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ORIGINAL PAPER

EGCG decreases binding of calcium oxalate monohydrate crystals onto renal tubular cells via decreased surface expression of alpha-enolase

Rattiyaporn Kanlaya[1,2] · **Nilubon Singhto**[1,2] · **Visith Thongboonkerd**[1,2]

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Abstract Crystal retention on tubular cell surface inside renal tubules is considered as the earliest and crucial step for kidney stone formation. Therapeutics targeting this step would cease the development of kidney stone. This study thus aimed to investigate the potential role of epigallocatechin-3-gallate (EGCG), a major antioxidant found in green tea leaves, in the reduction of calcium oxalate monohydrate (COM) crystal binding onto renal tubular cells. Pretreatment of the cells with EGCG for up to 6 h significantly diminished crystal-binding capability in a dose-dependent manner. Indirect immunofluorescence assay without and with cell permeabilization followed by laser-scanning confocal microscopy revealed that EGCG significantly reduced surface expression of alpha-enolase, whereas its intracellular level was increased. Western blot analysis confirmed such contradictory changes in membrane and cytosolic fractions of EGCG-treated cells, whereas the total level in whole cell lysate remained unchanged. Moreover, overexpression of surface alpha-enolase and enhancement of cell–crystal adhesion induced by 10 mM sodium oxalate were completely abolished by EGCG. Taken together, these data indicate that EGCG decreases binding of COM crystals onto renal tubular cells by decreasing the surface expression of alpha-enolase via re-localization or inhibition of alpha-enolase shuttling from the cytoplasm to the plasma membrane. These findings may also explain the effects of EGCG in reducing COM crystal deposition in previous animal models of kidney stone disease. Thus, EGCG may be useful for the prevention of new or recurrent stone formation.

Keywords Calcium oxalate · Crystal binding · EGCG · Enolase · Kidney stone · Renal tubular cells

Introduction

Calcium oxalate monohydrate (COM) is the most common type of kidney stones found in stone formers [1]. It is widely accepted that kidney stones initially occur as a result of multi-step processes, including crystallization, aggregation and retention in the kidney during crystalluria [2]. Several lines of evidence have shown that binding of COM crystals to renal tubular cells results in diverse cellular deteriorations, including oxidative stress, mitochondrial dysfunction, cytotoxicity and cell death [3, 4]. Therefore, investigations of the interaction between COM crystals and renal tubular cells, particularly in the initial step of crystal binding to the cell surfaces, would have high potential to provide therapeutic targets to prevent kidney stone formation.

Recently, epigallocatechin gallate (EGCG), the most potent antioxidant in green tea leaves (*Camellia sinensis*), has been evidenced as one of the promising natural compounds to inhibit many human diseases related to oxidative stress and cancers [5]. In addition, the antioxidant property of green tea (by its ability to reduce lipid peroxidation) could attenuate cell damage in rats treated with sodium oxalate [6]. Interestingly, in animal models of kidney stone disease, supplementation of green tea extract or EGCG in drinking water and diets could exert inhibitory effects on

✉ Visith Thongboonkerd
thongboonkerd@dr.com; vthongbo@yahoo.com

[1] Medical Proteomics Unit, Office for Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University, 6th Floor-SiMR Building, 2 Wanglang Road, Bangkoknoi, Bangkok 10700, Thailand

[2] Center for Research in Complex Systems Science (CRCSS), Mahidol University, Bangkok, Thailand

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urinary stone formation, as it could decrease urinary oxalate excretion and lower crystal deposition [7, 8]. Likewise, administration of green tea in glyoxylate-treated mice caused a significant decrease in crystal formation [3]. The molecular basis underlying the inhibitory effect of EGCG has been thought to be most likely due to its antioxidative property [3, 7, 8]. However, direct evidence showing the contribution of EGCG to reduce retention of COM crystals in tubular lumens is lacking. To provide additional insights into the potential role of EGCG in the reduction of COM crystal binding onto renal tubular cell surfaces, we performed COM crystal-binding assay and quantified the remaining crystals adhered on the cell monolayer after pretreatment with EGCG. Moreover, we examined the expression level of a known COM crystal-binding protein on the cell surface. Our findings have indicated that pretreatment of EGCG could significantly lower COM crystal binding onto renal tubular cell surface via suppressing surface expression of alpha-enolase.

Materials and methods

Cell cultivation

Madin–Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium (MEM) (GIBCO, Invitrogen Corporation; Grand Island, NY) supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine and 1.2 % penicillin/streptomycin. The cells were maintained at 37 °C with 5 % CO_2 in a humidified incubator. The cells were passaged by trypsinization when they reached 80–90 % confluence.

