Proteomic Analysis of Calcium Oxalate Monohydrate Crystal-Induced Cytotoxicity in Distal Renal Tubular Cells

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Calcium oxalate monohydrate (COM) is the major crystalline component found in kidney stones and its adhesion to renal tubular cells provokes tubular injury, which in turn enhances COM crystal adhesion. However, COM-induced toxic effects in these tubular cells remain largely unknown. We performed a proteomics study to characterize changes in the cellular proteome in MDCK distal renal tubular cells after an exposure to high-dose (1000 $\mu\text{g/mL}$) COM crystals for 48 h, at which percentage of cell death was significantly increased. Proteins were extracted from MDCK cells cultured with COM-containing or COM-free medium ($n = 5$ individual flasks per group), resolved in individual 2-D gels, and stained with SYPRO Ruby fluorescence dye. Quantitative and statistical analyses revealed 53 proteins whose abundance levels were altered (25 were increased, whereas other 28 were decreased) by COM-induced toxicity. Among these, 50 were successfully identified by quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) and/or tandem MS (MS/MS) analyses. The proteomic data were clearly confirmed by 2-D Western blot analysis. While three chaperones (GRP78, Orp150 and Hsp60) were increased, other proteins involved in protein biosynthesis, ATP synthesis, cell cycle regulator, cellular structure, and signal transduction were decreased. These data provide some novel mechanistic insights into the molecular mechanisms of COM crystal-induced tubular toxicity.

Keywords: Calcium oxalate • Cellular responses • Crystal adhesion • Kidney • Proteome • Proteomics • Stone • Tubular toxicity

Introduction

Kidney stone disease (nephrolithiasis) remains a common health problem worldwide.^{1,2} Among all types of kidney stones, calcium oxalate monohydrate (COM) is the major crystalline compound found in the stone mass (with a frequency of up to 77.5%).³ In addition to crystallization, crystal growth and crystal aggregation, the other crucial mechanism for COM kidney stone formation is adhesion of COM crystals to renal tubular epithelial cells.^{4,5} Adhesion of COM crystals can induce injury and apoptosis of renal tubular epithelial cells, and vice versa, COM-induced cellular injury can facilitate COM crystal adhesion.^{6–9} This vicious cycle therefore accelerates kidney stone formation. Understanding alterations in renal tubular

cells induced by COM crystals may lead to an identification of molecular targets for the prevention of kidney stone formation. However, changes in renal tubular epithelial cells during COM crystal-induced toxicity remain largely unknown. Several previous studies, which had dissected this vicious cycle, unfortunately focused mainly on redox or oxidative stress pathways,^{9–11} particularly in mitochondria.^{12,13} Clearer image of global changes in COM crystal-induced renal tubular toxicity is thus required.

During the postgenomic era, proteomics has become one of the most promising tools in nephrology and urology fields to unravel pathogenic mechanisms underlying renal and urological diseases.^{14–16} The advantage of the proteomics approach is that both the previously determined and undetermined proteins that are involved in the disease mechanisms can be simultaneously examined. In the present study, we employed a classical proteomics approach to identify changes in the cellular proteome as a response to COM crystal-induced toxicity in MDCK distal renal tubular cells. The rationale of using MDCK cell line was that it exhibits several features of tubular cells in the distal nephron,¹⁷ which has been thought to be the primary site of kidney stone formation. Moreover, MDCK cell line has been frequently used in several of previous

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Figure 1. COM crystal adhesion and cell-crystal interactions. Images were taken using phase-contrast microscopy (A) and scanning electron microscopy (SEM) (B) after an incubation of COM crystals (1000 $\mu\text{g/mL}$) with MDCK cells for 48 h. An inset in (A) is a zoom-in phase-contrast microscopic image demonstrating the COM crystals adhered on the MDCK cell monolayer. SEM image in (B) shows blurred or interrupted borders (illustrated with arrows) of COM crystals (which indicate cell-crystal interactions) with the background of the monolayer of MDCK cells. Original magnification power was 100 $\times$ in (A) and 2000 $\times$ in (B).

studies on COM crystal-induced renal tubular cell injury.¹⁸ A high-dose (1000 $\mu\text{g/mL}$) of COM crystals used in the present study caused significantly increased cell death at 48-h after the exposure to the crystals. Two-dimensional electrophoresis (2-DE) followed by quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) and/or tandem MS (MS/MS) analyses revealed significant changes in abundance levels of several proteins in these COM-induced toxic MDCK cells compared to controls. The functional roles and subcellular localizations of these altered proteins indicated that these changes were related, at least in part, to COM crystal-induced tubular injury and ultimately to kidney stone formation.

Materials and Methods

Preparation of COM Crystals. COM crystals were prepared as previously described.¹⁹ Briefly, 10 mM calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) was mixed with 10 mM sodium oxalate ($\text{Na}_2\text{C}_2\text{O}_4$) to make final concentrations of 5 mM and 0.5 mM, respectively, in Tris buffer containing 90 mM NaCl (pH 7.4). The mixture was incubated at 25 $^\circ\text{C}$ overnight and COM crystals were harvested by centrifugation at 3000 rpm for 5 min. Supernatant was discarded and the crystals were resuspended in methanol. After another centrifugation at 3000 rpm for 5 min, methanol was discarded and the crystals were dried at 37 $^\circ\text{C}$ overnight. COM crystals were then decontaminated by UV light radiation for 30 min. They were then added to a complete Eagle's minimum essential medium (MEM) (GIBCO, Invitrogen Corporation; Grand Island, NY) to achieve the final concentration of 1000 $\mu\text{g/mL}$. These *in vitro* COM crystals had similar size and shape as those of *in vivo* samples found in the urine of kidney stone patients.

