รายงานวิจัยฉบับสมบูรณ์

โครงการ กระบวนการสลายของผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตโดยเซลล์ท่อไต

โดย ดร.ศักดิเทพ ไชยฤทธิ์

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ผู้วิจัย ดร.ศักดิเทพ ไชยฤทธิ์
หน่วยโปรตีโอมิกส์ทางการแพทย์ สถานส่งเสริมการวิจัย
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และมหาวิทยาลัยมหิดล

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Dr. Sakdithep Chaiyarit

Abstract

Project Code: TRG5480008

Project Title: The role of endosomes and lysosomes in degradation/dissolution of internalized calcium oxalate monohydrate crystals by renal tubular epithelial cells

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Project Period: 2 years

To refine the assay for degradation/dissolution COM crystals examination and determine the degradation/dissolution mechanisms of calcium oxalate monohydrate (COM) crystals by renal tubular epithelial cells. Novel fluorescent-label COM crystals were successfully produced and treated with MDCK cells. Consequently, the degradation/dissolution internalized fluorescence COM crystals after 24-h were studied by crystal size and intracellular and extracellular fluorescent intensity. Internalized COM crystals were decreased their size and fluorescent intensity in contrast to extracellular fluorescent intensity by time dependent manner after 24-48h incubation. Localization of intracellular COM crystals was analyzed by immunofluorescence with early (Rab5) and late (Rab7) endosome marker. Accordingly, Rab5 and Rab7 were found in enlarge early endosomes with contain COM or degraded COM crystals. Therefore, Rab5 regulated enlarge endosome and endosome fusion might be the main mechanism of degradation/ dissolution of COM crystals by renal tubular epithelial cells. Further studies in the enlarge endosome molecular mechanism will lead to increase understanding of these mechanism.

Keywords: Nephrolithiasis, calcium oxalate monohydrate crystals, degradation, dissolution, endosome, lysosome

บทคัดย่อ

รหัสโครงการ: TRG5480008

ชื่อโครงการ:กระบวนการสลายของผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตโดยเซลล์ท่อไต

ชื่อนักวิจัย: ดร.ศักดิเทพ ไชยฤทธิ์ สังกัดหน่วยโปรตีโอมิกส์ทางการแพทย์ สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

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ระยะเวลาโครงการ : 2 ปี

เพื่อศึกษากระบวนการสลายผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตโดยเซลล์ท่อไต โดยพัฒนาปรับปรุงวิธีใช้ผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตที่ติดฉลากด้วยสารเรืองแสง วิเคราะห์การเข้าและการสลายของผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตในเซลล์ท่อไต Madin-Darby Canine Kidney (MDCK) เซลล์ โดยใช้ผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตที่ติดฉลากด้วยสารเรืองแสง และเทคนิคทางฟลูออเรสเซนต์ ถ่ายภาพและวัดขนาดที่เปลี่ยนแปลงของผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตที่ถูกนำเข้าไปภายในเซลล์ พบว่าผลึกขนาดมีเล็กลงแปรผันตามเวลาที่ 24 และ 48 ชั่วโมง และได้ทำการวิเคราะห์ปริมาณสารเรืองแสงที่อยู่ภายในเซลล์และที่ถูกหลั่งออกมาภายนอกเซลล์ พบว่ามีการลดลงของปริมาณสารเรืองแสงภายในเซลล์ และในทางกลับกันมีการเพิ่มขึ้นของปริมาณสารเรืองแสงที่ถูกขับออกมาจากเซลล์ซึ่งแปรผันตามระยะเวลาเช่นกัน จากนั้นทดสอบหากระบวนการสลายผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตโดยใช้เทคนิคการติดฉลากโปรตีนที่จำเพาะต่อ early (Rab5) and late (Rab7) เอนโดโซม ด้วยสารเรืองแสง ซึ่งพบว่าอยู่ร่วมกันในเอนโดโซมขนาดใหญ่ จึงสรุปได้ว่ากระบวนการสลายของผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตของเซลล์ท่อไตส่วนปลายเกี่ยวข้องกับการเกิดเอนโดโซมขนาดใหญ่และอาจเกี่ยวข้องกับการรวมตัวของ early and late เอนโดโซม โดยการศึกษานี้ยังได้เกิดวิธีการใหม่ในการประยุกต์ใช้ผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตที่ติดฉลากด้วยสารเรืองแสงเพื่อวิเคราะห์การสลายของผลึกได้ ดังนั้นการศึกษาเพิ่มเติมเกี่ยวกับความสัมพันธ์ของการสลายผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรตและการเกิดเอนโดโซมขนาดใหญ่ในระดับโมเลกุล อาจจะนำไปสู่ความเข้าใจพยาธิสรีรวิทยาของโรคนิ่วในไตที่ดีขึ้นต่อไป

คำหลัก : โรคนิ่วในไต, ผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรต, กระบวนการสลายผลึก, เอนโดโซม

1. INTRODUCTION

Kidney stones disease is yet, still public health problem world wild. Although, there are effective methods to remove kidney stone, for example extracorporeal shock wave lithotripsy and percutaneous nephrolithotomy, but recurrence stone are quiet high (1, 2). The pathogenesis of kidney stone disease has been studied for several years; however, the mechanisms of stone formation remain poorly understood. Accordingly, poorly soluble salts like calcium oxalate are commonly supersaturated in concentrated urine and generally precipitated as crystals. Calcium oxalate monohydrate (COM) crystal is found more frequent than any other crystals in the kidney stone disease (3). These crystalline particles can be retained in renal tubules and undergone impressive interaction with renal tubular epithelial cells, resulting in stone formation. In addition, crystal retention in kidney could be depending on the interaction between crystals and the renal tubular epithelial cells, even crystals are small (4-6). The interaction between crystal and cell has been proposed as the key mechanism of stone formation. Several investigators have proposed that calcium oxalate crystals adhere in specific manner of the apical membrane of renal tubular epithelial cells and this progression is rapidly followed by internalization (7, 8). Moreover, Lieske et al. found COM crystals were taken up and then degraded afterward by renal tubular cells (9). Additionally, the recent finding have shown that the interaction between COM crystals and renal tubular cells can promote numerous responses such as cell proliferation (8, 10, 11), cell death (12-14), cellular injury (15, 16), formation of reactive oxygen species (17, 18), mitochondria dysfunction (19, 20) and inflammation (21, 22).

Although massive effort has been made on the investigation on the adherence and endocytosis of COM crystals to renal tubular epithelial cells, the manner after internalization and mechanism of crystal degradation or dissolution has been slightly consideration. Thus, intracellular mechanism of COM crystal degradation, dissolution and elimination are the remarkable process. Previous study demonstrated COM crystals could be dissolved in lysosome 5-7 weeks after internalization (9). However, the subsequence processes of COM crystal digestion, including early and late endosomes formation, the fusion of late endosome with lysosomes and intracellular trafficking of crystal particles are not elucidated. The degradation and dissolution COM crystals products were secreted to apical sites have been proposed, thus, theses might be the regular defense against this stone disease (23, 24). Alternatively, degraded/dissolved products might promote inflammatory response at interstitial site (the plaque and stone formation region) (25-29). Therefore, the increasingly of crystal degraded fragments,

calcium and oxalate ion in basolateral area of epithelial cell after internalization process might be involved in stone or plaque formation at interstitial tissue.

This study assumes that the degradation/dissolution processes and trafficking molecules of internalized COM crystals would be the key process of cellular response and these processes might be the defense mechanism to eliminate retention crystal. Consequently, this study has been proposed to investigate the potential role of crystal digestion and trafficking by renal epithelial tubular cells. The fluorescence-COM crystals were used in this work, not only simple and effective techniques for imaging and quantification crystal but also more safety than using radioactive crystal for study crystal-cell interaction. The degradation of fluorescence-COM crystals were examined and quantified by fluorescent imaging and image software analysis. Moreover, dissolution of fluorescence-COM crystals was investigated by spectrofluorometer. The development of imaging was very useful tool to investigate mechanisms of crystal degradation/dissolution. Additionally, immunofluorescence staining examinations were performed to observe intracellular crystal trafficking and endosome-lysosome mechanism. The information achieved from this study would be lead to more understanding in the crystal digestion mechanism and the pathogenesis of kidney stone formation.

2. MATERAILS AND METHODS

2.1. MDCK cell culture

Mardin-Darby Canine kidney (MDCK), a distal tubular epithelial cells were be cultured in Eagle's minimal essential medium (MEM) (Gibco; Grand Island, NY) containing 10% heat inactivated fetal bovine serum (FBS) (Gibgo), 2mM glutamine (Sigma) in the presence of 100 U/ml penicillin G and 100 mg/ml streptomycin (Sigma, St.Louis, MO). The cells were be maintained in a humidified incubator 37°C with 5% CO_2. The medium was refreshed every other day. Morphology of the cells was checked under an inverted phase-contrast microscope

2.2. Preparation of fluorescence-labelled COM crystals

Fluorescence-labelled COM crystals were prepared as described previously (30). 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 FITC (Thermo Scientific Pierce, Rockford, IL). The solutions were incubated at 25°C overnight and COM crystals were harvested by a centrifugation at 2000 x g for 5 min. The supernatant was discarded and COM crystals were washed 3 times with methanol. After another centrifugation at 2000 x g for 5 min, methanol was discarded and the crystals were air-dried overnight at room temperature. COM crystals were imaged by phased-contrast microscopy (Olympus CKX41, Olympus Co. Ltd.; Tokyo, Japan) and fluorescent microscopy (Nikon ECLIPSE 80i, Nikon Corp.; Tokyo, Japan). Note that all of the reagents were sterilized before use for subsequent intervention with cells.

2.3. Internalization examination of fluorescence-labelled COM crystals

Mardin-Darby Canine kidney (MDCK) $1x10^5$ cells were cultured with media containing 1000 μg/ml fluorescence-labelled COM crystals. After a subsequent 1h incubation, 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 examined by fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corp.; Tokyo, Japan) and flow cytometer (FACScan, Becton Dickinson Immunocytometry System; San Jose, CA).

2.4. Fluorescence-labelled COM crystal degradation/dissolution analysis

After fluorescence-labelled COM crystals were internalized as describe above, and let degraded/dissolved by Mardin-Darby Canine kidney (MDCK) cells for 24, and 48 h. Thereafter, the culture supernatant (extracellular fraction) and cells (intracellular fraction) were collected at

0, 24 and 48h. Thereafter, cells were lysed and centrifugation at 2000 x g for 5 min. The fluorescent intensities of intracellular and extracellular supernatant were measured by spectrofluorometer and the degraded fluorescence-labelled COM crystals were investigated by fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corp.; Tokyo, Japan). The degraded fluorescence-labelled COM crystal size was analyzed by ImageMaster 2D Platinum software (GE Healthcare; Uppsala, Sweden).

2.5. Immunofluorescence examination of intracellular vesicles holding internalized COM crystal

Intracellular COM crystal localization was studied by immunofluorescence examination. Briefly, after to internalized fluorescent COM crystals and allow to degraded /dissolved for 16h, MDCK cells were then fixed and permealized. Thereafter, coverslips was probed with mouse anti-Rab5 (early endosomal marker) or anti-Rab7 (late endosomal marker) antibodies at a dilution 1:100 for 1h and probe with secondary antibody conjugated Alexa 546 and Alexa 555, respectively. Cell border were be determined by staining with fluorescent-labelled phalloidin. After being wash, the images were taken by fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corp.; Tokyo, Japan).

3. RESULTS

3.1. MDCK cell culture and fluorescence-labelled COM crystals preparation

To define the mechanism of kidney stone formation and their pathogenesis, renal tubular epithelial cell lines are regular model for crystal-cell interaction studies. Especially, MDCK cells are used as the model of distal tubular epithelium in several *in vitro* experiments (23). This study, MDCK cells were cultured and well maintained for further studies, as appearance the honeycomb-liked confluent monolayer cells in **Figure1**.

Previously, we successfully labelled COM crystals by fluorescent-conjugated immunoglobulin (IgG). Although, immunoglobulin are not effect COM crystal properties and crystal-cell interaction, but the cost of fluorescent-conjugated immunoglobulin are high. Thus, we produced novel fluorescence-labelled COM crystals with fluorescein isothiocyanate (FITC). **Figure2** represents typical monoclinic prismatic shape of fluorescence-labelled COM crystals and typical size (approximately 10-20 μm in length) similar to our previous study (30). Flow cytometric analysis also demonstrated that the percentage of fluorescent signal intensity above the cut off level was much greater in the fluorescent COM crystals compared to the plain crystals (98.27±1.24 vs. 1.22±0.36%; $p<0.0001$) (note that 1.22±0.36%; of the plain crystals represented background of flow cytometric analysis) (**Figure3**). So, these fluorescence-labelled COM crystals were suitable for our following experimentations.

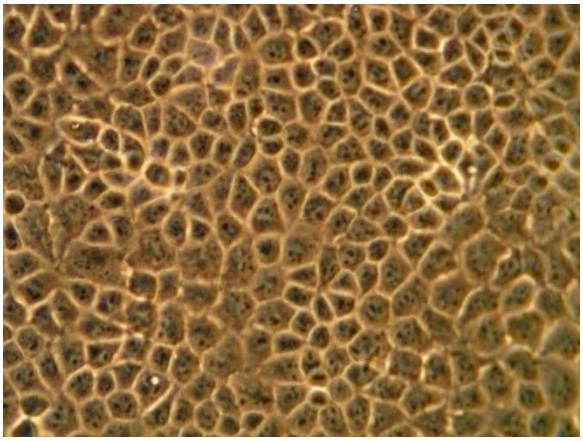


Figure1. The morphology of confluent monolayer of culture MDCK cells was visualized under Phase-contrast microscope. Original magnification = 400X.