Crystal-binding assay

COM crystals were generated as previously described [9]. The cells were seeded at a density of 5×10^5 cells/well and grown in the complete medium (as aforementioned) for 24 h prior to the experiment. Thereafter, the cells were pretreated with 0 or 25 μM EGCG (Sigma-Aldrich; St. Louis, MO) for 1, 3 or 6 h (time-course study), and with 0, 6.25, 12.5, 25 or 50 μM EGCG for 3 h (dose-dependent study), followed by an incubation with COM crystals (100 μg/mL of culture medium) for 30 min. The concentrations of EGCG used in this study were based on the study by Jeong et al. [8]. After treatment, unbound crystals were removed and the cells were washed twice with plain medium (without FBS supplementation). The remaining crystals that adhered on the cell monolayer were then imaged for at least 20 high-power fields (HPF) in each well using a phase-contrast microscope (Olympus CKX41, Olympus Co. Ltd.; Tokyo, Japan) and the crystal number was then counted using NIS-Elements D V.4.11 (Nikon; Tokyo, Japan).

Examination of surface and intracellular alpha-enolase expression by indirect immunofluorescence assay followed by laser-scanning confocal microscopy

Subcellular localization of alpha-enolase was determined by indirect immunofluorescence assay, followed by laser-scanning confocal microscopy. To differentiate between surface and intracellular expression of alpha-enolase, the cells were processed without and with cell permeabilization, respectively. MDCK cells were grown on a coverslip at a density of 1×10^6 cells for 24 h prior to the experiment. The cells were incubated with 25 μM EGCG for 3 h. After washing with PBS, the cells were fixed with 3.7 % formaldehyde/PBS for 10 min without permeabilization or with permeabilization by 0.1 % triton X-100/PBS for 10 min. The cells were then probed with rabbit polyclonal anti-alpha-enolase antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA) at a dilution of 1:50 in 1 % BSA/PBS at 37 °C for 1 h. Thereafter, the cells were incubated with the corresponding secondary antibody conjugated with Alexa 488 (Invitrogen-Molecular Probes; Burlington, ON, Canada) at a dilution of 1:2000 in 1 % BSA/PBS for 1 h. The nuclei were counterstained with 0.1 μg/ml Hoechst dye (Invitrogen; Paisley, UK). Finally, the coverslip was mounted onto a slide using 50 % glycerol/PBS. 3-D planes of *X–Y*, *X–Z* and *Y–Z* scanning were captured under ECLIPSE Ti-Clsi4 Laser Unit (Nikon) equipped with NIS-Elements D V.4.11 (Nikon). Fluorescence intensity representing protein level was measured and analyzed from ten random high-power fields (HPF) and at least ten cells/HPF of each sample using NIS-Elements D V.4.11 (Nikon).

Subcellular localization of alpha-enolase by Western blot analysis

Membrane, cytosolic, and total alpha-enolase levels were evaluated by subcellular fractionation of cells followed by Western blot analysis. After treatment with 25 μM EGCG for 3 h, the cells were washed with ice-cold PBS and incubated with cytosolic extraction buffer (10 mM PIPES pH 6.8, 0.02 % digitonin, 0.3 mM sucrose, 15 mM NaCl, and 0.5 mM EDTA) at 4 °C for 10 min with gentle agitation. The supernatant containing cytosolic proteins was then collected and named as the "cytosolic fraction". The remaining parts of the cells were washed three times with ice-cold PBS, extracted by Laemmli's buffer, and named as "membrane fraction". In parallel, whole cell lysate was extracted from the intact cells using Laemmli's buffer. Protein concentration in each fraction was determined by Bradford's method using the Bio-Rad Protein Assay (Bio-Rad Laboratories;

Hercules, CA). Equal amount of total protein (30 μg/lane) from each fraction was separated by 12 % SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking non-specific bindings with 5 % skim milk/PBS for 1 h, the membrane was incubated with rabbit polyclonal anti-alpha-enolase (1:1000), mouse monoclonal anti-GAPDH (1:2000), or rat polyclonal anti-E-cadherin (1:500) (all were purchased from Santa Cruz Biotechnology and diluted in 1 % skim milk/PBS) at 4 °C overnight. After washing with PBS three times, the membrane was incubated with the corresponding secondary antibody conjugated with horseradish peroxidase (1:2000 in 1 % skim milk/PBS; DAKO Glostrup, Denmark) at room temperature (25 °C) for 1 h. Immunoreactive bands were developed by SuperSignal West Pico chemiluminescence substrate (Pierce Biotechnology; Rockford, IL, USA) and then visualized by autoradiogram.