Cell Culture and COM Crystal Adhesion. Approximately 3×10^6 MDCK cells were inoculated in each 75 cm^2 tissue culture flask containing MEM supplemented with 10% fetal bovine serum (FBS), 1.2% penicillinG/streptomycin and 2 mM glutamine. The cultured cells were maintained in a humidified incubator at 37 $^\circ\text{C}$ with 5% CO_2 for 24 h. A total of 10 semiconfluent flasks were then divided into two groups ($n = 5$ per group) and the culture medium was replaced by either COM-containing (with 1000 $\mu\text{g/mL}$ COM crystals) or COM-free medium. For the COM-free medium, COM crystals with an equal amount of 1000 $\mu\text{g/}$

mL were added into the medium for 30 min, but were finally removed from the medium by centrifugation at 3000 rpm for 5 min.

Imaging by Phase-Contrast Microscopy and Scanning Electron Microscopy (SEM). Phase-contrast microscopy was simply performed on an inverted light microscope (Olympus CKX41, Olympus Co. Ltd.; Tokyo, Japan) after three washes (with PBS) of the MDCK cells incubated with COM crystals (1000 $\mu\text{g/mL}$) in 75 cm^2 tissue culture flasks, as mentioned above. For the evaluation by SEM, the cell culture and COM crystal adhesion were performed exactly the same as aforementioned, but on coverslips, instead of culture flasks (with approximately 1×10^6 cells per slide at an initial inoculation). After incubation with COM crystals (1000 $\mu\text{g/mL}$), the coverslips were rinsed with normal saline solution (NSS) three times and fixed with 2% glutaraldehyde in NSS at room temperature for 2 h. The coverslips were then rinsed again with NSS three times and dehydrated by a graded ethanol series of 50%, 70%, 95% and 100% before being air-dried overnight. The coverslips were finally mounted on aluminum stubs and coated with gold particles. The crystal morphology and adhesion were then examined under a scanning electron microscope (JSM-25S, Jeol; Kyoto, Japan).

Cell Death Assay. Apoptosis was detected by the determination of surface phosphatidylserine, which was translocated from the inner side of plasma membranes to outer layer of the cell during apoptotic cell death. After trypsinization, FITC-labeled annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine, was added to discriminate healthy from apoptotic cells. Additionally, propidium iodide, a DNA stain, was used simultaneously to detect necrotic cells. MDCK cells from the monolayer were detached with 0.1% trypsin in 2.5 mM EDTA and resuspended in 10 mL of MEM. The harvested cells were centrifuged at 1500 rpm, 4 $^\circ\text{C}$ for 5 min, and washed with PBS. Cell pellets were resuspended with annexin V buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; pH 7.4) at a final concentration of 5×10^5 cells/mL and then incubated with FITC-labeled annexin V (BD Biosciences; San Jose, CA) on ice for 15 min in the dark. Propidium iodide (BD Biosciences) was added into the samples at a final concentration of 10 $\mu\text{L/mL}$ prior to analysis. The cells were then analyzed by flow cytometry (FACScan, Becton Dickinson Immunocytometry System; San

Figure 2. Flow cytometric data of cell death during exposure to COM crystals. MDCK cells were cultured without (control) or with COM crystals (1000 $\mu\text{g}/\text{mL}$). Apoptosis and necrosis of these cells were evaluated using annexin V (FL1-H)/propidium iodide (FL2-H) double staining. The percentage of cell death was determined by a summation of the number of cells in the right upper quadrant (late apoptotic cells and/or necrotic cells), left upper quadrant (necrotic cells), and right lower quadrant (early apoptotic cells) compared to total cell count in all four quadrants. Lower panel shows Mean ($\pm$ SD) of cell death data obtained from 3 independent experiments.

Jose, CA) and a monolayer of MDCK cells treated with 2 $\mu\text{g}/\text{mL}$ camptothecin was used as a positive control. This experiment was performed in triplicates. Percentage of cell death (% cell death) = [(number of both apoptotic and necrotic cells/number of all cells) $\times$ 100%].

Protein Extraction. After incubation with or without COM crystals, the monolayer of MDCK cells was harvested by directly scraping into the tube containing 0.5 M EDTA in PBS to dissolve the adherent CaOx crystals followed by an incubation at 4 $^{\circ}\text{C}$ for 30 min. EDTA was then removed by washing with PBS three times. After a centrifugation at 10 000 rpm for 2 min, the cell pellet was washed with PBS three times and was then resuspended in a buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS), 120 mM dithiothreitol (DTT), 2% ampholytes (pH 3–10) and 40 mM Tris-HCl, and further incubated at 4 $^{\circ}\text{C}$ for 30 min. Unsolubilized debris and particulate matters were

removed by centrifugation at 10 000 rpm for 2 min. Protein concentrations were determined using the Bradford method.

2-DE and Staining. Protein solutions (each of 200 μg total protein) derived from individual culture flasks ($n = 5$ for each group) were premixed with a rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 120 mM DTT, 40 mM Tris-base, 2% ampholytes (pH 3–10) and a trace of bromophenol blue to make the final volume of 150 μL per sample. The mixtures were rehydrated onto Immobiline DryStrip (nonlinear pH gradient of 3–10, 7 cm long; GE Healthcare, Uppsala, Sweden) at room temperature for 10–15 h. The first-dimensional separation or isoelectric focusing (IEF) was performed in Ettan IPGphor II IEF System (GE Healthcare) at 20 $^{\circ}\text{C}$, using a stepwise mode to reach 9000 Vh. After completion of the IEF, the strips were first equilibrated for 15 min in an equilibration buffer containing 6 M urea, 130 mM DTT, 112 mM Tris-base, 4% SDS, 30% glycerol and 0.002% bromophenol blue, and then

Figure 3. Representative 2-D gel images of proteins derived from the control and COM-exposed MDCK cells. Proteins were resolved with 2-DE using a pH gradient of 3–10 (nonlinear) for the IEF and 12% polyacrylamide SDS-PAGE for the second-dimensional separation. The resolved protein spots were then visualized with SYPRO Ruby stain. Each gel contained 200 μ g of total protein extracted from each culture flask ($n = 5$ for each group; total $n = 10$ gels were used for quantitative intensity analysis). The spots labeled with numbers are those whose intensity levels significantly differed between the two groups. These differentially expressed proteins were subsequently identified by Q-TOF MS and/or MS/MS analyses (see Table 1).

in another similar buffer, which replaced DTT with 135 mM iodoacetamide, for further 15 min. The second-dimensional separation or SDS-PAGE was performed in 12% polyacrylamide gel using SE260 mini-Vertical Electrophoresis Unit (GE Healthcare) at 150 V for approximately 2 h. Separated proteins were visualized with SYPRO Ruby fluorescence staining (Invitrogen-Molecular Probes; Eugene, OR). Gel images were taken using a Typhoon laser scanner (GE Healthcare).