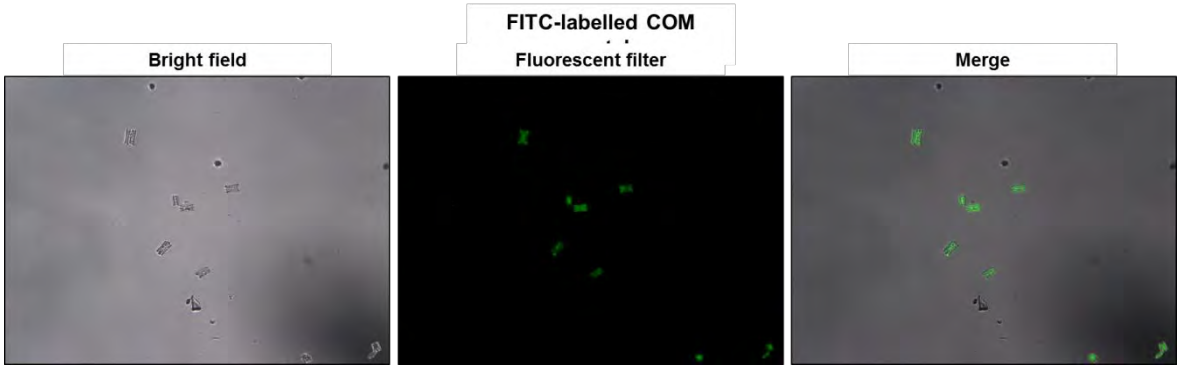


Figure2. The fluorescence images of typical monoclinic prismatic shapes of fluorescence-labelled COM crystals (FITC). The images in all panels were taken from Nikon ECLIPSE 80i (Nikon Corp.; Tokyo, Japan) with original magnification = 400X.

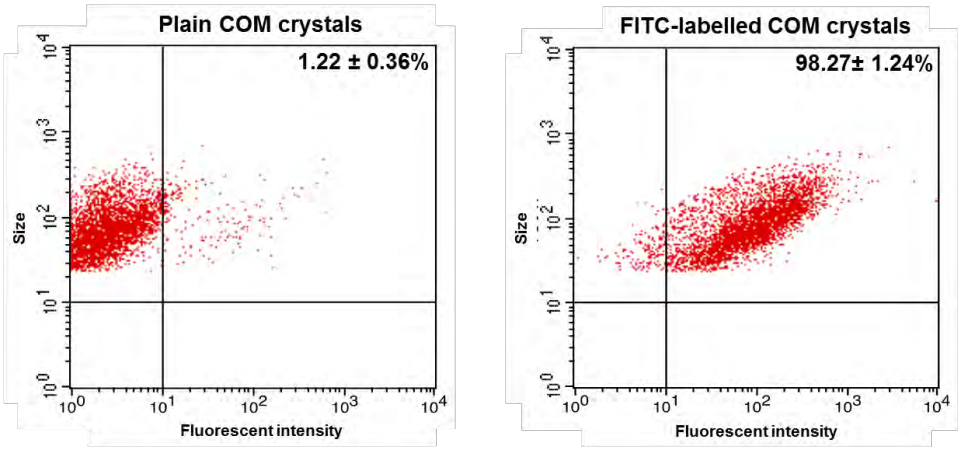


Figure3. Flow cytometric dot plots of fluorescence-labelled COM crystals and non-fluorescence plain COM crystals. Fluorescent intensities of COM crystals in “Upper right quadrant” demonstrated the percentages of fluorescent intensities above the threshold of fluorescence-labelled and and plain crystals with (98.27±1.24 vs. 1.22±0.36%, respectively; $p<0.0001$).

3.2. Internalization of COM Crystals with MDCK Cells

After the MDCK cells were incubated with 1000 μg/ml fluorescence-labelled COM crystals for 1 h, and removed non-internalized (both adherent and non-adherent crystals) COM crystals. **Figure4** shows the morphologies of MDCK cells internalized fluorescence-labelled COM crystals. Moreover, internalized crystals were then successfully quantitated by flow cytometry using the MDCK cells with plain crystals as the control (**Figure5**). Additionally, dot plot of non-fluorescent COM crystals treated MDCK cells were also presented same pattern as florescent COM crystals but also had less fluorescent signal intensity. The percentage of cells with the internalized crystals could be efficiently increased (14.83±0.85 vs. 0.52±0.38% for cells with internalized fluorescent COM crystals vs. cells with plain crystals, respectively; $p<0.0001$) (note that 0.52±0.38% in the cells with plain crystals represented background of flow cytometric analysis).

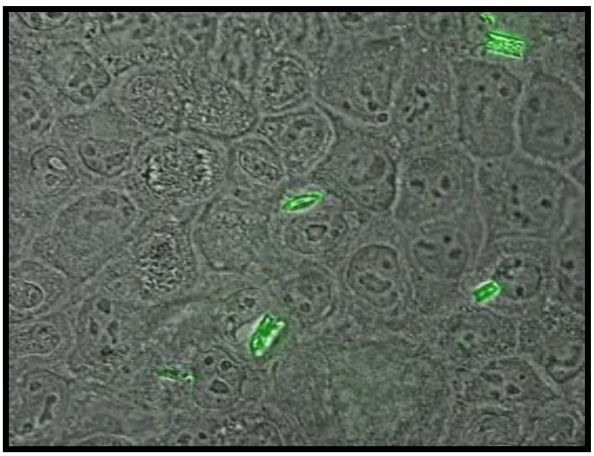


Figure4. The fluorescence image of intracellular fluorescence-labelled COM crystals. The images in all panels were taken from Nikon ECLIPSE 80i (Nikon Corp.; Tokyo, Japan) with original magnification = 400X.

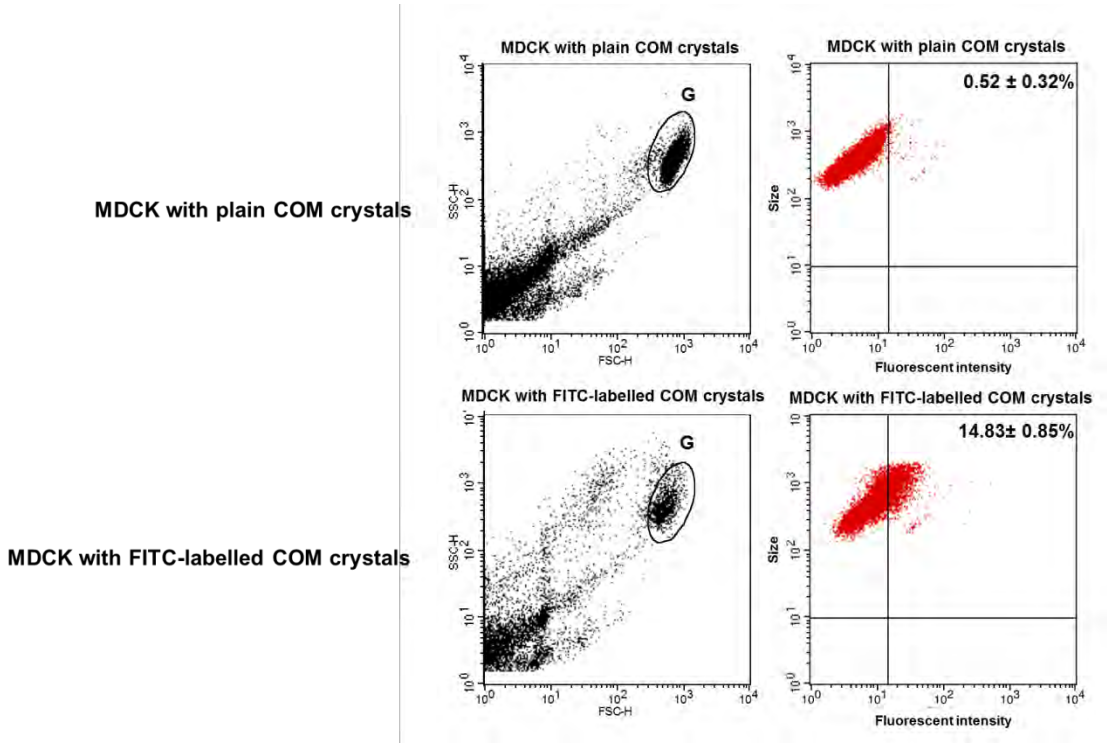
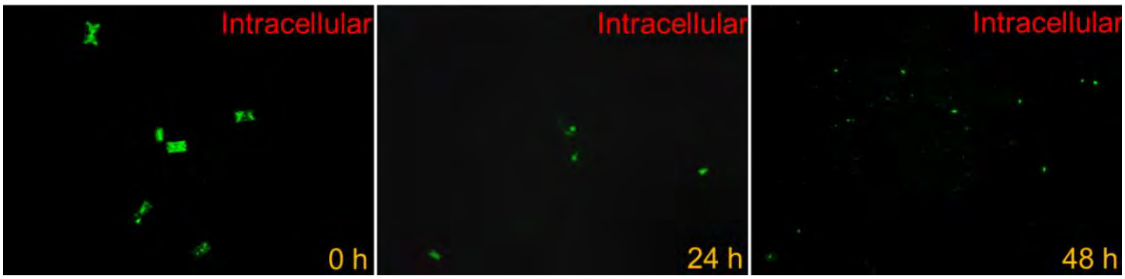


Figure5. These represented dot plots of MDCK cells with internalized of plain crystals or fluorescence-labelled COM crystals. Fluorescent intensities of population MDCK cells in “G” gate in left panel dot plots were obtained and presented in right panel dot plots. Percentages of MDCK cells with fluorescent intensities above the threshold (upper right quadrant) were then compared (14.83±0.85 vs. 0.52±0.32% for cells with internalized fluorescent COM crystals vs. cells with plain crystals, respectively; $p<0.0001$). Note that all the data in this figure were taken from a flow cytometer.

3.3. Degradation of internalized COM crystal

After 1h incubation of fluorescence-labelled COM crystals and eliminated all extracellular crystals, and then allow MDCK cells degraded/ dissolved intracellular fluorescence-labelled COM crystals for 24 and 48h. Cells were harvested and broken at 0, 24 and 48 after internalization. Thereafter, intracellular fluorescence-labelled COM crystals and their degraded/dissolved crystals were collected by centrifugation. Consequently, intracellular fluorescent COM crystal images were taken by fluorescent microscope and analyzed by ImageMaster 2D Platinum software. We successfully demonstrated smaller fluorescence-labelled COM crystals at 24h and most minuscule sizes at 48h after internalization (**Figure6A**). Moreover, quantitative analysis by software confirmed the significantly reduced of crystal size by time dependent manner as illustrated in **Figure6B**. Hence, internalized COM crystals were digested by renal tubular epithelial cells before 24h and almost degraded/ dissolved at 48h after endocytosis.

(A)



(B)

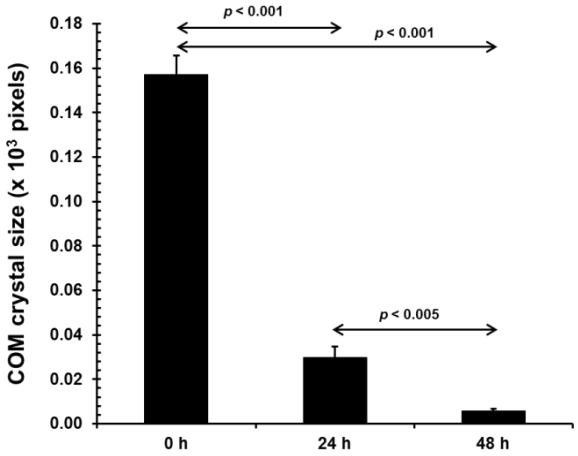


Figure6. The fluorescence images of degraded fluorescence-labelled COM crystals (Original magnification = 400X) (A) and the quantitatively analysis of degraded COM crystal size by ImageMaster 2D Platinum software (B) at 0, 24 and 48h after internalization.

3.4. Dissolution of internalized COM crystals and trafficking of degraded/dissolved products

Previous studies, (^{14}C)-oxalic acid labelled COM crystals were used as tool for quantitative crystals dissolution and following secreted products (31). However, the problem of a long-half-life (5730 years) radioisotope (^{14}C) and simply transfer of carbon compound to environment are disadvantage of these radiolabelled crystals (30). We therefore applied our novel fluorescence-labelled COM together with spectrofluorometer to measured dissolve crystal by fluorescent intensity and investigate the dissolve COM crystal trafficking. Afterward, allow MDCK cells degraded/ dissolved intracellular fluorescence-labelled COM crystals for 24 and 48h as aforementioned. Extra and intra cellular fractions at 0, 24 and 48 after internalization were collected by centrifugation and subsequently examined by spectrofluorometer. **Figure7** demonstrated fluorescent intensity of intracellular fraction (dissolved products) was significantly decreased at 24 and 48h after internalization. In contrast to extracellular fraction (secretory products) was extremely increased that might be COM crystal were rapidly digested and secreted by MDCK cells before 24h after internalization. Thus, we successfully generated new assay for quantitative analysis of dissolution that could be the useful tool for improvement of COM crystal studies. Moreover, extremely size reduction of fluorescence-labelled COM crystals as well as increase of extracellular fluorescent intensity at 24 after internalization indicated that foremost COM crystals were digested before 24h. Therefore, earlier (16 h after internalization) time might be the appropriate period for examining digestion mechanism of COM crystals by renal tubular epithelial cells.