Overexpression of surface alpha-enolase by high oxalate treatment

Overexpression of surface alpha-enolase was induced by high oxalate treatment as previously described [10]. The cell monolayers were divided into four groups: (1) without any treatment; (2) treated with 25 μM EGCG for 3 h; (3) treated with 10 mM sodium oxalate (Sigma-Aldrich) for 3 h; and (4) treated with 10 mM sodium oxalate for 3 h followed by medium refreshment and incubation with 25 μM EGCG for further 3 h. Surface expression of alpha-enolase was evaluated by indirect immunofluorescence assay (without cell permeabilization) followed by laser-scanning confocal microscopy as mentioned previously. Also, crystal-binding capacity of the cells was evaluated by a COM crystal-binding assay as described above.

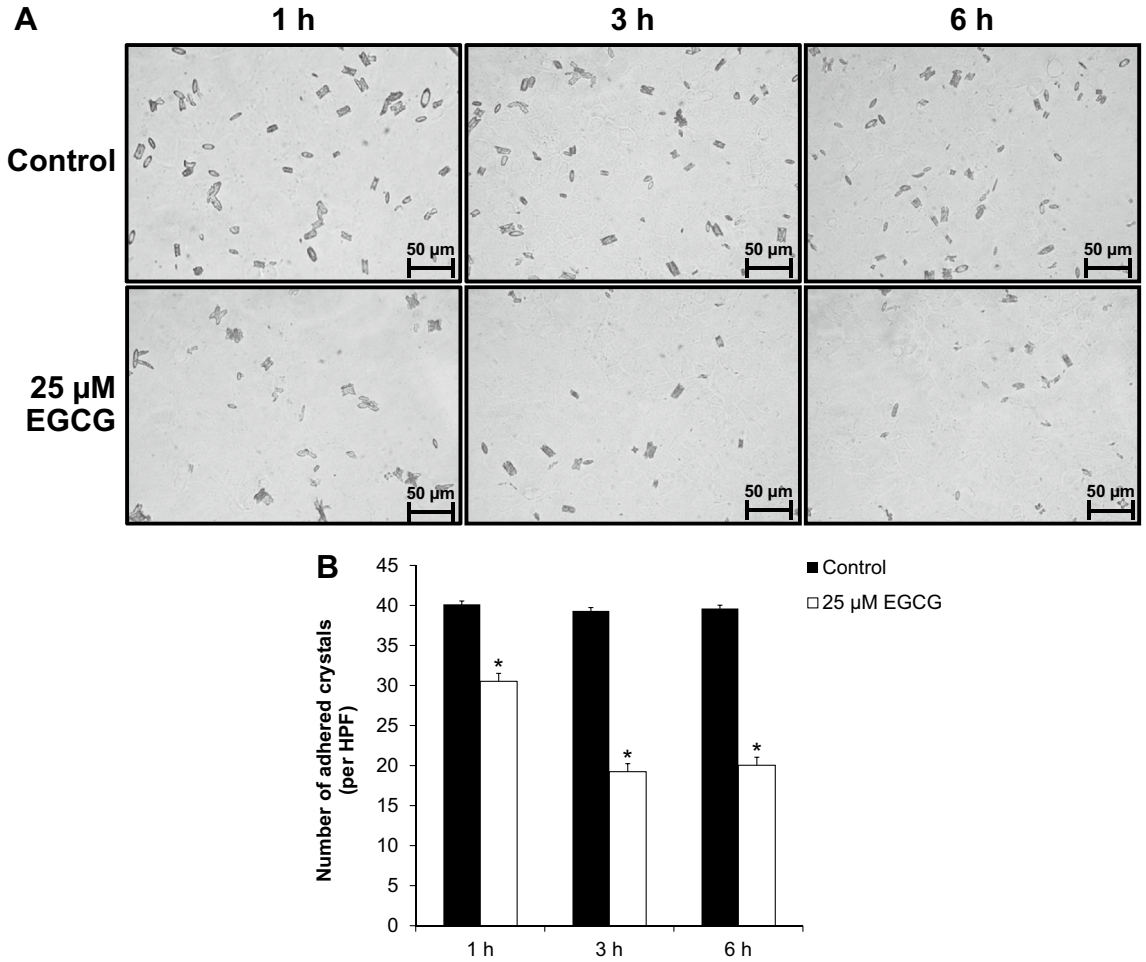

Fig. 1 Pretreatment of EGCG-reduced COM crystal binding onto the cell surface. MDCK cells were treated with 0 or 25 μM EGCG for 1, 3 or 6 h prior to incubation with COM crystals (100 μg/mL of culture medium) for 30 min. **a** After washing with PBS, images of the remaining crystals were captured under a phase-contrast microscope for at least 20 high-power fields (HPF) in each well. The original magnification power was ×400. **b** The remaining crystals that adhered on the cell surface were then counted. $N = 3$ independent experiments. *$p < 0.05$ vs. control

Statistical analysis

All quantitative data are reported as mean ± SEM of three independent experiments. The mean difference between the two groups was analyzed by Student's *t* test, whereas multiple comparisons among several groups were performed by one-way ANOVA with Tukey's post hoc test. *P* values less than 0.05 were considered to be statistically significant.