Matching and Analysis of Protein Spots. Image Master 2D Platinum (GE Healthcare) software was used for matching and analysis of protein spots in 2-D gels. Parameters used for spot detection were (i) minimal area = 10 pixels; (ii) smooth factor = 2.0; and (iii) saliency = 2.0. A reference gel was created from an artificial gel combining all of the (common and uncommon) spots presenting in different gels into one image. This reference gel was then used for matching the corresponding protein spots among all different gels within the same group ($n = 5$ gels per group) and between the two different groups (i.e., control vs COM-exposed cells). The spots were automatically aligned and matched using some selected landmarks, which were the well-defined spots that were present in all gels. However, each of the matched spots by this automatic option was then confirmed or edited manually. Background subtraction was performed and the intensity volume of each spot was normalized with total intensity volume (summation of the intensity volumes obtained from all spots within the same 2-D gel).

Statistical Analysis. Comparisons between the two sets of the samples (i.e., control vs COM-exposed cells) of % cell death and intensity levels of corresponding protein spots were performed using unpaired Student's *t* test. *P*-values less than 0.05 were considered statistically significant. Differentially expressed protein spots, which were statistically significant, were subjected to in-gel tryptic digestion and identification by mass spectrometry.

In-Gel Tryptic Digestion. The protein spots whose intensity levels significantly differed between groups were excised from 2-D gels, washed twice with 200 μ L of 50% acetonitrile (ACN)/25 mM NH_4HCO_3 buffer (pH 8.0) at room temperature for 15 min, and then washed once with 200 μ L of 100% ACN. After washing, the solvent was removed, and the gel pieces were

dried by a SpeedVac concentrator (Savant; Holbrook, NY) and rehydrated with 10 μ L of 1% (w/v) trypsin (Promega; Madison, WI) in 25 mM NH_4HCO_3 . After rehydration, the gel pieces were crushed with siliconized blue stick and incubated at 37 $^\circ\text{C}$ for at least 16 h. Peptides were subsequently extracted twice with 50 μ L of 50% ACN/5% trifluoroacetic acid (TFA); the extracted solutions were then combined and dried with the SpeedVac concentrator. The peptide pellets were resuspended with 10 μ L of 0.1% TFA and purified using ZipTip_{C18} (Millipore; Bedford, MA). The peptide solution was drawn up and down in the ZipTip_{C18} 10 times and then washed with 10 μ L of 0.1% formic acid by drawing up and expelling the washing solution three times. The peptides were finally eluted with 5 μ L of 75% ACN/0.1% formic acid.

Protein Identification by MALDI-Q-TOF MS and MS/MS Analyses. The proteolytic samples were premixed 1:1 with the matrix solution (5 mg/mL α -cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN, 0.1% (v/v) TFA and 2% (w/v) ammonium citrate) and spotted onto the 96-well sample stage. The samples were analyzed by the Q-TOF Ultima mass spectrometer (Micromass; Manchester, U.K.), which was fully automated with predefined probe motion pattern and the peak intensity threshold for switching over from MS survey scanning to MS/MS, and from one MS/MS to another. Within each sample well, parent ions that met the predefined criteria (any peak within the m/z 800–3000 range with intensity above 10 count $\pm$ include/exclude list) were selected for CID MS/MS using argon as the collision gas and a mass dependent ± 5 V rolling collision energy until the end of the probe pattern was reached. The LM and HM resolution of the quadrupole were both set at 10 to give a precursor selection window of about 4-Da wide. Manual acquisition and optimization for individual samples or peaks was also possible.

The instrument was externally calibrated to <5 ppm accuracy over the mass range of m/z 800–3000 using a sodium iodide and PEG 200, 600, 1000 and 2000 mixtures and further adjusted with Glu-Fibrinopeptide B as the near-point lock mass calibrant during data processing. At a laser firing rate of 10 Hz, individual spectra from 5-s integration period acquired for each of the MS survey and MS/MS performed were combined,

Table 1. Summary of Differentially Expressed Proteins, Whose Abundance Levels Were Significantly Altered after an Exposure to 1000 μ g/mL COM Crystals^a