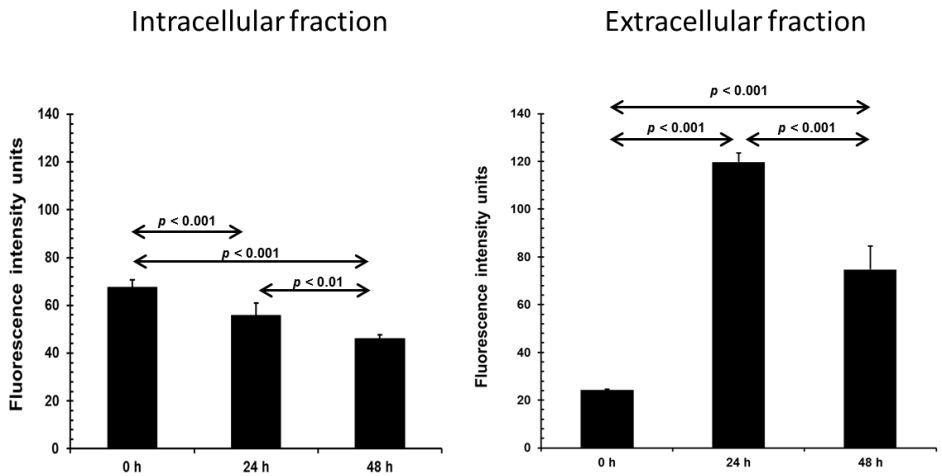


Figure7. This represents the quantitatively analysis intracellular fraction (Left panel) and extracellular fraction (Right panel) of COM crystal dissolution by fluorescence intensity at 0, 24 and 48 h after internalization.

3.5. Mechanism of intracellular COM crystal ingestion

To defined the mechanism of COM crystal digestion by renal tubular epithelial cell. Thus, we performed immunofluorescence examination at 16 h after internalization. Early endosome marker (Rab5) and late endosome marker (Rab7) were used for immunofluorescence staining. The enlargement endosome containing COM crystal was demonstrated localization with both Rab5 and Rab7 as illustrated in **Figure8**. Moreover, large endosome containing COM crystal (red signal) also found associated with F-actin (green signal) as show in yellow. Therefore, as enlarge endosome contain both early and late endosome marker, the mechanism of COM crystal digestion might be involved in giant endosomal biogenesis (32, 33) and Rab5 regulation of Kiss and run fusion of endosome and lysosome (34). Moreover, large endosome containing COM crystal (red signal) also found associated with F-actin (green signal) as show in yellow signal. This might be related to Rab5 regulate early endosome trafficking (35).

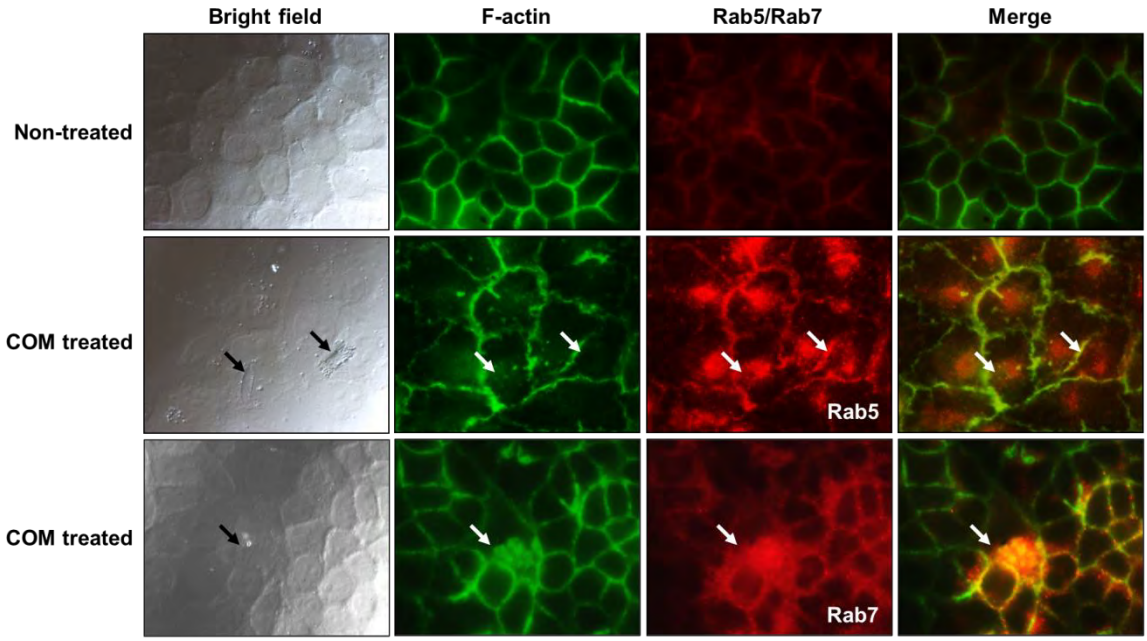


Figure8. The immunofluorescence staining of Non-treated and COM treated MDCK cells with early endosome marker (Rab5) and late endosome marker (Rab7) (arrow = intracellular COM crystals), Original magnification = 400X

4. DISCUSSION AND CONCLUSION

Kidney stone disease is an ongoing critical trouble in this time. Indeed, the effective medication with lithotripsy and surgery are useful; however, the recurrent stone formations are repeatedly occurred in stone former patient (36). Accordingly, the lacks of defensive manners lead to the cost-of-illness and economics problem in developing world. To resolve this problem, the pathogenic mechanisms of the disease become a critical issue of awareness. Additionally, the retention of crystals in tubular lumen has been focused as an important step to trigger a cascade of responses that is necessary for kidney stone developing (37, 38). Otherwise, the rapid uptake of crystals by the specific segment of renal tubular cells has been proposed to be the protective process to eliminate the retaining crystals (31). Although numerous evidences demonstrate the uptake of crystals into the renal tubular cells, up till now the processes of intracellular crystals localization and degradation/dissolution are yet poorly understand. Moreover, endosomes biogenesis and sorting of epithelial cells are involved in transcytosis process, including trafficking and degrading of uptake particle must be elucidated.

This study therefore analytically investigates on internalization, intracellular localization and degradation/dissolution of COM crystals by renal epithelial cells (MDCK cell line). The novel fluorescence-labeled COM crystals were created and applied in the present study instead of radiolabeled-COM crystals for reasons of safety and quantifiable, making the assay more accurate and simple. This will resolve the difficult of using a long-half-life radioisotope (^{14}C)-oxalic acid labelled COM crystals which carbon compound (^{14}C) could be easily transfused to environment (30). Additionally, tracking of internalized crystal inside the microsomes (endosomes/lysosomes) and their degraded/dissolved products were improved by using fluorescence-labelled COM crystals. Therefore, we successfully labelled COM crystals by fluorescein isothiocyanate (FITC) dye that are not similar too our previous fluorescent- labelled COM crystals (30). By using FITC dye instead of fluorescent- conjugated immunoglobulin (IgG) will reduce the price and problem of immunoglobulin (IgG) intervention experiment. Moreover, the morphology and properties of these fluorescence-labelled crystals were examined by fluorescence microscope and flow cytometric analysis (**Figure2, 3**). Thus, we successfully produced same quality and be able to applied fluorescence-labelled COM crystals for COM crystal internalization study as our previous work (**Figure4, 5**). Moreover, generation of fluorescence-labeled crystal also provides a promising approach to address other question regarding crystal-cell interaction especially the internalization for the critical ambition in protection of newly and/or repeated stone formation in kidney.

Additionally, combination of fluorescence-labelled COM crystals and image analysis software developed the different technique for quantify degraded COM crystals. The degradation products were collected and their images were capture by fluorescent microscope. We demonstrated the crystal degraded/dissolved (**figure6A**) and calculated crystal size (**figure6B**). This is the first endorsement of quantitative data of degraded/ dissolved internalized COM crystals. Moreover, quantitation of dissolution fluorescence-labelled COM crystals was then achieved to investigation intracellular dissolution and trafficking of their products. Previously, measurement of radioisotope (^{14}C)-oxalic acid in solution for (^{14}C)-oxalic acid labelled COM crystals dissolution and following secreted products were used for examination (31). Thus, we also developed novel assay for quantitative dissolution of fluorescence-labelled COM crystals (intracellular fraction) and secretion of dissolved products (extracellular fraction) by spectrofluorometer. Intracellular fluorescence intensity was decrease related to dissolution of fluorescence-labelled COM crystals and secreted from MDCK cells to extracellular fraction by time dependent manner (**figure7**). So, the extracellular fluorescence intensity was sharply increased at 24h associated with particularly decreased of crystal size (**figure6**) at 24h as well. The slightly drop fluorescence intensity of 48h extracellular fraction might be fade of FITC dye at that time point. We demonstrated that COM crystals were rapidly degraded/ dissolved before 24h after internalization as abundant increased of extracellular fluorescent intensity (**Figure7**). Hence, early time point before 24h should be the appropriation period for investigating degradation mechanism of COM crystal by renal tubular epithelial cells.

Afterward, we performed of immunofluorescence examination of endosome marker (Rab5 and Rab7) at 16h after internalization to identified the exactly mechanism of intracellular digestion of COM crystals. Rab5 and Rab7 were represented co -localized with giant endosome containing COM crystal. The enlarge endosome might be performed both early and late endosome role similar to previous studies (34). Rab5 containing enlarge endosome have been demonstrated, be able to regulate and fuse with late endosome or lysosome (39) and also found COM crystals degraded /dissolved at this period. Rab5 is also known as key regulatory protein of “Kiss and run” process for digest particle (34). Rab5 Enzyme are transferred or exchanged during every short time interacting of early endosome with late endosome and/ or lysosome. Subsequent, enlarge endosome could contain lysosomal enzyme and reduced to lower pH condition for digestion mechanism (32, 33, 40). Therefore, large endosome containing Rab5 could degrade particle and be broken to small vesicles and sorting out of cytoplasm. Thus, Rab5 regulated Kiss and run process might be the main mechanism of intracellular digestion of COM crystals by renal tubular epithelial cells.

In conclusion, we successfully demonstrated Rab5 containing enlarge or giant endosome with Kiss and Run" process is the main mechanism of intracellular COM crystal degradation/dissolution and endosome lysosome biogenesis. However, further investigation of enlarge endosome molecular mechanism in COM crystals digestion and trafficking would lead to increase more understanding in pathogenesis of kidney stone disease and find novel effective protection methods for stone formation and recurrent. Additionally, we also developed innovative simply method for COM crystal degradation and dissolution analysis. This could be powerful tool for quantified degraded and dissolved COM crystal and also crystal-cell interaction.

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   - สามารถพัฒนาวิธีการใหม่ในการวิเคราะห์สลาย และการขับออกของผลผลิตจากการสลายของผลึกแคลเซี่ยมอ๊อกซาเลตโมโนไฮเดรต ซึ่งเป็นวิธีที่ไม่ยุ่งยากและปลอดภัยกว่าวิธีที่ใช้ทั่วไปในปัจจุบัน ซึ่งก่อประโยชน์ต่องานวิจัยและศึกษาพยาธิสภาพและกลไกการเกิดของนิ่วในไต
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ORIGINAL PAPER

Macropinocytosis is the Major Mechanism for Endocytosis of Calcium Oxalate Crystals into Renal Tubular Cells

Rattiyaporn Kanlaya · Kitisak Sintiprungrat · Sakdithep Chaiyarit · Visith Thongboonkerd

Abstract During an initial phase of kidney stone formation, the internalization of calcium oxalate (CaOx) crystals by renal tubular cells has been thought to occur via endocytosis. However, the precise mechanism of CaOx crystal endocytosis remained unclear. In the present study, MDCK renal tubular cells were pretreated with inhibitors specific to individual endocytic pathways, including nystatin (lipid raft/caveolae-mediated), cytochalasin D (actin-dependent or macropinocytosis), and chlorpromazine (CPZ; clathrin-mediated) before exposure to plain (non-labeled), or fluorescence-labeled CaOx monohydrate (COM) crystals. Quantitative analysis by flow cytometry revealed that pretreatment with nystatin and CPZ slightly decreased the crystal internalization, whereas the cytochalasin D pretreatment caused a marked decrease in crystal uptake. Immunofluorescence study and laser-scanning confocal microscopic examination confirmed that the cytochalasin D-pretreated cells had dramatic decrease of the internalized crystals, whereas the total number of crystals interacted with the cells was unchanged (crystals could adhere but were not internalized). These data have demonstrated for the first time that renal tubular cells endocytose COM crystals mainly via macropinocytosis. These novel findings will be useful for further tracking the endocytosed crystals inside the cells during the course of kidney stone formation.

Keywords Calcium oxalate · Crystal · Endocytosis · Internalization · Macropinocytosis

Introduction

Calcium oxalate (CaOx) is the major causative crystalline composition of kidney stones, which lead to a common health problem around the globe [1]. Many lines of evidence from in vivo investigations and clinical studies have demonstrated that CaOx crystals can deposit inside tubular lumen [2] and renal interstitium [3, 4] during the course of kidney stone development and then aggravate renal tissue injury and dysfunction [5]. By adhesion, crystals can be retained on apical surface of renal tubular cells, which subsequently internalize the adherent crystals most likely by endocytosis [6, 7]. The internalized or endocytosed crystals are then translocated to basolateral site of renal tubular cells and finally to the interstitial space, leading to interstitial plaque formation [8, 9]. This plaque has been thought to serve as a crucial site for the stone development [10]. Despite this background, the precise mechanism for endocytosis of CaOx crystals by renal tubular cells had never been investigated previously and thus remained unknown.