Results and discussion

MDCK cells were pretreated with 25 μM EGCG for up to 6 h prior to incubation with COM crystals (100 μg/mL of culture medium). The concentrations of EGCG and COM crystals used in this study were based on previous studies by Jeong et al. [8] and by our group [10], respectively. The results from crystal-binding assay showed that pretreatment with 25 μM EGCG significantly decreased the number of the remaining COM crystals that adhered onto the cell surface as compared to the control at all time points (Fig. 1a, b). Such reduction of crystal binding was approximately a half of the control at 3- and 6-h pretreatment periods. To emphasize mainly on the crystal-binding step and to minimize the effect of crystal endocytosis, we performed our experiment at early time points (not exceeding 6 h). Since reduction in binding capacity was noticeably found at the 3-h pretreatment period and prolonged incubation to 6 h did not increase the degree of such change, we thus selected the 3-h pretreatment period for the subsequent experiment. In addition, we examined the effect of various doses (0–50 μM) of EGCG on the crystal-binding capacity of the cells at the 3-h time-point. The results showed that EGCG decreased the crystal-binding capability of the cells in a dose-dependent manner (Fig. 2a, b). However, this inhibitory effect reached a plateau at 25 μM, because increasing the dosage to 50 μM could not further lower the number of adhered crystals. From these findings, an optimal condition for EGCG treatment selected for all subsequent experiments in our present study was a dosage of 25 μM EGCG for 3 h.

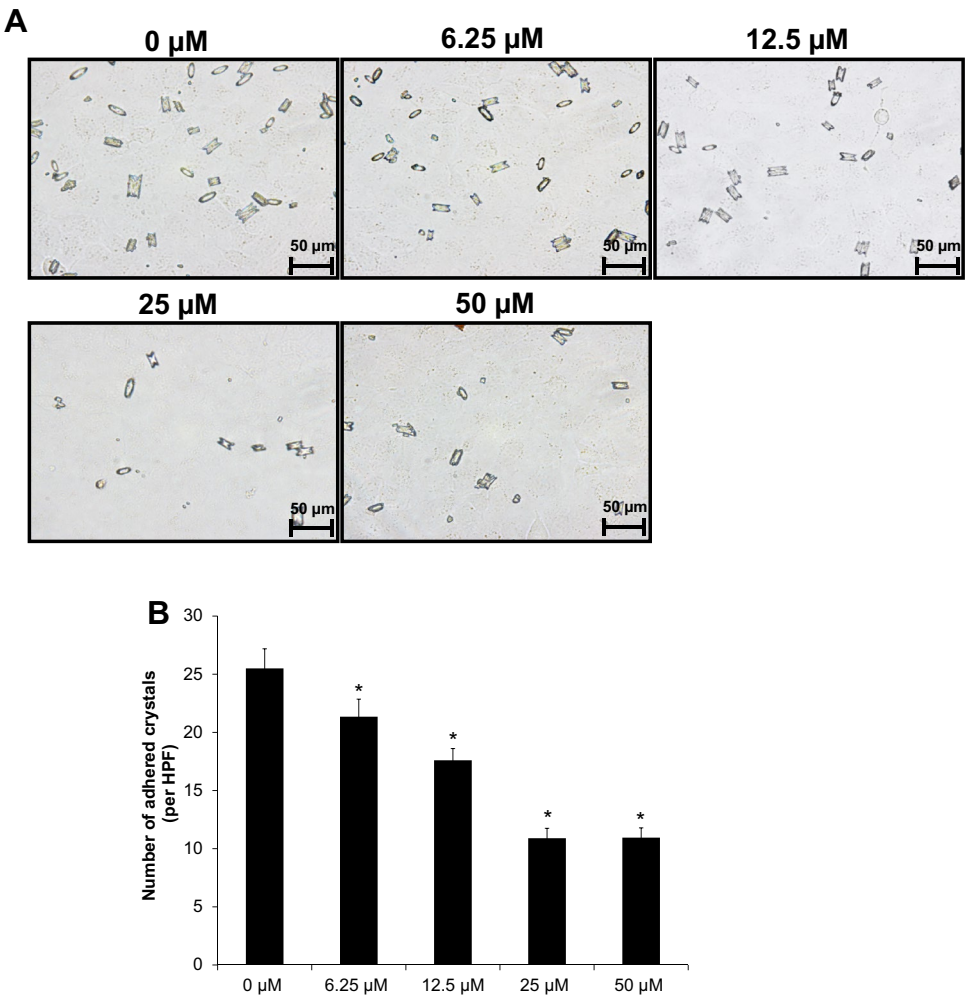

Fig. 2 Effect of EGCG on COM crystal-binding capability of the cells was dose dependent. MDCK cells were pretreated with various doses of EGCG (0, 6.25, 12.5, 25, and 50 μM) for 3 h followed by incubation with COM crystals (100 μg/mL of culture medium) for 30 min. **a** After washing with PBS, images of the remaining crystals were captured under a phase-contrast microscope for at least 20 high-power fields (HPF) in each well. The original magnification power was ×400. **b** The remaining crystals that adhered on the cell surface were then counted. $N = 3$ independent experiments. $^{*}p < 0.05$ vs. control