spot no.	protein	NCBI ^b ID	identified by	PMF ^c , MS/MS ions scores	%cov ^d (MS, MS/MS)	no. of matched peptides (MS, MS/MS)	intensity levels (pixel unit) (mean ± SEM)				ratio (COM/control)	p-values
							pI	MW (kDa)	control	COM		
1	150 kDa oxygen-regulated protein precursor (Orp150)	XP_536547	MS/MS	NA, 34	NA, 1	NA, 1	5.46	122.21	0.0155 ± 0.0098	0.0531 ± 0.0117	3.42	0.038
2	Major vault protein (MVP)	XP_536910	MS	78, NA	20, NA	12, NA	5.50	99.42	0.0498 ± 0.0050	0.0287 ± 0.0066	0.57	0.033
3	Cingulin isoform CRA_b	EAW53435	MS	57, NA	15, NA	11, NA	5.58	120.30	0.0188 ± 0.0060	0.0389 ± 0.0034	2.07	0.019
4	Progesterone-induced blocking factor 1 (PIBF1)	AAL37481	MS	74, NA	20, NA	15, NA	5.77	90.00	0.0347 ± 0.0021	0.0112 ± 0.0051	0.32	0.003
5	Glutamyl-tRNA synthetase (GlnRS) isoform 9	XP_862570	MS	83, NA	22, NA	15, NA	6.38	88.71	0.0291 ± 0.0037	0.0161 ± 0.0040	0.55	0.044
6	78 kDa glucose-regulated protein precursor (GRP78) isoform 5	XP_863385	MS	91, NA	26, NA	13, NA	5.09	72.25	0.0085 ± 0.0085	0.0629 ± 0.0131	7.40	0.008
7	CG1550-PA	XP_531703	MS, MS/MS	119, 142	29, 5	15, 3	5.22	74.48	0.0738 ± 0.0059	0.0510 ± 0.0078	0.69	0.049
8	Ezrin (Cyto villin) (Villin-2) isoform 5	XP_862432	MS, MS/MS	104, 93	33, 3	18, 3	5.90	70.86	0.5208 ± 0.0462	0.2588 ± 0.0752	0.50	0.018
9	Aconitase 2, mitochondrial isoform 7	XP_858265	MS, MS/MS	101, 140	23, 5	15, 3	8.61	85.64	0.0416 ± 0.0059	0.0902 ± 0.0133	2.17	0.010
10	Meningioma expressed antigen 6 (Mgea6) (Ctge5) protein	AAH24076	MS	73, NA	26, NA	11, NA	5.17	65.34	0.0443 ± 0.0033	0.0269 ± 0.0053	0.61	0.024
11	Beta tubulin	CAA43197	MS, MS/MS	114, 211	32, 7	20, 4	4.85	50.11	1.0862 ± 0.0634	0.6589 ± 0.0886	0.61	0.004
12	60 kDa heat shock protein (Hsp60)	XP_857240	MS, MS/MS	139, 310	38, 13	18, 5	5.47	57.84	0.8067 ± 0.0523	1.4488 ± 0.1902	1.80	0.012
13	Cyokeratin 8 (CK 8)	XP_543639	MS, MS/MS	118, 120	40, 9	15, 3	5.94	49.14	0.0951 ± 0.0079	0.0093 ± 0.0086	0.10	<0.001
14	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit precursor	NP_004037	MS/MS	NA, 142	NA, 7	NA, 3	9.16	59.83	0.0672 ± 0.0117	0.0333 ± 0.0050	0.50	0.029
15	Hypothetical protein LOC533555	NP_001095628	MS	71, NA	23, NA	12, NA	7.02	63.12	0.0287 ± 0.0024	0.0442 ± 0.0051	1.54	0.026
16	Cyokeratin 7 (CK 7)	XP_534795	MS, MS/MS	82, 36	28, 2	11, 1	6.21	51.65	0.0356 ± 0.0073	0.0683 ± 0.0120	1.92	0.047
17	Cyclin A1	XP_600212	MS	54, NA	23, NA	7, NA	5.73	48.16	0.0437 ± 0.0041	0.0131 ± 0.0064	0.30	0.004
18	Alpha enolase (Enolase 1) isoform 9	XP_859439	MS, MS/MS	119, 180	37, 7	13, 3	6.63	47.68	0.5842 ± 0.0273	0.3343 ± 0.0604	0.57	0.005
19	Unidentified	—	—	—	—	—	—	—	0.0990 ± 0.0124	0.2176 ± 0.0417	2.20	0.026
20	Alpha-centractin (centractin) (Actin-related protein)	XP_001499674	MS, MS/MS	58, 61	26, 6	7, 2	6.40	48.86	0.0858 ± 0.0059	0.0606 ± 0.0089	0.71	0.046
21	Tu translation elongation factor (EF-Tu), mitochondrial	XP_536924	MS, MS/MS	139, 349	38, 13	18, 5	9.03	63.60	0.2163 ± 0.0176	0.3604 ± 0.0552	1.67	0.038
22	Stomatin-like protein 2 (SLP-2) isoform 1	XP_531986	MS, MS/MS	47, 108	22, 12	7, 3	6.88	38.66	0.0692 ± 0.0033	0.0841 ± 0.0054	1.22	0.046
23	Elongation factor 1 (EF-1 delta) isoform 3	XP_856555	MS, MS/MS	61, 104	33, 16	6, 3	4.94	30.328	0.1489 ± 0.0222	0.0749 ± 0.0168	0.50	0.029
24	Annexin A1 (Anx I) (Lipocortin I) (Calpactin II) isoform 2	XP_533524	MS, MS/MS	141, 289	49, 16	13, 4	5.84	38.887	0.5712 ± 0.0439	0.3555 ± 0.0742	0.62	0.037
25	Casein kinase 1 (CK1), delta isoform 2	XP_533137	MS	63, NA	27, NA	11, NA	9.54	45.39	0.0555 ± 0.0150	0.2040 ± 0.0339	3.68	0.004
26	Nebulin-related anchoring protein (NRAP) isoform C	NP_006166	MS	76, NA	15, NA	21, NA	9.25	193.79	0.0884 ± 0.0150	0.3783 ± 0.0839	4.28	0.009
27	Branched-chain-amino-acid aminotransferase (BCAT(m)) (placenta protein 18) (PP18)	XP_541509	MS	47, NA	20, NA	8, NA	7.14	44.93	0.1139 ± 0.0137	0.1711 ± 0.0130	1.50	0.016
28	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 (GalNAc)	XP_345319	MS	56, NA	30, NA	8, NA	8.53	43.79	0.0789 ± 0.0200	0.0045 ± 0.0045	0.06	0.007
29	alpha-2,6-sialyltransferase V) Heterogeneous nuclear ribonucleoprotein H3 (hnRNP-H3) isoform 6	XP_860502	MS, MS/MS	56, 59	40, 4	8, 1	6.37	38.76	0.0504 ± 0.0049	0.0242 ± 0.0064	0.48	0.012