Endocytosis is a common cellular function pivotal for nutrient uptake, receptor recycling, regulation of cell shape for mitosis, antigen presentation, and cell migration [11]. Major endocytic mechanisms can be divided into three distinct pathways with differential sensitivities to chemical inhibitors [12]. These include (i) lipid raft/caveolae-mediated endocytosis, (ii) macropinocytosis/phagocytosis, and

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(iii) clathrin-mediated endocytosis [12, 13]. Lipid raft/caveolae-mediated pathway causes engulfment of cholesterol-enriched microdomains (also known as lipid rafts) of the plasma membranes containing glycosylphosphatidylinositol-anchored proteins [14, 15]. Macropinocytosis/phagocytosis is initiated by changes in the dynamics of cortical actin in order to uptake particles or aqueous solutions via pseudopodium formation. The plasma membranes from two sides of pseudopodia can fuse together to engulf such particles or foreign bodies into a specialized structure namely "phagosome" (mainly used for typical phagocytes) or "macropinosome" (used for non-phagocytes) [16]. Clathrin-mediated endocytosis is a result of clathrin-coated protein assembly beneath inner leaflet of the plasma membranes, which then form clathrin-coated pits [17].

Several chemical inhibitors have been employed to evaluate specific endocytic pathways in various models of in vitro and in vivo studies [12]. Nystatin can bind with cholesterol in plasma membranes and then alters the membrane function, including caveolae formation, thus can inhibit lipid raft engulfment [18]. Cytochalasin D is used as a specific inhibitor for macropinocytosis by binding to actin filaments, thus can interfere with actin polymerization and assembly [19]. Chlorpromazine (CPZ) is widely used as an inhibitor of clathrin-mediated endocytosis because it can be incorporated into lipid bilayers and later causes defective invagination of plasma membranes [20].

In this study, we aimed to define the specific endocytic pathways that renal tubular epithelial cells used to internalize CaOx crystals into the cells. Specific inhibitors for all three major endocytic pathways together and recently established fluorescence-labeled CaOx monohydrate (COM) crystals [21] were employed for quantitative analysis using flow cytometry. Moreover, immunofluorescence staining followed by laser-scanning confocal microscopic examinations were performed to confirm the flow cytometric data.

Materials and Methods

Preparation of Plain (Non-labeled) and Fluorescence-Labeled COM Crystals

Plain COM crystals were prepared as described previously [22, 23]. Briefly, 10 mM calcium chloride was mixed with 10 mM sodium oxalate to achieve final concentrations of 5 mM and 0.5 mM, respectively, in a buffer containing 90 mM Tris–HCl (pH 7.4) and 10 mM NaCl. The mixture was incubated at room temperature (RT) overnight and the COM crystals were harvested by a centrifugation at 3,000 rpm for 5 min. The crystal pellet was washed in absolute methanol and then collected by another centrifugation at 3,000 rpm for 5 min. The size of crystals generated by this protocol was approximately 5–20 μm. The crystals were then dried and decontaminated by UV light radiation for 30 min before intervention with the cells.

The fluorescence-labeled COM crystals were generated according to the protocol recently reported by our group [21]. Briefly, the crystals were prepared as aforementioned but in the presence of 10 μl of rabbit anti-mouse IgG conjugated with FITC (DAKO; Glostrup, Denmark) during the initial reaction between calcium chloride and sodium oxalate. The mixture was incubated at RT overnight in the dark and the crystals were harvested, followed by washing, drying, and decontaminating steps as for preparation of the plain COM crystals. The size of fluorescence-labeled crystals obtained was the same as of plain crystals.

Cell Cultivation and COM Crystal Treatment

MDCK renal tubular cells were cultivated in a growth medium [Eagle's minimum essential medium (MEM; Gibco, Grand Island, NY) supplemented with 10 % fetal bovine serum (FBS) (Gibco), 1.2 % penicillin-G/streptomycin, and 2 mM L-glutamine (Sigma; St. Louis, MO)]. The cells were maintained in a humidified incubator at 37 °C with 5 % CO_2. For crystal intervention, the cells (approximately 8×10^4 cells) were seeded in each well of a 24-well plate containing a maintenance medium (MEM supplemented with 1 % heat-inactivated FBS). After 24-h incubation, the cells were washed twice with plain medium and then pretreated with 50 μM nystatin, cytochalasin D, or CPZ in the maintenance medium for 15 min. After the pretreatment, the inhibitors were discarded and the cells were rinsed twice with plain medium. The cells were further incubated with plain (non-labeled) or fluorescence-labeled COM crystals at a dosage of 500 μg crystals/ml culture medium at 37 °C with 5 % CO_2. At 1, 2, and 6 h post-incubation with crystals, the cells were subjected to the flow cytometric measurements (as detailed below). The cells without crystal intervention served as the controls.

Quantitative Analysis of Endocytosis by Flow Cytometry

At 1, 2, and 6 h post-incubation with plain (non-labeled) or fluorescence-labeled crystals with or without the inhibitor pretreatment, the cells at indicated time-points were rinsed with PBS (to eliminate the unbound crystals) followed by trypsinization. The adherent crystals were then eliminated (dissolved) by 5 mM EDTA in PBS for 5 min. The cells with internalized crystals were then quantitated using a flow cytometer (FACScan, Becton-Dickinson Immunocytometry System; San Jose, CA). Quantitation of the internalized plain crystals was performed by analyzing side scattered (SSC) light parameter. Briefly, cell population

was gated by forward-scattered (FSC) and SSC lights, which reflected cell size and granularity, respectively. The controlled cells without any treatment were used to determine the cut-off level of normal cell granularity. The cells with SSC shift-up above this cut-off value were counted as the ones with internalized crystals. For FITC-labeled crystals, the cells with positive FITC signal were directly counted as those with internalized crystals.

Immunofluorescence Staining and Imaging by Laser-Scanning Confocal Microscopy

MDCK cells were cultivated on cover slips (cleaved mica disk diameter: 9.5 mm, SPI Supplies; Toronto, Canada) for 24 h. The cells were rinsed with plain medium before pretreatment with or without 50 μM cytochalasin D for 15 min followed by incubation with 500 μg/ml COM crystals in the maintenance medium for 2 h. After washing with PBS, the cells were fixed with 3.7 % formaldehyde for 10 min and permeabilized with 0.1 % Triton X-100 for another 10 min. After another wash with PBS, the cells were incubated with Phalloidin conjugated with Oregon Green (Invitrogen/Molecular Probes; Burlington, Canada) to stain F-actin (at a dilution of 1:50 in 1 % BSA/PBS) at 37 °C for 1 h. The nuclei were counterstained by Hoechst dye (Invitrogen/Molecular Probes) (at a dilution of 1:2,000 in 1 % BSA/PBS) at RT for 5 min. The cover slips were then mounted with 50 % glycerol/PBS and the cells were visualized using a laser-scanning confocal microscope equipped with LSM5 Image Browser (LSM 510 META, Carl Zeiss; Oberkochen, Germany). COM crystals adhered on the cell surface or internalized into the cells were visualized by light reflection (in red) at $\lambda = 633$ nm (λ633 nm) of the Kr laser as described elsewhere [21, 24]. The intracellular (endocytosed) crystals were also imaged along the vertical axis (*Z*-axis).

Statistical Analysis

All the aforementioned experiments were performed in triplicate and all the quantitative data are presented as mean ± SEM unless stated otherwise. Comparisons between two sets of the data were performed using unpaired Student's *t* test. Multiple-comparisons were performed by ANOVA with Tukey's HSD post-hoc test. *P* values <0.05 were considered statistically significant.

Results

In previous studies, electron microscopic examination had demonstrated that COM crystals could be internalized into MDCK renal tubular cells by endosome formation [6, 7, 25]. On this basis, the endocytosed COM crystals should result in the increase of cellular granularity because of the formed endosomes and crystalline compositions inside the cells. To address this point, we applied quantitative flow cytometric analysis to determine changes in cellular granularity after MDCK cells were incubated with or without plain (non-labeled) COM crystals at a dosage of 500 μg/ml. At 1, 2, and 6 h post-incubation, the cells incubated with COM crystals had marked increase of cellular granularity as compared to the controlled cells (Fig. 1). The data also showed that the increase of cellular granularity of the COM-exposed MDCK cells was time dependent (i.e., the degree of increase was greatest at 6 h).

To investigate specific endocytic pathway of COM crystal uptake into renal tubular cells, MDCK cells were pretreated with each of specific inhibitors for the three main endocytic pathways (including nystatin, cytochalasin D, and CPZ) before incubation with plain COM crystals. The results showed that the increase of cellular granularity induced by plain COM crystals was modestly reduced (or even unaffected in some conditions) by the pretreatment with nystatin and CPZ (Fig. 1). However, the COM crystal-induced increase of cellular granularity was dramatically decreased by the pretreatment with cytochalasin D (Fig. 1), suggesting that macropinocytosis might be the major pathway for endocytosis of COM crystals by renal tubular cells.

Since apoptotic cell death might also lead to the increase of cell granularity and could interfere with data interpretation, we thus performed trypan blue exclusion assay to determine cell viability after treatment with crystals and inhibitors. The results showed that >95 % of the cells remained survived under our experimental conditions. COM crystals at a dosage of 500 μg crystals/ml culture medium and 6-h incubation did not affect the cell survival. Additionally, there were no significant differences by using various inhibitors (Supplementary Methods and Fig. S1). We therefore assumed that changes in cell granularity observed was due to modifications of crystal internalization into the cells and was not caused by potential toxic effects of chemicals and inhibitors.

In addition to the increase of cellular granularity induced by plain COM crystals, flow cytometric quantification of the internalized fluorescence-labeled COM crystals was also performed. This recently established technique, based on the direct quantification of fluorescence signals inside the cells, allows more accurate quantification of the internalized COM crystals [21]. In concordance with the data obtained from the plain COM crystals, the cells incubated with fluorescence-labeled COM crystals had marked increase in percentage of FITC-positive cells, which indicated the internalized crystals, in a time-dependent manner (Fig. 2). Also, pretreatment with nystatin and CPZ slightly decreased the number of the FITC-positive cells with internalized crystals. However,

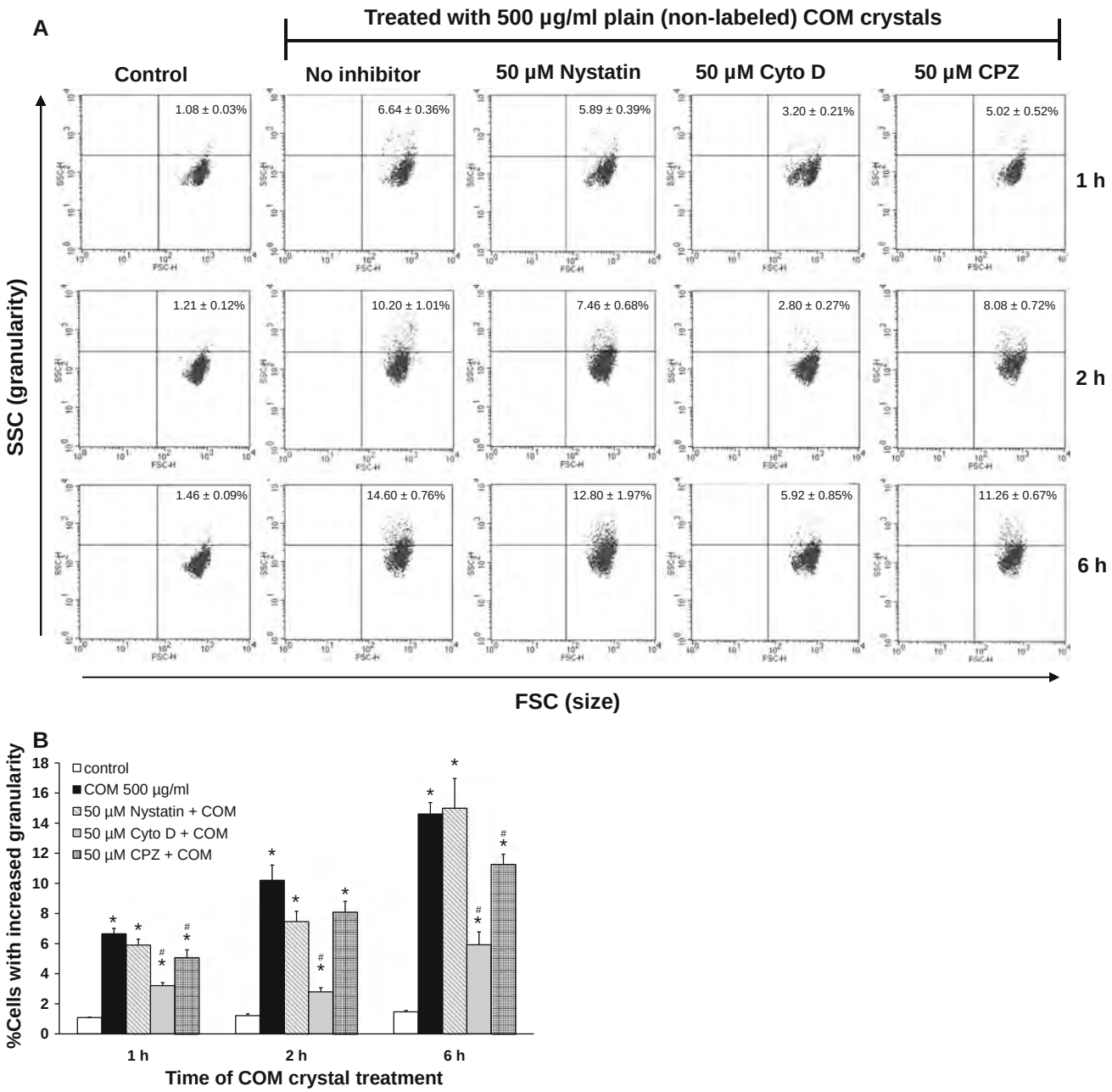

Fig. 1 Flow cytometric analysis of cellular granularity of MDCK cells incubated with plain (non-labeled) COM crystals. Before incubation with or without 500 µg/ml plain COM crystals, MDCK cells were pretreated with 50 µM nystatin, cytochalasin D, or CPZ for 15 min. After 1–6 h of incubation with crystals, the cells were harvested and analyzed by flow cytometry. Dot-plot analysis of granularity (SSC; *y*-axis) and size (FSC; *x*-axis) of the cells is demonstrated in (**a**), whereas statistical analysis of the percentage of cells with increased granularity is shown in (**b**). Each *bar* was derived from 3 independent experiments. $^{*}P < 0.05$ vs. controls; $^{\#}P < 0.05$ vs. COM-treated cells without inhibitor pretreatment

pretreatment with cytochalasin D dramatically reduced the number of FITC-positive cells with internalized crystals (Fig. 2). These results strongly suggested that COM crystals were internalized into renal tubular cells mainly through macropinocytosis.