Recently, Lee et al. [11] showed that pre-coating COM crystals with gallotannin, a tannin found in green tea, could suppress COM binding onto human renal tubular cells. Similarly, pre-coating COM crystals with aluminum citrate could inhibit COM crystal binding onto renal tubular cells [12], supporting its role in the prevention of renal injury in rats caused by crystal deposition [13]. The most recent study showed that an aqueous extract of *Costus arabicus* inhibited COM crystal growth and adhesion to renal epithelial cells [14]. The inhibitory effect on crystal–cell adhesion was found only when using pre-coated COM crystals; however, pretreatment of the extract to the cells had no effect. Inhibition of COM crystal–cell interaction observed in these three studies might be due to the chemical modification on COM crystal surfaces, which subsequently interfered with the crystal-binding capability. Unlike those previous studies, we challenged EGCG directly to the cells followed by treatment with COM crystals. We thus presumed that our findings could be explained by the effects of EGCG on renal tubular cell membrane and/or a cascade of molecular events induced by EGCG.

We speculated that the possible mechanism underlying this phenomenon might be related to an ability of EGCG to reduce some particular COM crystal-binding proteins localized on the cell surfaces. Alpha-enolase is one of these potential COM crystal-binding proteins that has been well documented in our previous expression and functional studies [10, 15]. The study on molecular and physicochemical interactions between green tea catechins and cell membranes revealed that EGCG showed the strongest interaction with lipid bilayers due to a large number of hydrogen bonds formed [16]. The investigation of EGCG treatment in colon cancer cells revealed that EGCG could alter lipid organization of plasma membrane, causing internalization of epidermal growth factor receptor (EGFR) into endosomes and thus sequestering the activation of epidermal growth factor (EGF) [17]. In addition, EGCG could specifically interact with membrane lipid raft, a signal-processing platform, resulting in alterations in several signaling pathways and modulation of gene expression [18]. Interestingly, alpha-enolase has been deposited into a lipid raft proteome database (http://lipid-raft-database.di.uq.edu.au/). Most recently, it has been demonstrated that alpha-enolase was found in a specialized lipid raft caveolae, which was necessary for its trafficking to the cell surface [19]. Accordingly, by modulating the plasma membrane,