Table 1. Continued

spot no.	protein	NCBI ^b ID	identified by	PMF ^c , MS/MS ions scores	%cov ^d (MS, MS/MS)	no. of matched peptides (MS, MS/MS)	pI	MW (kDa)	intensity levels (pixel unit) (mean ± SEM)			p-values
									control	COM	ratio (COM/control)	
30	Mitochondrial import receptor subunit TOM40 homologue (translocase of outer membrane 40 kDa subunit homologue) isoform 1 (TOMM40)	XP_852633	MS/MS	NA, 96	NA, 7	NA, 2	6.79	37.99	0.0838 ± 0.0097	0.1444 ± 0.0238	1.72	0.046
31	Proteasome subunit p42	BAA11338	MS, MS/MS	114, 37	41, 3	15, 1	7.10	44.42	0.0871 ± 0.0062	0.0416 ± 0.0143	0.48	0.019
32	Ubiquinol-cytochrome-c reductase complex core protein 2, mitochondrial precursor (Complex III subunit II) isoform 1	XP_536942	MS, MS/MS	44, 81	23, 7	8, 2	9.04	48.41	0.1203 ± 0.0182	0.2232 ± 0.0402	1.86	0.048
33	Fructose-bisphosphate aldolase A (aldolase A) isoform 2	XP_849434	MS, MS/MS	107, 100	37, 13	13, 3	8.30	39.94	0.3415 ± 0.0411	0.0665 ± 0.0202	0.19	<0.001
34	Aspartate aminotransferase (AST) isoform 2	XP_851273	MS, MS/MS	77, 28	30, 3	10, 1	9.10	43.76	0.0080 ± 0.0080	0.0978 ± 0.0281	12.25	0.015
35	Eukaryotic translation initiation factor C2, 2 protein (Eif2c2) isoform 2	XP_001143064	MS	69, NA	26, NA	11, NA	9.29	56.53	0.0601 ± 0.0079	0.1464 ± 0.0193	2.44	0.003
36	Cell division cycle 2 (CDC2), G1 to S and G2 to M	AAI10152	MS	68, NA	38, NA	7, NA	8.38	34.06	0.1975 ± 0.0131	0.3729 ± 0.0729	1.89	0.045
37	Annexin A8 (Anx VIII) (Vascular anticoagulant-beta) isoform 1	XP_536412	MS, MS/MS	159, 97	63, 6	17, 2	5.53	36.83	0.1547 ± 0.0176	0.0977 ± 0.0130	0.63	0.031
38	Uridine phosphorylase 1 (UPP1)	XP_537889	MS, MS/MS	85, 53	39, 11	10, 2	6.59	34.50	0.1722 ± 0.0267	0.3016 ± 0.0361	1.75	0.020
39	Porin	AAA60145	MS, MS/MS	74, 171	35, 19	7, 3	5.87	35.62	0.0675 ± 0.0107	0.2257 ± 0.0490	3.34	0.014
40	Proteasome alpha 1 subunit	XP_001379009	MS, MS/MS	69, 147	36, 15	8, 3	7.64	33.61	0.1166 ± 0.0095	0.0717 ± 0.0127	0.62	0.022
41	Unnamed protein product	BAC05344	MS	70, NA	37, NA	8, NA	5.91	31.63	0.2460 ± 0.0255	0.1514 ± 0.0234	0.62	0.026
42	Adenylate kinase isoenzyme 2 (Ak2)	XP_535321	MS, MS/MS	43, 61	36, 7	7, 2	6.54	26.78	0.0682 ± 0.0139	0.1218 ± 0.0126	1.78	0.021
43	Rho GDP dissociation inhibitor alpha (RhoGDI alpha)	XP_849933	MS, MS/MS	112, 90	63, 19	13, 3	5.12	23.44	0.2278 ± 0.0134	0.1255 ± 0.0210	0.55	0.003
44	Peroxisome 3 (PRDX3)	NP_776857	MS/MS	NA, 114	NA, 9	NA, 2	7.15	28.41	0.1159 ± 0.0056	0.0667 ± 0.0137	0.58	0.011
45	Triosephosphate isomerase (TIM) isoform 5	XP_867371	MS, MS/MS	55, 89	40, 16	6, 2	8.29	23.18	0.2064 ± 0.0242	0.0452 ± 0.0205	0.22	0.001
46	Phosphatidylethanolamine binding protein 1 (PEBP1)	NP_001041557	MS	67, NA	50, NA	7, NA	7.10	21.08	0.1870 ± 0.0167	0.0866 ± 0.0199	0.46	0.005
47	Transgelin 2 (TAGLN2)	NP_003555	MS, MS/MS	78, 7	38, 7	10, 1	8.41	22.55	0.7400 ± 0.1872	0.2654 ± 0.0657	0.36	0.044
48	Heterogeneous nuclear ribonucleoprotein M isoform (hnRNP-M) isoform 12	XP_868371	MS	61, NA	24, NA	12, NA	8.74	75.93	0.0289 ± 0.0078	0.1182 ± 0.0151	4.09	0.001
49	Eukaryotic translation initiation factor 5A-1 (eIF-5A1)	P10160	MS, MS/MS	40, 101	43, 8	7, 2	5.08	16.98	0.4196 ± 0.0478	0.2217 ± 0.0336	0.53	0.010
50	TGF beta receptor type I (TGFR1)	BAC66064	MS	69, NA	57, NA	5, NA	6.36	13.86	0.1719 ± 0.0169	0.3219 ± 0.0545	1.87	0.030
51	Ribosomal protein L12 (RPL12) isoform 1	XP_001143119	MS/MS	NA, 48	NA, 30	NA, 2	9.15	14.60	0.1729 ± 0.0250	0.2618 ± 0.0269	1.51	0.042
52	Polyubiquitin	XP_851119	MS/MS	NA, 86	NA, 19	NA, 2	5.31	12.82	0.1408 ± 0.0166	0.2519 ± 0.0135	1.79	0.001
53	Peptidylprolyl isomerase A (PPIA)	BAC56500	MS, MS/MS	77, 178	47, 29	6, 3	8.46	17.09	0.9448 ± 0.1487	0.5269 ± 0.0932	0.56	0.044

^a These altered proteins were then identified by Q-TOF MS and/or MS/MS analyses. NA = Not applicable. ^b NCBI = National Center for Biotechnology Information. ^c PMF = Peptide mass fingerprinting. ^d %Cov = %Sequence coverage [(number of the matched residues/total number of residues in the entire sequence) × 100%].