To confirm the flow cytometric data, we performed immunofluorescence staining followed by laser-scanning confocal microscopy to directly visualize the endocytosed COM crystals with or without a specific inhibitor of macropinocytosis, cytochalasin D. This experiment was also to address whether cytochalasin D affected crystal adhesion or not. The data obtained from top-view captures revealed that the total number of crystals (adhered onto the cell surface and/or internalized into the cells) remained

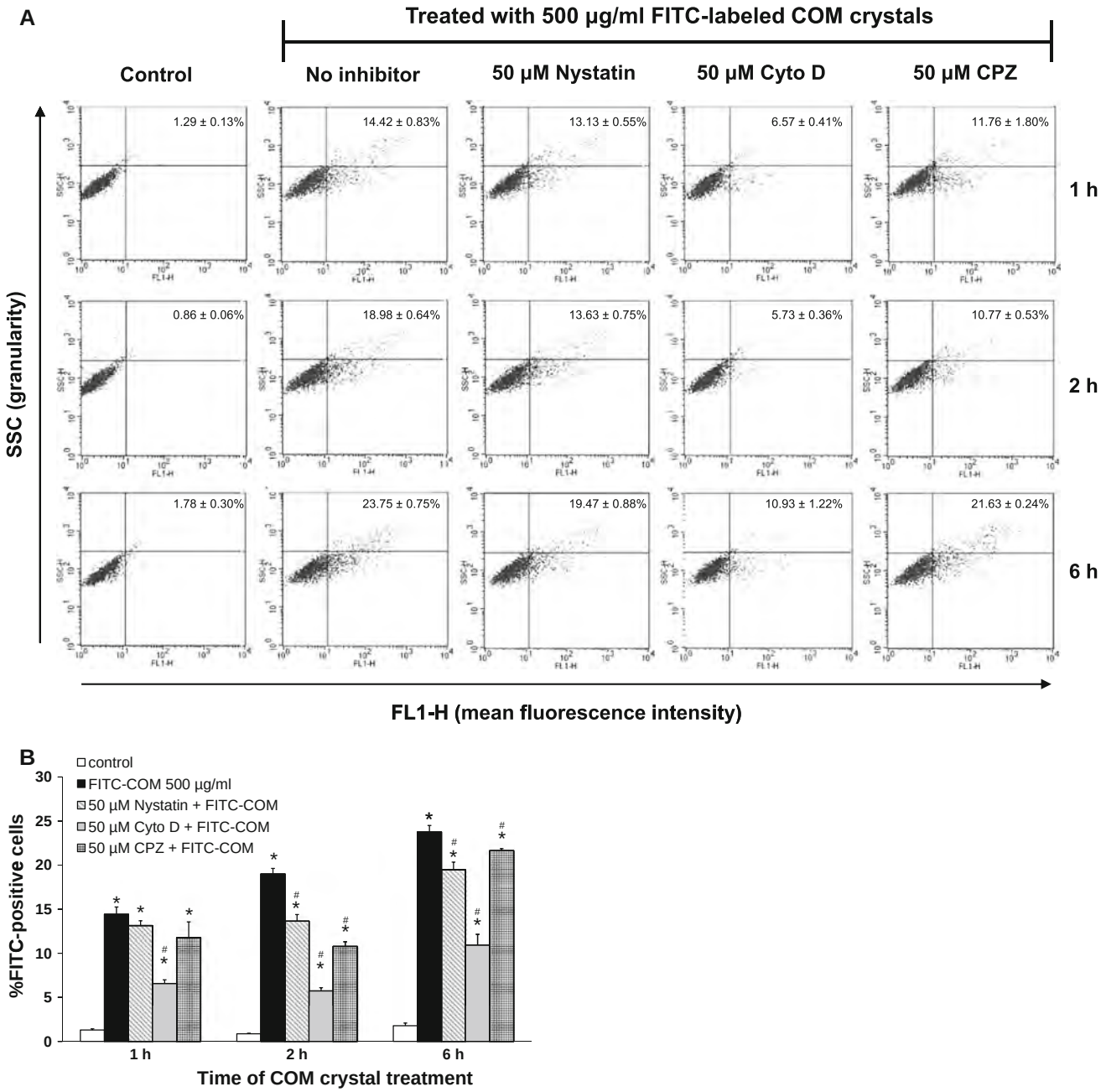

Fig. 2 Quantitative assay of the COM crystal endocytosis in MDCK cells using fluorescence-labeled crystals. Before incubation with or without 500 µg/ml FITC-labeled COM crystals, MDCK cells were pretreated with 50 µM nystatin, cytochalasin D, or CPZ for 15 min. After 1–6 h of incubation with crystals, the cells were harvested and analyzed by flow cytometry. Dot-plot analysis of granularity (SSC; *y*-axis) and mean fluorescence intensity (FL1-H; *x*-axis) of the cells is demonstrated in (**a**), whereas statistical analysis of the percentage of FITC-positive cells is shown in (**b**). Each *bar* was derived from 3 independent experiments. $^{*}P < 0.05$ vs. controls; $^{\#}P < 0.05$ vs. COM-treated cells without inhibitor pretreatment

unchanged by cytochalasin D (Fig. 3a, b). This data indicated that the inhibitory effect of cytochalasin D on endocytosis (macropinocytosis) was not a result of reduction of total number of crystals interacted with the cells, but was really a result of the specific inhibition of macropinocytosis. Similar results on number of adhered and/or internalized crystals were observed when the cells were treated with nystatin and CPZ (Supplementary Fig. S2A and S2B, respectively).

Consistently, the depth- and sagittal-views showed that the cells pretreated with cytochalasin D had the COM crystals mostly at their apical surfaces with rare crystals found inside the cells, whereas the cells without inhibitor pretreatment demonstrated many of the internalized

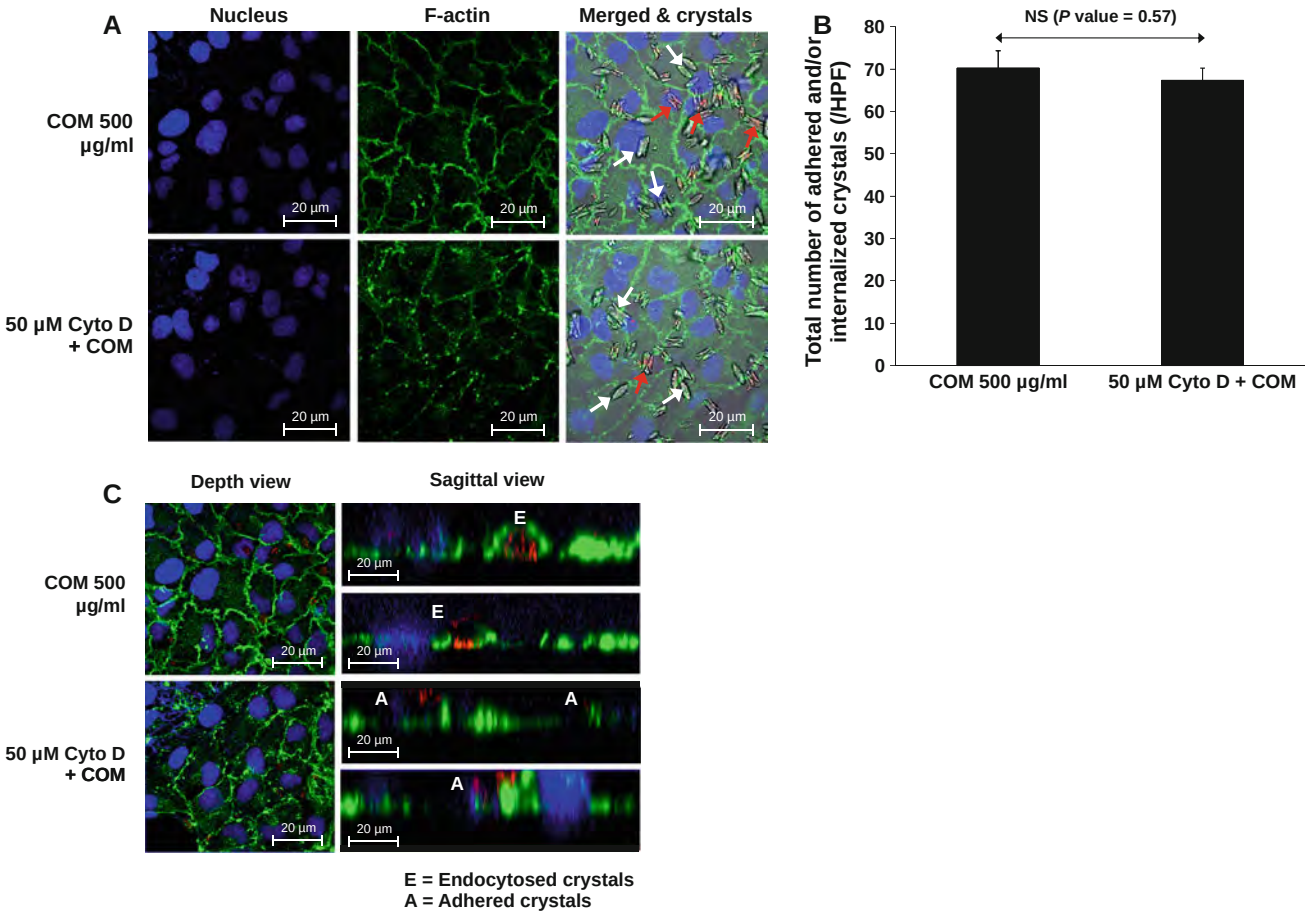

Fig. 3 Confirmation of the inhibitory effects of cytochalasin D on COM crystal endocytosis. MDCK cells grown on cover slips were pretreated with or without 50 µM cytochalasin D for 15 min before incubation with COM crystals for 2 h. **a** F-actin was stained in *green* with Oregon Green-conjugated phalloidin, whereas nuclei were counterstained with Hoechst dye. The images were taken by *top-view*. The *last column* shows merged view of bright field and fluorescence staining. The internalized crystals are shown in red color (by using light reflection at λ633 nm) and marked with *red arrows*, whereas the adhered crystals (by bright field) on cell surfaces are located with *white arrows*. **b** Quantitative data and statistical analysis of the total number of COM crystals interacted with MDCK cells by adhesion and/or internalization. Each *bar* was derived from 3 independent experiments (*HPF* high-power field). **c** Depth- and sagittal-views of cells with adhered (labeled as "*A*") or endocytosed (labeled as "*E*") crystals. The images were captured under a laser-scanning confocal microscope at an original magnification of ×630. COM crystals were visualized in *red* by a light reflection at λ633 nm using the Kr laser (Color figure online)

crystals (Fig. 3c). These findings indicated that cytochalasin D pretreatment did not interfere with crystal adhesion on the cell surface (which is a crucial step required before crystal internalization) and confirmed the flow cytometric data that cytochalasin D dramatically prevented the cells to internalize COM crystals.

Discussion

Nephrolithiasis (kidney stone disease) is a common disease affecting general adult population worldwide. Although physicochemical mechanisms of stone formation have been partially described, little is known for the interaction between renal tubular cells and crystalline compound of the stones. To unravel pathogenic mechanisms of the disease, the interaction between renal tubular cells and crystals has become one of major research topics of this field during the past decade. The fixed-particle model of the disease has suggested that crystal retention is an important step prior to the stone development [26, 27]. Several macromolecules (in free forms or anchored on the cell surfaces) have been demonstrated to involve in this step [26]. Subsequently, the crystals are rapidly engulfed or endocytosed into renal tubular cells. The endocytosis of crystals has been shown to be a crucial step during the stone initiation [6, 7, 28]. In many previous studies, animal models of kidney stone disease and clinical samples obtained from stone formers have demonstrated that CaOx crystals can be found inside renal tubular cells [29, 30]. In addition, several in vitro studies on renal tubular cell lines exposed to COM crystals have shown that the crystals could adhere on microvilli of

the cell surface and were sequentially internalized into the cells within an hour [6, 7, 25, 31]. The term "endocytosis" has been generally used for internalization or engulfment of the crystals adhered on the cell surface. However, there had been no available data to address molecular pathway underlying crystal internalization through endocytosis.