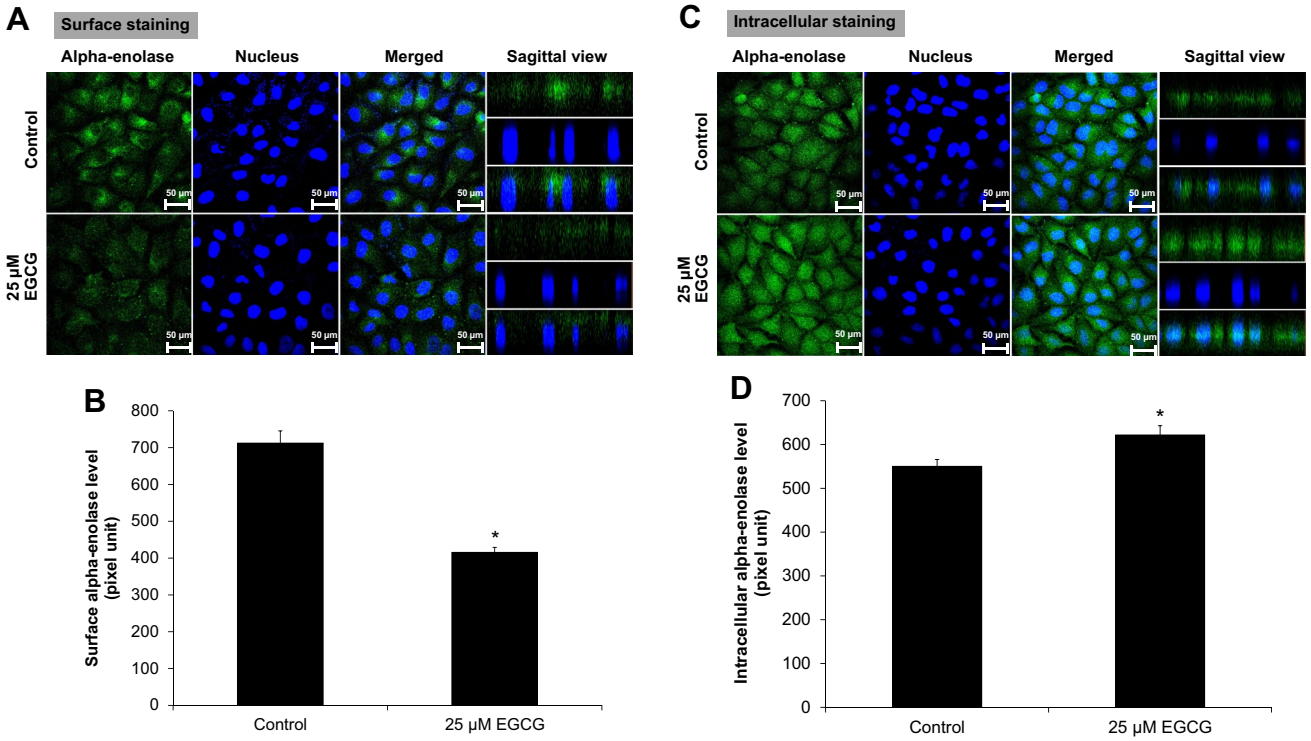

Fig. 3 EGCG reduced the surface expression of alpha-enolase, but increased its intracellular level. **a**, **b** Surface expression of alpha-enolase (without cell permeabilization). **c**, **d** Intracellular expression of alpha-enolase (with cell permeabilized). MDCK cells were pretreated with 25 μM EGCG for 3 h and processed for indirect immunofluorescence study using anti-alpha-enolase as a primary antibody and secondary antibody conjugated with Alexa 488 (shown in *green*), whereas nuclei were counterstained with Hoechst dye (shown in *blue*). Original magnification power was ×400. Fluorescence intensity representing protein level was measured and analyzed from ten random high-power fields (HPF) and at least ten cells/HPF of each sample. $N = 3$ independent experiments. $^*p < 0.05$ vs. control

EGCG might decrease the expression of surface alpha-enolase through transcriptional suppression or alter molecular trafficking or shuttling of the protein from cytoplasm to the cell surface.

We therefore addressed whether alpha-enolase was decreased in its surface expression on MDCK cells pretreated with EGCG. As expected, the result from indirect immunofluorescence assay and laser-scanning confocal microscopy showed that surface expression of alpha-enolase was markedly decreased by pretreatment with 25 μM EGCG by almost half of the control (Fig. 3a, b). In contrast, intracellular expression of alpha-enolase was increased in EGCG-treated cells (Fig. 3c, d). We further confirmed these findings by Western blot analysis of alpha-enolase in subcellular fractions and whole cell lysate. In concordance with the indirect immunofluorescence data, Western blot analysis revealed that EGCG could dramatically reduce alpha-enolase level in the membrane fraction, whereas its level in the cytosolic fraction was increased (Fig. 4a, b). However, the total level of alpha-enolase in whole cell lysate remained unchanged (Fig. 4a, b). These findings indicated that EGCG reduced the surface expression of alpha-enolase by re-localization or inhibition of its shuttling from cytoplasm to plasma membrane.

Glycolytic enzyme enolase is a multifunctional protein expressed in three different isoforms (alpha, beta and gamma). Apart from its role in glycolysis, alpha-enolase (ENO1) expressed on cellular surface acts as a plasminogen receptor enabling proteolytic cleavage of plasmin, resulting in the promotion of tumor cell invasion and inflammatory response [20]. We have previously identified alpha-enolase as one of the potential COM crystal-binding proteins on MDCK cell surface and reported that high oxalate caused an increased level of alpha-enolase on MDCK cell surface [10]. Using a specific antibody to block alpha-enolase on the cell surfaces, we have confirmed a novel role of this protein in facilitating COM crystal binding [10]. To strengthen our hypothesis that the inhibitory effect of EGCG on COM crystal-binding was mediated by surface expression of alpha-enolase, we thus induced overexpression of surface alpha-enolase by high oxalate condition. The expression and functional data revealed that overexpression of surface alpha-enolase and enhancement of