Figure 4. Validation of the proteomic data by 2-D Western blot analysis. Proteins derived from MDCK cells (100 μ g of total protein for each sample; extraction was performed as for 2-DE analysis) were resolved with 2-DE and transferred onto nitrocellulose membranes. After blocking nonspecific bindings, the membranes were incubated with mouse polyclonal anti-ezrin (A), rabbit polyclonal anti-enolase 1 (B), and mouse monoclonal anti-Anx I (C) primary antibodies, and then with their respective secondary antibodies conjugated with horseradish peroxidase. Immunoreactive protein spots were then visualized with chemiluminescence substrate.

smoothed, deisotoped (fast option) and centroided using the ProteinLynx GlobalSERVER 2.0 data processing software (Micromass). The MS and MS/MS data were extracted and outputted as the searchable *.txt* and *.pkl* files, respectively, for independent searches using the public MASCOT search engine (<http://www.matrixscience.com>). The data were searched against the NCBI mammalian protein database (with approximately 5.7×10^6 entries) assuming that peptides were monoisotopic. Fixed modification was carbamidomethylation at cysteine residues, whereas variable modification was oxidation at methionine residues. Only 1 missed trypsin cleavage was allowed, and peptide mass tolerances of 100 and 50 ppm were allowed for peptide mass fingerprinting and MS/MS ions search, respectively.

2-D Western Blotting. To confirm the results of MS and/or MS/MS protein identification, 2-D Western blot analysis was performed. Proteins derived from MDCK cells (100 μ g of total protein for each sample; extraction was performed as for 2-DE

analysis) were resolved by 2-DE (as described above) and transferred onto nitrocellulose membranes (Whatman, Dassel, Germany) using a semidry transfer apparatus (Bio-Rad, Milano, Italy) at 75 mA for 1 h. Nonspecific bindings were blocked with 5% milk in PBS at room temperature for 1 h. The membranes were then incubated with mouse polyclonal anti-ezrin (1:500 in 5% milk/PBS) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-enolase 1 (1:200 in 5% milk/PBS) (Santa Cruz Biotechnology, Inc.) and mouse monoclonal anti-Anx I (1:500 in 5% milk/PBS) (CHEMICON International, Inc., Temecula, CA) primary antibodies at 4 °C overnight. After washing, the membranes were further incubated with respective secondary antibodies conjugated with horseradish peroxidase (with dilutions of 1:2000 to 1:5000 in 5% milk/PBS) at room temperature for 1 h. Immunoreactive protein spots were then visualized with SuperSignal West Pico chemiluminescence substrate (Pierce Biotechnology, Inc., Rockford, IL).

Table 2. Functional Roles and Subcellular Localizations of the Altered Proteins Identified from the COM-Exposed MDCK Cells^a

altered proteins	subcellular localization	function
<i>Proteins Whose Abundance Levels Were Significantly Increased during COM Crystal Adhesion</i>		
Aconitase2, mitochondrial isoform 7	Mitochondria	Metabolic enzyme
Ak 2 isoform 1	Mitochondrial intermembrane space	Metabolic enzyme
AST isoform 2	Mitochondria	Metabolic enzyme
BCAT(m)	Mitochondria	Metabolic enzyme
CDC2	Nucleus	Metabolic enzyme
Cingulin isoform CRA_b	Tight junction	Cellular structure
CK1delta isoform 2	Cytosol-Golgi and ER-Golgi transport vesicles	Vesicular trafficking
CK7	Intermediate filament	Cellular structure
EF-Tu	Cytoplasm	Protein biosynthesis
Eif2c2 isoform 2	Cytoplasm	Protein biosynthesis
GRP78	ER	Chaperone
hnRNP-M, a isoform 12	Nucleus	RNA metabolism
Hsp60	Mitochondria	Chaperone
NRAP isoform C	Cell membrane	Cellular structure
Orp150	ER	Chaperone
Polyubiquitin	Cytoplasm	Protein degradation
Porin	Cell membrane	Channel
RPL 12 isoform 1	Large ribosomal subunit	Translation
SLP-2 isoform 1	Cell membrane	Cellular structure
TGFR1	Cell membrane	Receptor for TGF-beta
TOMM40 isoform 1	Mitochondrial outer membrane	Import of protein precursors
Ubiquinol-cytochrome-c reductase	Mitochondria	Metabolic enzyme
UPP1	Cytoplasm	Metabolic enzyme
<i>Proteins Whose Abundance Levels Were Significantly Decreased during COM Crystal Adhesion</i>		
Annexin A8 isoform 1	Cell membrane	Signal transduction
Annexin A1	Cell membrane	Signal transduction
ATP synthase alpha subunit precursor	Mitochondria	ATP synthesis
Beta tubulin	Microtubule	Cellular structure
Centractin (Actin-related protein)	Associated with centrosome, cytosol	Cellular structure
CG1550-PA	Cytoplasm	Metabolic enzyme
CK8	Intermediate filament	Cellular structure
Cyclin A1	Nucleus	Cell cycle regulator
EF-1, delta isoform 3	Cytoplasm	Protein biosynthesis
eIF-5A1	Cytoplasm	Protein biosynthesis
Enolase 1	Cytoplasm	Metabolic enzyme
Ezrin isoform 5	Cell membrane	Cellular structure
Fructose-bisphosphate aldolase A isoform 2	Cytoplasm	Metabolic enzyme
GalNAc alpha-2,6-sialyltransferase	Golgi apparatus membrane	Metabolic enzyme
GlnRS isoform 9	Cytoplasm	Protein biosynthesis
hnRNP-H3, a isoform 6	Nucleus	RNA metabolism
Mgea6 (Ctage5) protein	Cell membrane	Signal transduction
MVP	Nucleus/Cytosol	Nucleocytoplasmic transport
PEBP1	Cytoplasm	Signal transduction
PIBF1	Nucleus	Immune response
PPIA	Cytoplasm	Protein biosynthesis
PRDX3	Mitochondrial membrane	Antioxidant enzyme
Proteasome alpha 1 subunit	Cytoplasm	Protein degradation
Proteasome subunit p42	Cytoplasm	Protein degradation
RhoGDI alpha	Cytoplasm	Inhibitor of Rho protein degradation
TAGLN2	Cytoplasm	Cellular structure
TIM isoform 5	Cytoplasm	Metabolic enzyme

^a Three altered proteins: hypothetical protein (spot no. 15), unnamed protein product (spot no. 41), and unidentified protein (spot no. 19), of which function remains unknown are not included.