Several methods have been used for the investigations of COM crystal endocytosis. The simplest one is using a phase contrast microscope. Nevertheless, the data obtained are not reliable since this technique cannot distinguish the internalized crystals from those adhered on the cell surface. Therefore, most of the studies have employed [^{14}C]-labeled COM crystals to determine the cells with the engulfed crystals [6, 32]. Although it provides higher sensitivity, this method may be hazardous to health and environment. Another approach is to distinguish cell size and granularity between the controlled cells and those with endocytosed crystals. Flow cytometry allows for such discrimination by using FSC and SSC signals, which correlate very well with the cell size and the complexity inside the cells (e.g., granules, vesicles), respectively. The granularity of cells with endocytosed crystals was expected to increase compared to the controlled cells [33]. Furthermore, we have recently established a novel non-radioactive method based on fluorescence labeling for visualizing and tracking the crystals [21].

In the present study, we applied both flow cytometry and fluorescence-labeled crystals for the investigations of COM crystal endocytosis in renal tubular cells. It has been hypothesized that distal nephron was the priming site for stone nidus formation. Therefore, MDCK cells, which were derived from normal canine kidneys (particularly from distal renal tubule and collecting duct [34]), were employed in the present study. We first used the plain or non-labeled COM crystals and found the increased granularity of the cells with endocytosed crystals (Fig. 1). However, the obtained data could not be referred directly to the cells with the endocytosed or internalized crystals. We then employed the FITC-labeled COM crystals in combination with flow cytometry for the more reliable analyses [21]. To avoid the false positive generated by the adhered crystals, we completely dissolved such adherent crystals by using EDTA before the analysis [32]. Thus, the positive signals would directly refer to the engulfed COM crystals inside the cells. The results showed that the data obtained from fluorescence-labeled COM crystals (Fig. 2) were consistent with those obtained from the plain COM crystals (Fig. 1).

The analysis revealed that pretreatment with cytochalasin D provided the greatest degree of reduction of the endocytosed crystals, whereas nystatin and CPZ had only modest degree of such inhibition (Figs. 1, 2). However, higher degree of inhibition was observed when using fluorescence-labeled crystals. The reason behind was that detection of changes in fluorescence signal (%FITC-positive cells) is more sensitive as compared to the detection of changes in cell granularity. This means that under the same situation, if the cell engulfed only a tiny fluorescence-labeled crystal, it could be counted as a FITC-positive cell although such uptake might not affect the cell granularity.

Various drugs and chemical reagents were used to define the specific mechanisms involved in COM crystal endocytosis by MDCK cells. Previous findings suggested that increased intracellular calcium concentration, blockade of actin polymerization, inhibition of protein kinase C, and cyclo-oxygenase activity were involved in the reduction of COM crystal uptake [35]. We obtained similar findings that cytochalasin D significantly reduced the number of crystals uptake most likely via inhibition of actin polymerization. In concordance with others [6, 25], using laser-scanning confocal microscopy, we found that more than one crystal could be engulfed into the cell. Microscopic examination revealed that microvilli of the cells played a key role in crystal internalization process [36]. Lieske et al. [6] demonstrated that the adhered crystals were engulfed into vesicles by the cellular projection. Using laser-scanning confocal microscope, we also observed that the endocytosed crystals were surrounded by actin vesicles. Pretreatment with cytochalasin D did not affect crystal adhesion on MDCK cell surface. As macropinosomes were derived from actin-rich plasma membranes, so called ruffles, it was unsurprising that disruption by cytochalasin D markedly inhibited COM crystal uptake.

Although macropinosome shared some features with other organelles, e.g., endosome, lysosome, and phagolysosome, however, it has distinct organization process. While phagosome formation is initiated and maintained by interaction between Fc receptors and the internalized particles (opsonized particles), macropinosome formation is rather independent of the external stimuli [16]. In addition, maturation of macropinosome requires the exchange of membrane components with those of other organelles, including endolysosomal system (i.e., Rab7), making it more difficult to define the unique molecular markers of this process [37, 38].

Finally, it should be emphasized that the inhibition of crystal endocytosis using cytochalasin D to block macropinocytosis was not perfectly complete. This might be simply explained that endocytosis of COM crystals by renal tubular cells was also mediated partially through lipid raft/caveolae- and clathrin-dependent pathways (as the minor or alternative mechanisms to uptake COM crystals) (Figs. 1, 2). Theoretically, using inhibitors specific to all three endocytic pathways should completely inhibit the crystal uptake. However, it may not be practical because overdose of inhibitors will cause cytotoxicity and ultimately cell death, which affects interpretation of the

results. Also, the experimental condition used in our study might not be optimal to completely inhibit the COM crystal uptake. However, these findings could highlight that endocytosis of COM crystals by MDCK renal tubular cells was mainly actin cytoskeleton-dependent process (i.e., macropinocytosis).

In summary, we report herein for the first time that renal tubular cells endocytose COM crystals mainly via macropinocytosis. These novel findings will pave the way for further tracking the endocytosed crystals inside renal tubular cells during the course of kidney stone formation. Further in-depth studies on subsequent signal transduction cascade following macropinocytosis of COM crystals and the fate of the endocytosed crystals after such uptake will definitely provide valuable information to better understand the precise mechanisms of kidney stone formation.

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The role of endosomes and lysosomes in degradation/dissolution of internalized calcium oxalate monohydrate crystals by renal tubular epithelial cells

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Running Title: **"COM crystal degradation/dissolution"**

Keywords: Nephrolithiasis, calcium oxalate monohydrate crystals, degradation, dissolution, endosome, lysosome

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ABSTRACT

The lack of protection manner and repeated stone formation following medication, are still existing serious problems in kidney stone disease. Furthermore, stone formation mechanism must be elucidated to determine and improve the defensive procedures. The interaction among calcium oxalate crystals and renal tubular epithelial cells are recognized as key mechanisms of stone formation. However, the mechanisms of crystal degradation and/or dissolution after internalization are yet, still ambiguous. To explore these mechanisms, we successfully produced newly fluorescent calcium oxalate monohydrate (COM) crystal and applied to assessed crystal degradation/dissolution. Therefore, fluorescence-labeled COM crystals were degraded and/ or dissolved as demonstrated by the reduction of crystal size and intracellular fluorescent intensity by time dependent manner (0-48h after internalization). Consequently, in contrast to extracellular fluorescent intensity was increased. Moreover, the association of endosome and lysosome containing COM crystal were examined by immunofluorescence microscopy with early (Rab5) and late (Rab7) endosome marker. Accordingly, Rab5 containing enlargement early endosome were predominantly found associated with intracellular COM crystals. Enthusiastically, we continue to strive to explore this degradation/dissolution mechanism.

Keywords: Nephrolithiasis, calcium oxalate monohydrate crystals, degradation, dissolution, endosome, lysosome

INTRODUCTION

Kidney stones disease is yet, still public health problem world wild. Although, there are effective methods to remove kidney stone, for example extracorporeal shock wave lithotripsy and percutaneous nephrolithotomy, but recurrence stone are quiet high (1, 2). The pathogenesis of kidney stone disease has been studied for several years; however, the mechanisms of stone formation remain poorly understood. Accordingly, poorly soluble salts like calcium oxalate are commonly supersaturated in concentrated urine and generally precipitated as crystals. Calcium oxalate monohydrate (COM) crystal is found more frequent than any other crystals in the kidney stone disease (3). These crystalline particles can be retained in renal tubules and undergone impressive interaction with renal tubular epithelial cells, resulting in stone formation. In addition, crystal retention in kidney could be depending on the interaction between crystals and the renal tubular epithelial cells, even crystals are small (4-6). The interaction between crystal and cell has been proposed as the key mechanism of stone formation. Several investigators have proposed that calcium oxalate crystals adhere in specific manner of the apical membrane of renal tubular epithelial cells and this progression is rapidly followed by internalization (7, 8). Moreover, Lieske et al. found COM crystals were taken up and then degraded afterward by renal tubular cells (9). Additionally, the recent finding have shown that the interaction between COM crystals and renal tubular cells can promote numerous responses such as cell proliferation (8, 10, 11), cell death (12-14), cellular injury (15, 16), formation of reactive oxygen species (17, 18), mitochondria dysfunction (19, 20) and inflammation (21, 22).

Although massive effort has been made on the investigation on the adherence and endocytosis of COM crystals to renal tubular epithelial cells, the manner after internalization and mechanism of crystal degradation or dissolution has been slightly consideration. Thus,

intracellular mechanism of COM crystal degradation, dissolution and elimination are the remarkable process. Previous study demonstrated COM crystals could be dissolved in lysosome 5-7 weeks after internalization (9). However, the subsequence processes of COM crystal digestion, including early and late endosomes formation, the fusion of late endosome with lysosomes and intracellular trafficking of crystal particles are not elucidated. The degradation and dissolution COM crystals products were secreted to apical sites have been proposed, thus, theses might be the regular defense against this stone disease (23, 24). Alternatively, degraded/dissolved products might promote inflammatory response at interstitial site (the plaque and stone formation region) (25-29). Therefore, the increasingly of crystal degraded fragments, calcium and oxalate ion in basolateral area of epithelial cell after internalization process might be involved in stone or plaque formation at interstitial tissue.

This study assumes that the degradation/dissolution processes and trafficking molecules of internalized COM crystals would be the key process of cellular response and these processes might be the defense mechanism to eliminate retention crystal. Consequently, this study has been proposed to investigate the potential role of crystal digestion and trafficking by renal epithelial tubular cells. The fluorescence-COM crystals were used in this work, not only simple and effective techniques for imaging and quantification crystal but also more safety than using radioactive crystal for study crystal-cell interaction. The degradation of fluorescence-COM crystals were examined and quantified by fluorescent imaging and image software analysis. Moreover, dissolution of fluorescence-COM crystals was investigated by spectrofluorometer. The development of imaging was very useful tool to investigate mechanisms of crystal degradation/dissolution. Additionally, immunofluorescence staining examinations were performed to observe intracellular crystal trafficking and endosome-lysosome mechanism. The

information achieved from this study would be lead to more understanding in the crystal digestion mechanism and the pathogenesis of kidney stone formation.

MATERIALS AND METHODS

Cell culture

Mardin-Darby Canine kidney (MDCK), a distal tubular epithelial cells were be cultured in Eagle's minimal essential medium (MEM) (Gibco; Grand Island, NY) containing 10% heat inactivated fetal bovine serum (FBS) (Gibgo), 2mM glutamine (Sigma) in the presence of 100 U/ml penicillin G and 100 mg/ml streptomycin (Sigma, St.Louis, MO). The cells were be maintained in a humidified incubator 37°C with 5% CO2. The medium was refreshed every other day. Morphology of the cells was checked under an inverted phase-contrast microscope

Preparation of fluorescence-labelled COM crystals

Fluorescence-labelled COM crystals were prepared as described previously (30). Briefly, 5 mM CaCl2·2H2O was mixed with 0.5 mM Na2C2O4 in a buffer containing 90 mM Tris-HCl and 10 mM NaCl (pH 7.4) in the presence of 0.01 μg/mL FITC (Thermo Scientific Pierce, Rockford, IL). The solutions were incubated at 25°C overnight and COM crystals were harvested by a centrifugation at 2000 x g for 5 min. The supernatant was discarded and COM crystals were washed 3 times with methanol. After another centrifugation at 2000 x g for 5 min, methanol was discarded and the crystals were air-dried overnight at room temperature. COM crystals were imaged by phased-contrast microscopy (Olympus CKX41, Olympus Co. Ltd.; Tokyo, Japan) and fluorescent microscopy (Nikon ECLIPSE 80i, Nikon Corp.; Tokyo, Japan). Note that all of the reagents were sterilized before use for subsequent intervention with cells.

Internalization examination of fluorescence-labelled COM crystals

Mardin-Darby Canine kidney (MDCK) 1x105 cells were cultured with media containing 1000 μg/ml fluorescence-labelled COM crystals. After a subsequent 1h incubation, 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 examined by fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corp.; Tokyo, Japan) and flow cytometer (FACScan, Becton Dickinson Immunocytometry System; San Jose, CA).

Fluorescence-labelled COM crystal degradation/dissolution analysis

After fluorescence-labelled COM crystals were internalized as describe above, and let degraded/dissolved by Mardin-Darby Canine kidney (MDCK) cells for 24, and 48 h. Thereafter, the culture supernatant (extracellular fraction) and cells (intracellular fraction) were collected at 0, 24 and 48h. Thereafter, cells were lysed and centrifugation at 2000 x g for 5 min. The fluorescent intensities of intracellular and extracellular supernatant were measured by spectrofluorometer and the degraded fluorescence-labelled COM crystals were investigated by fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corp.; Tokyo, Japan). The degraded fluorescence-labelled COM crystal size was analyzed by ImageMaster 2D Platinum software (GE Healthcare; Uppsala, Sweden).

