognized as one of the key mechanisms for kidney stone formation. While crystal adhesion and internalization have been extensively investigated, subsequent phenomena (i.e. crystal degradation and dissolution) remained poorly understood. To explore these mechanisms, we used fluorescein isothiocyanate (FITC)-labelled calcium oxalate monohydrate (COM) crystals (1000 μg/ml of crystals/culture medium) to confirm crystal internalization into MDCK (Type II) renal tubular cells after exposure to the crystals for 1 h and to trace the internalized crystals. Crystal size, intracellular and extracellular fluorescence levels were measured using a spectrofluorometer for up to 48 h after crystal internalization. Moreover, markers for early endosome (Rab5), late endosome (Rab7) and lysosome (LAMP-2) were examined by laser-scanning confocal microscopy. Fluorescence imaging and flow cytometry confirmed that FITC-labelled COM crystals were internalized into MDCK cells (14.83 ± 0.85%). The data also revealed a reduction of crystal size in a time-dependent manner. In concordance, intracellular and extracellular fluorescence levels were decreased and increased, respectively, indicating crystal degradation/dissolution inside the cells and the degraded products were eliminated extracellularly. Moreover, Rab5 and Rab7 were both up-regulated and were also associated with the up-regulated LAMP-2 to form large endolysosomes in the COM-treated cells at 16-h after crystal internalization. We demonstrate herein, for the first time, that COM crystals could be degraded/dissolved by endolysosomes inside renal tubular cells. These findings will be helpful to better understand the crystal fate and protective mechanism against kidney stone formation.

1. Introduction

Kidney stone disease remains an important public health problem in both developing and developed countries [1,2]. Although extracorporeal shock wave lithotripsy and percutaneous nephrolithotomy are the effective therapeutics to remove kidney stone, its recurrence is considerably high after the stone removal. This is due to a lack of an effective method to prevent any new or recurrent stone formation. Hence, finding the way to prevent stone formation is one of the utmost priorities in kidney stone research.

Calcium oxalate, particularly its monohydrate form (COM), is the most common chemical composition found in kidney stones [3]. COM can be crystallized in the concentrated renal tubular fluid and interacted with renal tubular epithelial cells, leading to a stone nidus, which is the origin of stone formation. Recent findings have shown that COM crystal–cell interaction can lead to several subsequent phenomena, including cell proliferation [4,5], death [6] and injury [7], formation of reactive oxygen species [8], mitochondrial dysfunction [9,10], and tissue inflammation [11]. After adhesion, the crystal can then be internalized into renal tubular epithelial cells [12]. Recently, we have reported that macropinocytosis is the major mechanism for endocytosis of calcium oxalate crystals into renal tubular cells [13]. However, subsequent phenomena after crystal internalization (e.g. whether the internalized crystals are degraded or further enlarged, and by what mechanisms) remain unclear.

We thus hypothesized that the internalized COM crystals can be degraded or dissolved by a particular response in renal tubular cells, as a defense mechanism, to eliminate the retained crystals. Accordingly, this study aimed to address the involvement of

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endolysosome in crystal degradation/dissolution in renal tubular epithelial cells. The fluorescence-labelled COM crystals were generated and then used for tracking the internalized COM crystals. Crystal size, intracellular and extracellular fluorescence levels were measured using a spectrofluorometer for up to 48 h after crystal internalization. Moreover, markers for early endosome (Rab5), late endosome (Rab7) and lysosome (LAMP-2) were examined by laser-scanning confocal microscopy.

2. Materials and methods

2.1. Cell cultivation

High-passage parental Mardin-Darby Canine kidney (MDCK) cells (which displayed many characteristics of MDCK Type II, including low transepithelial resistance) were cultivated in Eagle's minimal essential medium (MEM) (Gibco; Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibgo), 2 mM L-glutamine (Sigma; St.Louis, MO), and 1.2% penicillin/streptomycin (Sigma). To subculture these cells, the confluent monolayer was trypsinized with 0.1% trypsin in 2.5 mM EDTA/PBS at 37 °C for 5 min. Thereafter, the detached cells were collected and seeded in a new tissue culture flask (approximately 3×10^6 cells/75 cm^2 flask) containing MEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1.2% penicillin/streptomycin. The cells were maintained in a humidified incubator at 37 °C with 5% CO_2 and the medium was refreshed every other day.

2.2. COM crystal internalization into MDCK cells

Fluorescence-labelled COM crystals were freshly prepared as described previously [14]. Briefly, 5 mM $CaCl_2 \cdot 2H_2O$ was mixed with 0.5 mM $Na_2C_2O_4$ in a buffer containing 90 mM Tris–HCl and 10 mM NaCl (pH 7.4) in the presence of 0.01 μg/ml fluorescein isothiocyanate (FITC) dye (Thermo Scientific Pierce; Rockford, IL) at 25 °C overnight. Thereafter, FITC-labelled COM crystals were washed 3 times with methanol and collected by a centrifugation at 2000 *g* for 5 min. After supernatant was discarded, the crystals were air-dried overnight at room temperature (RT) (25 °C). FITC-labelled COM crystals were examined by a fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corp.; Tokyo, Japan) and flow cytometer (FACScan, Becton Dickinson Immunocytometry System; San Jose, CA).