Results

Approximately 3-million MDCK cells were inoculated in each culture flask and maintained for 24 h. A total of 10 semiconfluent flasks were then divided into two groups (*n* = 5 per group) and the culture medium was replaced by either COM-containing (with 1000 μg/mL COM crystals) or COM-free medium. The cells were grown further for 48 h. Crystal adhesion on the cell surface was clearly demonstrated using phase-contrast microscopy (Figure 1A) and SEM (Figure 1B). These crystals adhered tightly onto the cells as they remained adherent even after several washes with PBS. Interestingly, the borders of these crystals became blurred or interrupted, which indicated cell–crystal interactions. In a parallel experiment, time-course cell death assay using annexin V/propidium iodide double staining showed that % cell death was gradually increased in MDCK cells exposed to COM crystals. At 48 h, %

cell death was significantly greater in the COM-exposed MDCK cells compared to the control MDCK cells (28.04 ± 0.89 vs 16.71 ± 1.89%, *p* < 0.001) (Figure 2).

After the culture with or without COM crystals for 48 h, MDCK cells were harvested from individual flasks and cellular proteins from each flask were extracted and resolved in each 2-D gel (*n* = 5 gels per group). The resolved proteins were then visualized by SYPRO Ruby fluorescence stain and the visualized spots were analyzed by Image Master 2D Platinum (GE Healthcare) software, as detailed in Materials and Methods. From approximately 1200–1400 spots visualized in each 2-D gel, quantitative intensity analysis and statistics revealed significant changes in abundance levels of totally 53 protein spots in the COM-exposed cells (Figure 3). These altered proteins were then identified by Q-TOF MS (peptide mass fingerprinting; PMF) and/or MS/MS (peptide sequencing). Table 1 summarizes

Figure 5. Numbers of the altered proteins in MDCK cells treated with COM crystals (1000 $\mu\text{g/mL}$) with respect to individual subcellular compartments (A) and functional categories (B).

identities, identification scores and other information related to protein identification and changes in their abundance levels. 2-D Western blot analysis was performed on randomly selected altered proteins to confirm the proteomic data. Figure 4 illustrates that the decreased levels in ezrin, enolase 1, and annexin A1 (Anx I) were clearly confirmed by 2-D Western blot analysis.

In addition, bioinformatic analysis using the Swiss-Prot protein knowledgebase and TrEMBL computer-annotated supplement to Swiss-Prot (<http://www.expasy.org/sprot/>) was performed to obtain additional information (specifically, subcellular localization and functional significance) of the altered proteins; (Table 2). Numbers of the altered proteins, with respect to individual subcellular compartments and functional categories, are summarized in Figure 5, panels A and B, respectively.

Discussion

We demonstrated that COM crystals could adhere tightly to MDCK cells with subsequent detrimental effects to the cells (Figures 1 and 2). Exposure to COM crystals for 48 h (which might be considered as an acute phase response *in vivo*) caused increased cell death and alterations in abundance levels of several cellular proteins (25 proteins were increased, whereas other 28 were decreased). These data clearly indicate that COM crystals are toxic to distal renal tubular cells. Table 2 provides functional insights and subcellular localizations of these altered proteins. Regarding function, proteins involved in metabolic enzymatic pathways were mostly affected (7 proteins were increased, whereas 5 were decreased) (Figure 5B). The second and third most common functional groups affected by COM-induced toxicity included those involved in cell structure and cytoskeletal assembly (4 proteins were increased, whereas 5

were decreased) and those involved in protein biosynthesis (2 proteins were increased, whereas 4 were decreased), respectively (Figure 5B). Regarding subcellular localization, the most affected compartment was cytoplasm (4 proteins were increased, whereas 13 were decreased) (Figure 5A). Mitochondria (7 proteins were increased, whereas 2 were decreased) and cell membrane (4 proteins were increased, whereas 4 were decreased) were the second and third most common compartments, respectively, affected by COM-induced toxicity (Figure 5A). Our data on subcellular compartments of renal tubular cells that were frequently affected by COM crystal-induced toxicity were consistent with those reported in other previous studies.^{12,20,21} The functional significance and potential roles of some of these altered proteins in the renal tubular toxicity induced by COM crystals are highlighted as follows.

Three chaperones (GRP78, Orp150 and Hsp60) had increased levels in MDCK cells after the exposure to COM crystals. GRP78 (or immunoglobulin-binding protein, BiP) is an ER luminal chaperone that can be activated by unfolded/misfolded proteins in the ER.²² Orp150, another ER chaperone, belongs to Hsp70 family and can be triggered by oxygen deprivation or hypoxia.²³ While GRP78 and Orp150 are ER chaperones, Hsp60 (or mitochondrial matrix protein P1) is a mitochondrial chaperone that can prevent misfolding of proteins and promote refolding and proper assembly of unfolded/misfolded proteins in the mitochondrial matrix.²⁴ Numerous studies have demonstrated an important role of these chaperones in defense mechanism against cellular toxicity and apoptosis.^{23,25–31} Our present study identified increased levels of these three chaperones in response to COM crystal adhesion, suggesting that their increases might be for protecting further cellular damage induced by COM crystals. However, the time-course cell death study implicated that their increases were not sufficient to protect COM crystal-induced cellular injury as the cell death was progressively increased when the exposure time was prolonged (Figure 2) and there were too many detrimental changes in cellular proteins that counterbalanced and finally overcame the protecting effects of these three chaperones (Table 2 and Figure 5B).