Immunofluorescence examination of intracellular vesicles holding internalized COM crystal

Intracellular COM crystal localization was studied by immunofluorescence examination. Briefly, after to internalized fluorescent COM crystals and allow to degraded /dissolved for 16h, MDCK cells were then fixed and permealized. Thereafter, coverslips was probed with mouse anti-Rab5 (early endosomal marker) or anti-Rab7 (late endosomal marker) antibodies at a dilution 1:100 for 1h and probe with secondary antibody conjugated Alexa 546 and Alexa 555, respectively. Cell border were be determined by staining with fluorescent-labelled phalloidin. After being wash, the images were taken by fluorescence microscope (Nikon ECLIPSE 80i, Nikon Corp.; Tokyo, Japan).

RESULTS

MDCK cell culture and fluorescence-labelled COM crystals preparation

To define the mechanism of kidney stone formation and their pathogenesis, renal tubular epithelial cell lines are regular model for crystal-cell interaction studies. Especially, MDCK cells are used as the model of distal tubular epithelium in several in vitro experiments (23). This study, MDCK cells were cultured and well maintained for further studies, as appearance the honeycomb-liked confluent monolayer cells in **Figure1**.

Previously, we successfully labelled COM crystals by fluorescent-conjugated immunoglobulin (IgG). Although, immunoglobulin are not effect COM crystal properties and crystal-cell interaction, but the cost of fluorescent-conjugated immunoglobulin are high. Thus, we produced novel fluorescence-labelled COM crystals with fluorescein isothiocyanate (FITC). **Figure2** represents typical monoclinic prismatic shape of fluorescence-labelled COM crystals and typical size (approximately 10-20 μm in length) similar to our previous study (30). Flow cytometric analysis also demonstrated that the percentage of fluorescent signal intensity above the cut off level was much greater in the fluorescent COM crystals compared to the plain crystals (98.27±1.24 vs. 1.22±0.36%; $p<0.0001$) (note that 1.22±0.36%; of the plain crystals represented background of flow cytometric analysis) (**Figure3**). So, these fluorescence-labelled COM crystals were suitable for our following experimentations.

Internalization of COM Crystals with MDCK Cells

After the MDCK cells were incubated with 1000 μg/ml fluorescence-labelled COM crystals for 1 h, and removed non-internalized (both adherent and non-adherent crystals) COM

crystals. **Figure4** shows the morphologies of MDCK cells internalized fluorescence-labelled COM crystals. Moreover, internalized crystals were then successfully quantitated by flow cytometry using the MDCK cells with plain crystals as the control (**Figure5**). Additionally, dot plot of non-fluorescent COM crystals treated MDCK cells were also presented same pattern as florescent COM crystals but also had less fluorescent signal intensity. The percentage of cells with the internalized crystals could be efficiently increased (14.83±0.85 vs. 0.52±0.38% for cells with internalized fluorescent COM crystals vs. cells with plain crystals, respectively; $p<0.0001$) (note that 0.52±0.38% in the cells with plain crystals represented background of flow cytometric analysis).

Degradation of internalized COM crystal

After 1h incubation of fluorescence-labelled COM crystals and eliminated all extracellular crystals, and then allow MDCK cells degraded/ dissolved intracellular fluorescence-labelled COM crystals for 24 and 48h. Cells were harvested and broken at 0, 24 and 48 after internalization. Thereafter, intracellular fluorescence-labelled COM crystals and their degraded/dissolved crystals were collected by centrifugation. Consequently, intracellular fluorescent COM crystal images were taken by fluorescent microscope and analyzed by ImageMaster 2D Platinum software. We successfully demonstrated smaller fluorescence-labelled COM crystals at 24h and most minuscule sizes at 48h after internalization (**Figure6A**). Moreover, quantitative analysis by software confirmed the significantly reduced of crystal size by time dependent manner as illustrated in **Figure6B**. Hence, internalized COM crystals were digested by renal tubular epithelial cells before 24h and almost degraded/ dissolved at 48h after endocytosis.

Dissolution of internalized COM crystals and trafficking of degraded/dissolved products

Previous studies, (^{14}C)-oxalic acid labelled COM crystals were used as tool for quantitative crystals dissolution and following secreted products (31). However, the problem of a long-half-life (5730 years) radioisotope (^{14}C) and simply transfer of carbon compound to environment are disadvantage of these radiolabelled crystals (30). We therefore applied our novel fluorescence-labelled COM together with spectrofluorometer to measured dissolve crystal by fluorescent intensity and investigate the dissolve COM crystal trafficking. Afterward, allow MDCK cells degraded/ dissolved intracellular fluorescence-labelled COM crystals for 24 and 48h as aforementioned. Extra and intra cellular fractions at 0, 24 and 48 after internalization were collected by centrifugation and subsequently examined by spectrofluorometer. **Figure7** demonstrated fluorescent intensity of intracellular fraction (dissolved products) was significantly decreased at 24 and 48h after internalization. In contrast to extracellular fraction (secretory products) was extremely increased that might be COM crystal were rapidly digested and secreted by MDCK cells before 24h after internalization. Thus, we successfully generated new assay for quantitative analysis of dissolution that could be the useful tool for improvement of COM crystal studies. Moreover, extremely size reduction of fluorescence-labelled COM crystals as well as increase of extracellular fluorescent intensity at 24 after internalization indicated that foremost COM crystals were digested before 24h. Therefore, earlier (16 h after internalization) time might be the appropriate period for examining digestion mechanism of COM crystals by renal tubular epithelial cells.

Mechanism of intracellular COM crystal ingestion

To defined the mechanism of COM crystal digestion by renal tubular epithelial cell. Thus, we performed immunofluorescence examination at 16 h after internalization. Early endosome marker (Rab5) and late endosome marker (Rab7) were used for immunofluorescence staining. The enlargement endosome containing COM crystal was demonstrated localization with both Rab5 and Rab7 as illustrated in **Figure8**. Moreover, large endosome containing COM crystal (red signal) also found associated with F-actin (green signal) as show in yellow. Therefore, as enlarge endosome contain both early and late endosome marker, the mechanism of COM crystal digestion might be involved in giant endosomal biogenesis (32, 33) and Rab5 regulation of Kiss and run fusion of endosome and lysosome (34). Moreover, large endosome containing COM crystal (red signal) also found associated with F-actin (green signal) as show in yellow signal. This might be related to Rab5 regulate early endosome trafficking (35).

4. DISCUSSION AND CONCLUSION

Kidney stone disease is an ongoing critical trouble in this time. Indeed, the effective medication with lithotripsy and surgery are useful; however, the recurrent stone formations are repeatedly occurred in stone former patient (36). Accordingly, the lacks of defensive manners lead to the cost-of-illness and economics problem in developing world. To resolve this problem, the pathogenic mechanisms of the disease become a critical issue of awareness. Additionally, the retention of crystals in tubular lumen has been focused as an important step to trigger a cascade of responses that is necessary for kidney stone developing (37, 38). Otherwise, the rapid uptake of crystals by the specific segment of renal tubular cells has been proposed to be the protective process to eliminate the retaining crystals (31). Although numerous evidences demonstrate the uptake of crystals into the renal tubular cells, up till now the processes of intracellular crystals localization and degradation/dissolution are yet poorly understand. Moreover, endosomes biogenesis and sorting of epithelial cells are involved in transcytosis process, including trafficking and degrading of uptake particle must be elucidated.

This study therefore analytically investigates on internalization, intracellular localization and degradation/dissolution of COM crystals by renal epithelial cells (MDCK cell line). The novel fluorescence-labeled COM crystals were created and applied in the present study instead of radiolabeled-COM crystals for reasons of safety and quantifiable, making the assay more accurate and simple. This will resolve the difficult of using a long-half-life radioisotope (^{14}C)-oxalic acid labelled COM crystals which carbon compound (^{14}C) could be easily transfused to environment (30). Additionally, tracking of internalized crystal inside the microsomes (endosomes/lysosomes) and their degraded/dissolved products were improved by using

fluorescence-labelled COM crystals. Therefore, we successfully labelled COM crystals by fluorescein isothiocyanate (FITC) dye that are not similar too our previous fluorescent- labelled COM crystals (30). By using FITC dye instead of fluorescent- conjugated immunoglobulin (IgG) will reduce the price and problem of immunoglobulin (IgG) intervention experiment. Moreover, the morphology and properties of these fluorescence-labelled crystals were examined by fluorescence microscope and flow cytometric analysis (**Figure2, 3**). Thus, we successfully produced same quality and be able to applied fluorescence-labelled COM crystals for COM crystal internalization study as our previous work (**Figure4, 5**). Moreover, generation of fluorescence-labeled crystal also provides a promising approach to address other question regarding crystal-cell interaction especially the internalization for the critical ambition in protection of newly and/or repeated stone formation in kidney.

Additionally, combination of fluorescence-labelled COM crystals and image analysis software developed the different technique for quantify degraded COM crystals. The degradation products were collected and their images were capture by fluorescent microscope. We demonstrated the crystal degraded/dissolved (**figure6A**) and calculated crystal size (**figure6B**). This is the first endorsement of quantitative data of degraded/ dissolved internalized COM crystals. Moreover, quantitation of dissolution fluorescence-labelled COM crystals was then achieved to investigation intracellular dissolution and trafficking of their products. Previously, measurement of radioisotope (^{14}C)-oxalic acid in solution for (^{14}C)-oxalic acid labelled COM crystals dissolution and following secreted products were used for examination (31). Thus, we also developed novel assay for quantitative dissolution of fluorescence-labelled COM crystals (intracellular fraction) and secretion of dissolved products (extracellular fraction) by spectrofluorometer. Intracellular fluorescence intensity was decrease related to dissolution of

fluorescence-labelled COM crystals and secreted from MDCK cells to extracellular fraction by time dependent manner (**figure7**). So, the extracellular fluorescence intensity was sharply increased at 24h associated with particularly decreased of crystal size (**figure6**) at 24h as well. The slightly drop fluorescence intensity of 48h extracellular fraction might be fade of FITC dye at that time point. We demonstrated that COM crystals were rapidly degraded/ dissolved before 24h after internalization as abundant increased of extracellular fluorescent intensity (**Figure7**). Hence, early time point before 24h should be the appropriation period for investigating degradation mechanism of COM crystal by renal tubular epithelial cells.

Afterward, we performed of immunofluorescence examination of endosome marker (Rab5 and Rab7) at 16h after internalization to identified the exactly mechanism of intracellular digestion of COM crystals. Rab5 and Rab7 were represented co -localized with giant endosome containing COM crystal. The enlarge endosome might be performed both early and late endosome role similar to previous studies (34). Rab5 containing enlarge endosome have been demonstrated, be able to regulate and fuse with late endosome or lysosome (39) and also found COM crystals degraded /dissolved at this period. Rab5 is also known as key regulatory protein of "Kiss and run" process for digest particle (34). Rab5 Enzyme are transferred or exchanged during every short time interacting of early endosome with late endosome and/ or lysosome. Subsequent, enlarge endosome could contain lysosomal enzyme and reduced to lower pH condition for digestion mechanism (32, 33, 40). Therefore, large endosome containing Rab5 could degrade particle and be broken to small vesicles and sorting out of cytoplasm. Thus, Rab5 regulated Kiss and run process might be the main mechanism of intracellular digestion of COM crystals by renal tubular epithelial cells.

In conclusion, we successfully demonstrated Rab5 containing enlarge or giant endosome with Kiss and Run" process is the main mechanism of intracellular COM crystal degradation/dissolution and endosome lysosome biogenesis. However, further investigation of enlarge endosome molecular mechanism in COM crystals digestion and trafficking would lead to increase more understanding in pathogenesis of kidney stone disease and find novel effective protection methods for stone formation and recurrent. Additionally, we also developed innovative simply method for COM crystal degradation and dissolution analysis. This could be powerful tool for quantified degraded and dissolved COM crystal and also crystal-cell interaction.

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Figure legends

Figure1. The morphology of confluent monolayer of culture MDCK cells was visualized under Phase-contrast microscope. Original magnification = 400X

Figure2. The fluorescence images of typical monoclinic prismatic shapes of fluorescence-labelled COM crystals (FITC). The images in all panels were taken from Nikon ECLIPSE 80i (Nikon Corp.; Tokyo, Japan) with original magnification = 400X.

Figure3. Flow cytometric dot plots of fluorescence-labelled COM crystals and non-fluorescence plain COM crystals. Fluorescent intensities of COM crystals in "Upper right quadrant" demonstrated the percentages of fluorescent intensities above the threshold of fluorescence-labelled and and plain crystals with (98.27±1.24 vs. 1.22±0.36%, respectively; $p<0.0001$).

Figure4. The fluorescence image of intracellular fluorescence-labelled COM crystals. The images in all panels were taken from Nikon ECLIPSE 80i (Nikon Corp.; Tokyo, Japan) with original magnification = 400X.

Figure5. These represented dot plots of MDCK cells with internalized of plain crystals or fluorescence-labelled COM crystals. Fluorescent intensities of population MDCK cells in "G" gate in left panel dot plots were obtained and presented in right panel dot plots. Percentages of MDCK cells with fluorescent intensities above the threshold (upper right quadrant) were then compared (14.83±0.85 vs. 0.52±0.32% for cells with internalized fluorescent COM crystals vs.

cells with plain crystals, respectively; $p<0.0001$). Note that all the data in this figure were taken from a flow cytometer.

Figure6. The fluorescence images of degraded fluorescence-labelled COM crystals (Original magnification = 400X) (A) and the quantitatively analysis of degraded COM crystal size by ImageMaster 2D Platinum software (B) at 0, 24 and 48h after internalization.