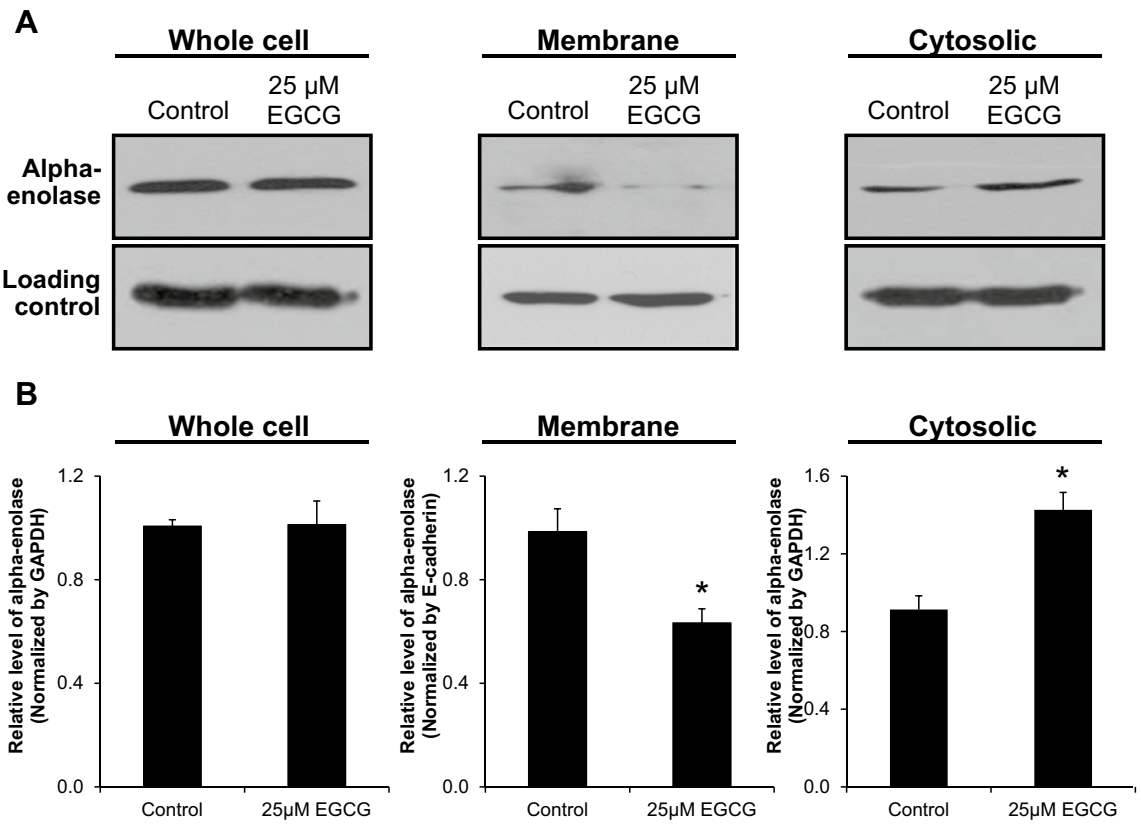

Fig. 4 EGCG caused re-localization or inhibition of shuttling of alpha-enolase from cytoplasm to plasma membrane. MDCK cells were incubated with 25 μM EGCG for 3 h and subjected to subcellular fractionation. **a** Western blot analysis of alpha-enolase in whole cell lysate, membrane and cytosolic fractions. **b** Relative band intensity of alpha-enolase normalized with loading control (GAPDH for whole cell lysate/cytosolic fraction and E-cadherin for membrane fraction). $N = 3$ independent experiments. *$p < 0.05$ vs. control