COM crystals caused several detrimental effects in MDCK cells. In particular, four proteins involved in protein biosynthesis [glutamyl-tRNA synthetase (GlnRS) isoform 9, elongation factor 1 (EF-1) delta isoform 3, eukaryotic translational initiator factor 5A-1 (eIF-5A1), and peptidylprolyl isomerase A (PPIA)], a protein involved in ATP synthesis (ATP synthase alpha subunit precursor), and a cellular cycle regulator (cyclin A1) were significantly decreased after the exposure to COM crystals. These detrimental changes might clearly explain the reason that the three chaperones with increased levels (GRP78, Orp150 and Hsp60) could not completely protect the cells from further injury or apoptosis/necrosis induced by COM crystal adhesion.

Moreover, several proteins involved in cellular structure [beta tubulin, contractin, cytokeratin 8 (CK8), ezrin isoform 5, and transgelin 2 (TAGLN2)] were also decreased. While tubulin^{32,33} and contractin^{34,35} are very important for cell cycle and division, ezrin^{36–38} and transgelin 2^{39,40} are essential for other several cellular functions. The decreases in these proteins involved in cellular structure can definitely reduce cell integrity and also induce cellular vulnerability to stimuli or toxicants. Ezrin (also known as p81, cytovillin and villin-2) is a PDZ-domain phosphoprotein, belongs to an ezrin/radixin/moesin (ERM) family, and is a linker protein for the association between plasma

membrane and actin cytoskeleton assembly.³⁶ These ERM proteins govern cellular function through the Rho GTPase pathway.^{41,42} Ezrin also participates in other signal transduction pathways.^{37,38} Additionally, our data also showed the coordinated changes in several proteins involved in signal transduction, including RhoGDI alpha, meningioma expressed antigen 6 (Mgea6), annexin A1 (Anx I), annexin A8 (Anx VIII) isoform 1, and phosphatidyl ethanolamine binding protein 1 (PEBP1), all of which were also decreased. All these decreases were likely to worsen the cellular toxicity induced by COM crystals.

While several proteins involved in cellular structure and cytoskeletal assembly were decreased, some of these proteins on the other hand were increased. The mechanism underlying these disparate results remains unclear. Interestingly, most of these increased cellular structure proteins were plasma membrane [nebulin-related anchoring protein (NRAP) isoform C, and stomatin-like protein 2 (SLP-2) isoform 1] and tight junction (cingulin isoform CRA_b) proteins. Perhaps, these proteins might play important role in facilitating crystal binding or serve as adhesion molecules to bind with COM crystals. However, further investigation is required to confirm our hypothesis.

Interestingly, one antioxidative protein [peroxiredoxin 3 (PRDX3)] was significantly decreased during COM crystal adhesion. PRDX3 is a mitochondrial protein involved in the redox pathway and can protect cells from oxidative damage. Its reduction can decline the threshold of cells to the oxidative stress.⁴³ This finding was consistent with the results reported in previous studies, which indicated that COM can induce oxidative damage to renal tubular epithelial cells.^{44–46} The decrease in PRDX3 might explain tubular oxidative injury induced by COM crystals.

It should be noted that we evaluated changes in the cellular proteome in MDCK cells after 48-h exposure to a high dose (1000 μ g/mL) of COM crystals. Although we successfully identified several proteins that were altered in this setting, evaluation of changes in the cellular proteome induced by lower doses of COM crystals and at earlier time-points would lead to an understanding of “early responses” in distal renal tubular cells during cell–crystal interactions. Moreover, the study of proteome changes in proximal renal tubular cells would provide more insights into the molecular mechanisms of COM crystal-induced tubulotoxicity.

In summary, we successfully identified a set of proteins in MDCK cells that were altered by COM crystal-induced toxicity. Alterations in several of these proteins could explain the mechanistic pathways of COM crystal-induced toxicity in renal tubular epithelial cells. However, the functional significance of some altered proteins remains unclear and should be further elucidated to better understand the detrimental changes and adaptive responses in these cells during COM crystal adhesion, and to unravel the pathogenic mechanisms of kidney stone disease.

Abbreviations: 2-DE, two-dimensional electrophoresis; ACN, acetonitrile; Ak2, adenylate kinase isoenzyme 2; Anx I, annexin A1; Anx VIII, annexin A8; AST, aspartate aminotransferase; BCAT(m), branched-chain-amino-acid aminotransferase; CDC2, cell division cycle 2; CHAPS, 3-[(3-cholamidopropyl) dimethyl-amino]-1-propanesulfonate; CHCA, cyano-4-hydroxycinnamic acid; CK1, casein kinase 1; CK 7, cytokeratin 7; CK 8, cytokeratin 8; COM, calcium oxalate monohydrate; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EF-1, elongation factor 1; EF-Tu, Tu translation elongation factor; Eif2c2, eukaryotic

translation initiation factor C2, 2 protein; eIF-5A1, eukaryotic translation initiation factor 5A-1; FBS, fetal bovine serum; GalNAG, alpha-N-acetylgalactosaminide; GlnRS, glutamyl-tRNA synthetase; GRP78, 78 kDa glucose-regulated protein; hnRNP-H3, heterogeneous nuclear ribonucleoprotein H3; hnRNP-M, heterogeneous nuclear ribonucleoprotein M isoform; Hsp60, 60 kDa heat shock protein; IEF, isoelectric focusing; MDCK, Madin-Darby Canine Kidney; MEM, Eagle's minimum essential medium; Mgea6, meningioma expressed antigen 6; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MVP, major vault protein; NRAP, nebulin-related anchoring protein; Orp150, 150 kDa oxygen-regulated protein precursor; PEBP1, phosphatidylethanolamine binding protein 1; PIBF1, progesterone-induced blocking factor 1; PMF, peptide mass fingerprinting; PPIA, peptidylprolyl isomerase A; PRDX3, peroxiredoxin 3; Q-TOF, quadrupole time-of-flight; RhoGDI alpha, Rho GDP dissociation inhibitor alpha; RPL12, ribosomal protein L12; SEM, scanning electron microscopy; SLP-2, stomatin-like protein 2; TAGLN2, transgelin 2; TFA, trifluoroacetic acid; TGFRI, TGF beta receptor type I; TIM, triosephosphate isomerase; TOMM40, translocase of outer membrane 40 kDa subunit; UPP1, uridine phosphorylase 1; UPR, unfolded protein response.

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