Figure7. This represents the quantitatively analysis intracellular fraction (Left panel) and extracellular fraction (Right panel) of COM crystal dissolution by fluorescence intensity at 0, 24 and 48 h after internalization.

Figure8. The immunofluorescence staining of Non-treated and COM treated MDCK cells with early endosome marker (Rab5) and late endosome marker (Rab7) (arrow = intracellular COM crystals), Original magnification = 400X

Figure1.

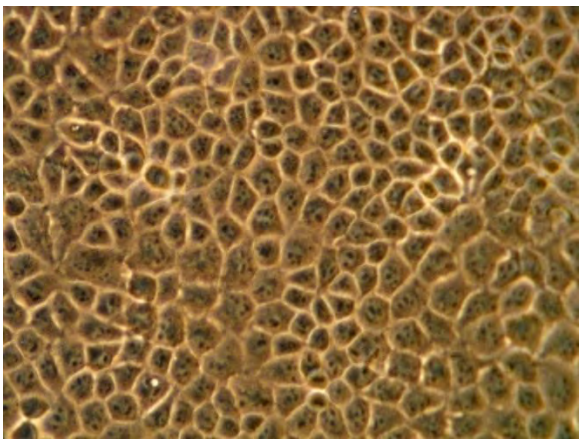


Figure2.

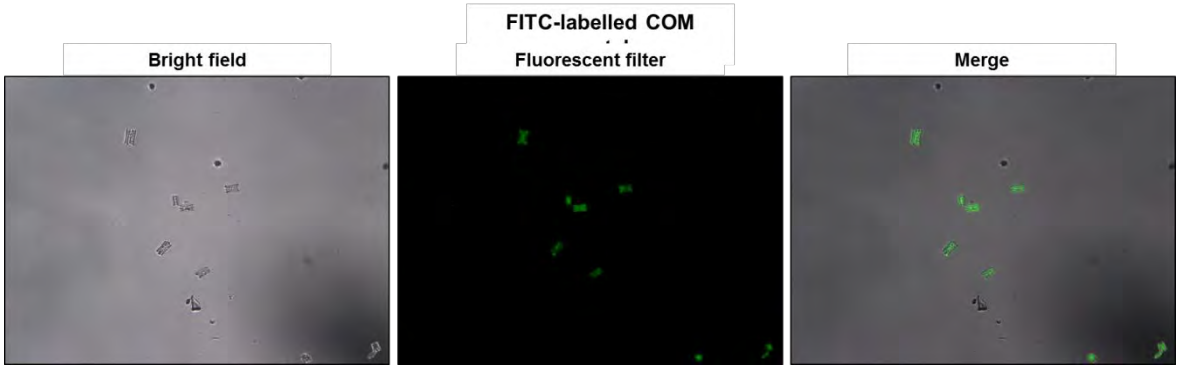


Figure3.

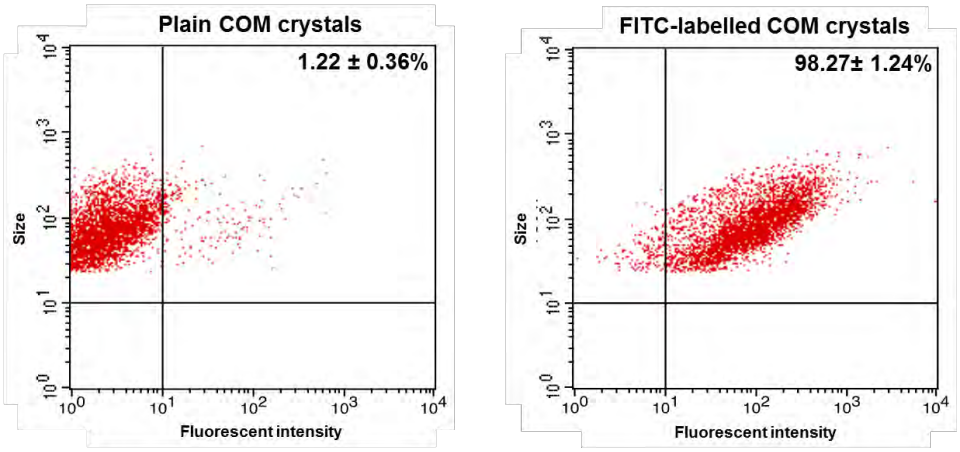

Plain COM crystals
1.22 ± 0.36%
Size
Fluorescent intensity
FITC-labelled COM crystals
98.27± 1.24%
Size
Fluorescent intensity

Figure4.

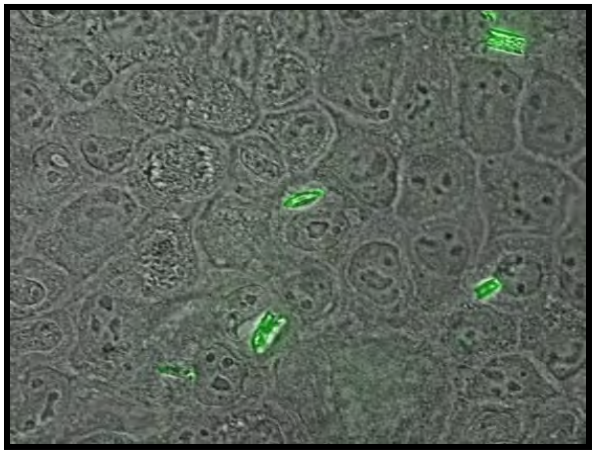


Figure5.

MDCK with plain COM crystals

MDCK with FITC-labelled COM crystals

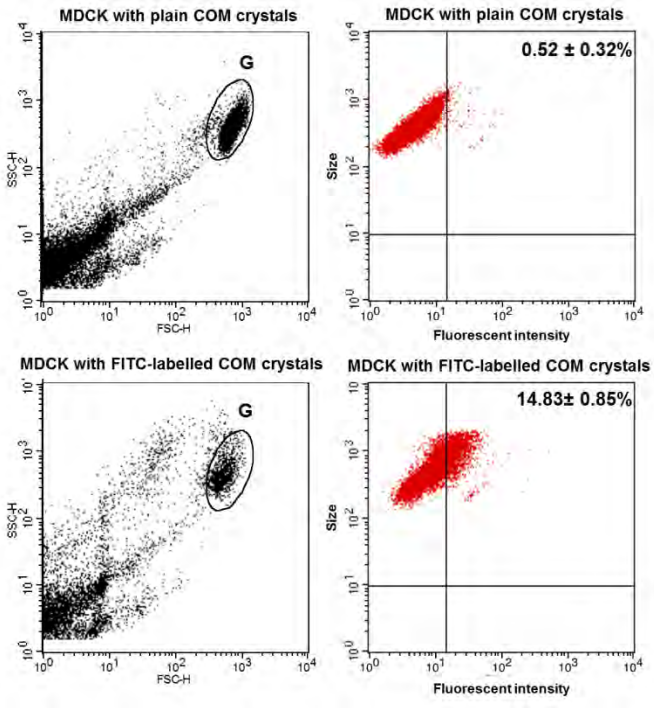
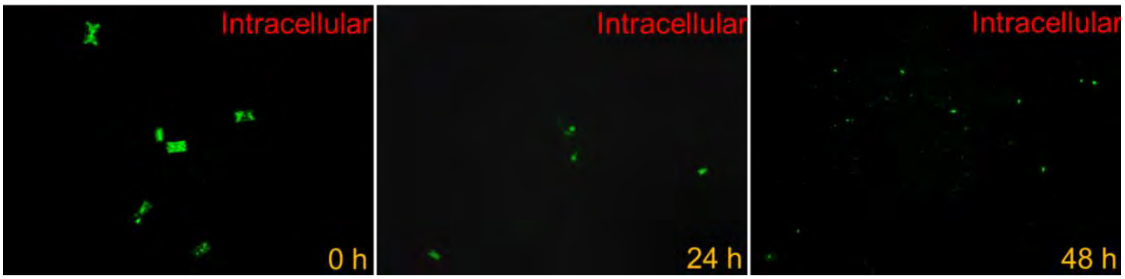


Figure6.

(A)


Intracellular
0 h
Intracellular
24 h
Intracellular
48 h

(B)

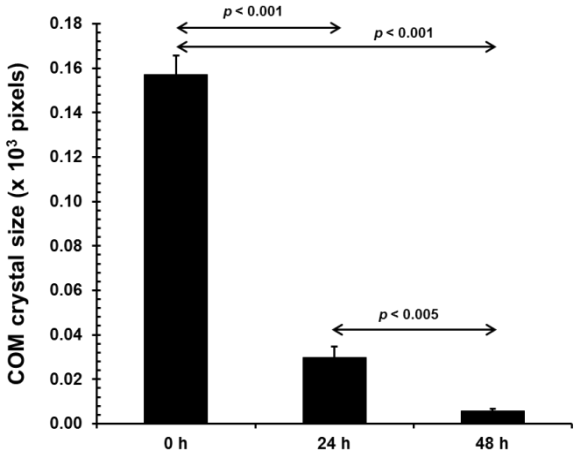

p < 0.001
p < 0.001
p < 0.005
COM crystal size (x 10³ pixels)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0 h
24 h
48 h

Figure7.

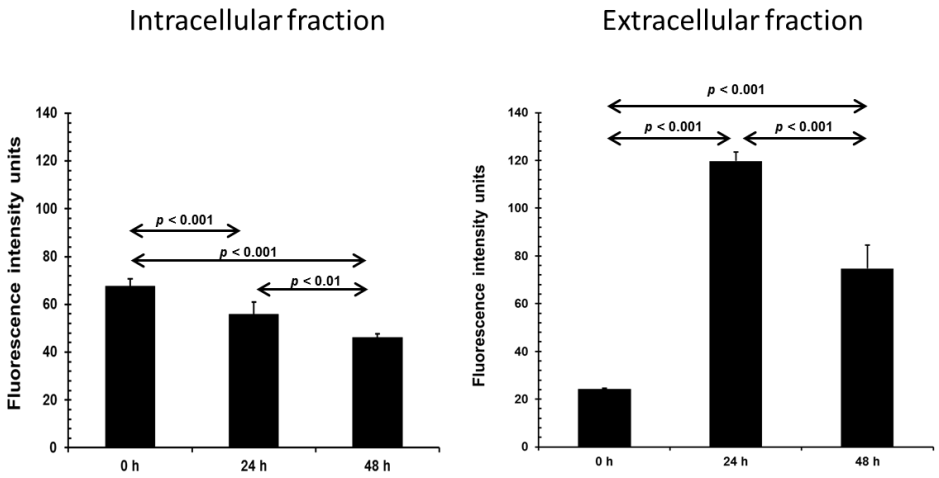
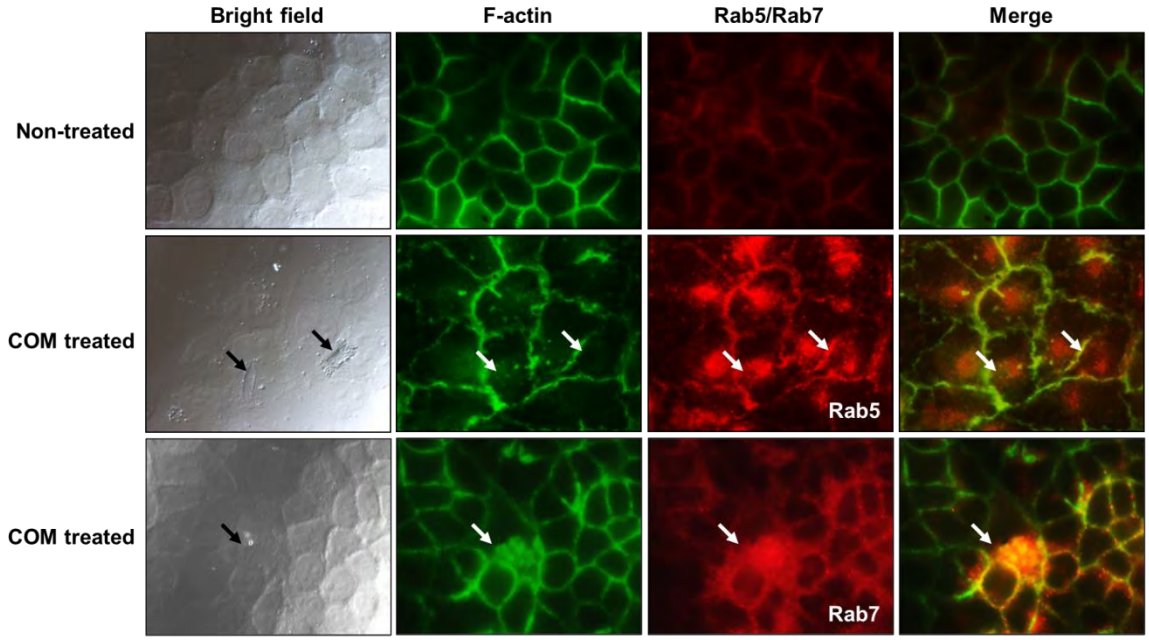

Intracellular fraction
Extracellular fraction
Fluorescence intensity units
140
120
100
80
60
40
20
0
p < 0.001
p < 0.001
p < 0.01
0 h
24 h
48 h
p < 0.001
p < 0.001
p < 0.001

Figure8.


Bright field
F-actin
Rab5/Rab7
Merge
Non-treated
COM treated
COM treated
Rab5
Rab7