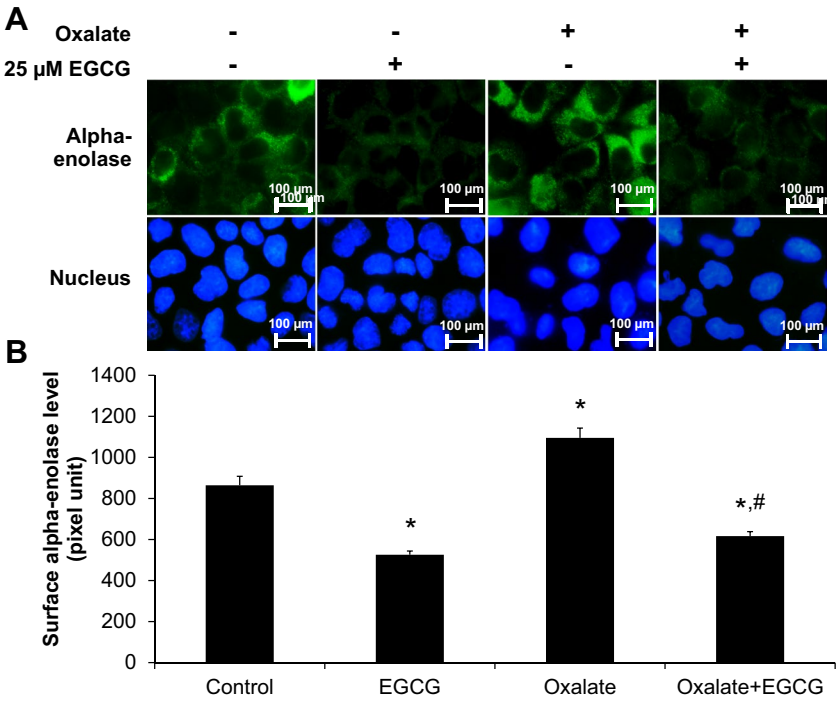

Fig. 5 EGCG abolished the effect of high oxalate on increased surface expression of alpha-enolase. MDCK cells were treated with 25 μM EGCG, 10 mM sodium oxalate or 10 mM sodium oxalate + 25 μM EGCG for 3 h. **a** The cells were then subjected to indirect immunofluorescence assay (without cell permeabilization) and laser-scanning confocal microscopy using anti-alpha-enolase as a primary antibody and secondary antibody conjugated with Alexa 488 (shown in *green*), whereas nuclei were counterstained with Hoechst dye (shown in *blue*). The original magnification power was ×1000. **b** Fluorescence intensity representing the protein level was measured and analyzed from ten random high-power fields (HPF) and at least ten cells/HPF of each sample. $N = 3$ independent experiments. $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. oxalate

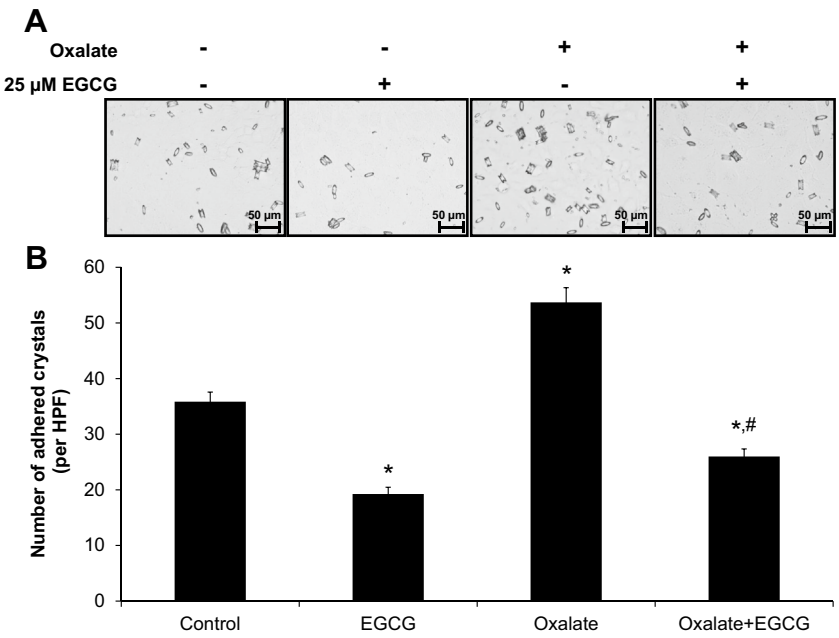

Fig. 6 EGCG abolished the effect of high oxalate on enhanced COM crystal-binding capability of the cells. MDCK cells were treated with 25 μM EGCG, 10 mM sodium oxalate or 10 mM sodium oxalate +25 μM EGCG for 3 h prior to incubation with COM crystals (100 μg/mL of culture medium) for 30 min. **a** After washing with PBS, images of the remaining crystals were captured under a phase-contrast microscope for at least 20 high-power fields (HPF) in each well. The original magnification power was ×400. **b** The remaining crystals that adhered on the cell surface were then counted. $N = 3$ independent experiments. $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. oxalate

crystal-binding capacity induced by 10 mM sodium oxalate were completely abolished by EGCG (Figs. 5, 6).

The present study supports the concept proposed by Verkoelen and Verhulst [21] that crystal binding may require specific changes in cell surface properties or components under defined conditions. Nonetheless, EGCG could not completely inhibit the COM crystal–cell interaction, suggesting that such interaction involves diverse mechanisms and other binding molecules, e.g., annexin II, osteopontin and hyaluronan [22–24]. Moreover, we have previously reported a set of COM crystal-binding proteins on the apical membrane of MDCK cells [15]. Therefore, it is plausible that other, yet unexplored key molecules might be also responsible, at least in part, for the inhibitory effects of EGCG on COM crystal-binding capacity of renal tubular cells. A large-scale, high-throughput study (i.e., proteomics) is required for further elucidations of those non-alpha-enolase-dependent mechanisms.

Herein we have reported, for the first time, the inhibitory effect of EGCG on COM crystal-binding capacity of renal tubular cells that was mediated, at least in part, via decreased expression of alpha-enolase on renal tubular cell surface. It is worth noting that alpha-enolase might be one of the key players in pathogenic mechanisms of kidney stone formation. Our findings may also explain the lower crystal deposition found in animal models of kidney stone disease treated with EGCG or green tea extract. Finally, our data support a potential role of using EGCG in the prevention of new or recurrent stone formation.

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