Subsequently, 1×10^6 MDCK cells were incubated in a medium containing 1000 μg/ml FITC-labelled COM crystals for 1 h in a humidified incubator at 37 °C with 5% CO_2. The cells were then washed with PBS and incubated with trypsin-EDTA solution to eliminate non-internalized (both adherent and non-adherent crystals) COM crystals. The internalized crystals were then observed by a fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corp.) and quantitated by a flow cytometer (FACScan, Becton Dickinson Immunocytometry System).

2.3. Examination, quantitation, and tracking of degraded/dissolved COM crystal

After FITC-labelled COM crystals were internalized into MDCK cells as described above, their degradation/dissolution (if any) was examined at 0, 24 and 48 h after internalization. The remaining crystals inside the cells were imaged by a fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corp.) and their size was measured by using ImageMaster 2D Platinum software (GE Healthcare; Uppsala, Sweden). In parallel, the culture supernatant (extracellular fraction) was collected (by a centrifugation at 2000 *g* for 5 min) and the cells were bursted by a hypotonic technique using deionized water (intracellular fraction) to measure fluorescence levels using a spectrofluorometer (Jasco FP-6300, Jasco Corp.; Tokyo, Japan) to track for the fate of the degraded/dissolved crystals.

2.4. Immunofluorescence co-staining and imaging

Immunofluorescence co-staining and imaging were performed to examine localization of the intracellular COM crystals and their association with early and late endosomes as well as lysosome. After the plain COM crystals were internalized for 16 h, the cells were fixed with 3.8% formaldehyde/PBS and permeabilized with 1% Triton X-100/PBS at RT for 10 min. Note that we used plain (non-labelled) crystals in this experiment as to avoid the interference of FITC signal on immunodetection. After washing with PBS, the cells were blocked with 1%BSA/PBS at RT for 1 h and then incubated with mouse monoclonal anti-Rab5 (early endosome marker), anti-Rab7 (late endosome marker) or anti-LAMP-2 (lysosome marker) antibody (all were purchased from Santa Cruz Biotechnology; Santa Cruz, CA) at a dilution of 1:100 in 1%BSA/PBS at 37 °C for 1 h. The cells were incubated further with secondary antibody conjugated with Alexa 546 or Alexa 555 (Invitrogen – Molecular Probes; Eugene, OR). Cell nucleus was localized by using Hoechst-dye 33342 (Invitrogen – Molecular Probes). Immunofluorescence images were then taken by a laser-scanning confocal microscope (Nikon A1R, Nikon Corp.; Tokyo, Japan).

2.5. Statistical analysis

Quantitative data are presented as mean ± SEM. Comparisons of the two data sets were performed by unpaired Student's t test (SPSS version 13.0). P values less than 0.05 were considered statistically significant.

3. Results

To define mechanisms of intracellular COM crystal degradation and dissolution, we employed an *in vitro* model of crystal–cell interaction. ^{14}C-oxalic acid labelled COM crystals have been employed to study crystal–cell interaction for quite some time [15,16]. However, the long half-life of ^{14}C (5730 years) and its ease to be transferred to environment were our concern. Recently, we have developed an effective fluorescence labelling method that can avoid radioactive exposure [14]. Non-radioactive labelled crystals were thus employed in our present study. Fig. 1A showed morphology of the FITC-labelled COM crystals with green fluorescent signal, which could be quantified by flow cytometry (Fig. 1B). Moreover, the fluorescence-labelled COM crystals could be applied for quantitation of cells with the internalized crystals (Fig. 1C). After incubation of MDCK cells with the FITC-labelled COM crystals for 1 h and removal of non-internalized crystals, 14.83 ± 0.85% of the cells had the internalized crystals (Fig. 1C).

The crystals were then further traced for up to 48 h after the internalization. Fig. 2A and B shows that size of the internalized crystals was progressively smaller in a time-dependent manner, indicating that the internalized crystals were degraded. Culture supernatants were collected and the cells were bursted at 0, 24 and 48 h after the internalization. Spectrofluorometry revealed progressive decrease of intracellular fluorescence level, consistent with the reduction in their size (Fig. 2C). In contrast, extracellular fluorescence level was increased at 24- and 48-h after internalization, indicating that the degraded crystals were dissolved and released extracellularly (Fig. 2D).

Additionally, we performed immunofluorescence study to investigate the mechanism of COM crystal degradation/dissolution by renal tubular cells. The data demonstrated that the internalized