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Cellular adaptive response of distal renal tubular cells to high-oxalate environment highlights surface alpha-enolase as the enhancer of calcium oxalate monohydrate crystal adhesion

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ABSTRACT

Hyperoxaluria is one of etiologic factors of calcium oxalate kidney stone disease. However, response of renal tubular cells to high-oxalate environment remained largely unknown. We applied a gel-based proteomics approach to characterize changes in cellular proteome of MDCK cells induced by 10 mM sodium oxalate. A total of 14 proteins were detected as differentially expressed proteins. The oxalate-induced up-regulation of alpha-enolase in whole cell lysate was confirmed by 2-D Western blot analysis. Interaction network analysis revealed that cellular adaptive response under high-oxalate condition involved stress response, energy production, metabolism and transcriptional regulation. Down-regulation of RhoA, which was predicted to be associated with the identified proteins, was confirmed by immunoblotting. In addition, the up-regulation of alpha-enolase on apical surface of renal tubular epithelial cells was also confirmed by immunoblotting of the isolated apical membranes and immunofluorescence study. Interestingly, blockage of alpha-enolase expressed on the cell surface by antibody neutralization significantly reduced the number of calcium oxalate monohydrate (COM) crystals adhered on the cells. These results strongly suggest that surface alpha-enolase plays an important role as the enhancer of COM crystal binding. The increase of alpha-enolase expressed on the cell surface may aggravate kidney stone formation in patients with hyperoxaluria.

1. Introduction

Many attempts have been made to better understand the initiation of kidney stone formation in patients with hyperoxaluria. Because calcium oxalate is the most common type of crystals found in kidney stones, effects of dicarboxylic anion (oxalate) have received wide attention [1,2]. The supersaturation of oxalate ion can initiate crystalline formation and induce renal cell injury, which promotes crystal adhesion on renal cell surface [3,4]. Oxalate exposure can activate several cellular events, including redistribution of membrane phospholipids, activation of membrane-bound phopholipases, and consequently, lipid signaling that is involved in cellular injury and death [5]. Overloading of oxalate ion can decrease antioxidant activity and increase reactive oxygen species (ROS) production by mitochondria, and ultimately, cell death. However, the increased ROS production and lactate dehydrogenase (LDH) release induced by oxalate are counteracted by several free radical scavengers, including vitamin E, superoxide dismutase (SOD), catalase and iron chelator, suggesting the

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promising way to prevent oxidative injury and subsequent crystal nucleation [6]. Moreover, oxalate can induce mitochondrial dysfunction as demonstrated in hyperoxaluric rats induced by ethylene glycol (EG) [7]. The results have shown that oxalate could induce permeability transition in this organelle by decreasing activity of mitochondrial respiratory enzyme complexes and increasing mitochondrial swelling [7]. A recent study by Thamilselvan, et al. [8] has demonstrated for the first time that activation of protein kinase C alpha and delta isoforms is the underlying mechanism for oxalate-induced renal epithelial cell injury through the increased ROS production and LDH release. In addition, oxalate exposure increases the production of urinary modulators, e.g. osteopontin (OPN), that can modulate crystal nucleation, growth and aggregation [9]. Moreover, tubular secretion of oxalate has been considered as the key event resulting to hyperoxaluria in calcium stone formers [10].

Nevertheless, response of renal tubular cells to high-oxalate environment remained largely unknown. Daily urinary oxalate excretion can be as high as 0.45–0.50 mg/24 h (equivalent to a concentration of approximately 40–45 mM) in patients with hyperoxaluria [1]. In this study, we investigated the global changes of proteins as a response of distal renal tubular cells under a high-oxalate environment using a gel-based proteomics approach. This *in vitro* condition mimicked hyperoxaluric feature in patients with hyperoxaluria [1,11]. The interacting protein network was then analyzed to obtain biological meanings of the altered proteins. Our findings highlighted alterations of proteins in many cellular processes for adaptation of distal renal tubular cells under a high-oxalate condition. Thereafter, functional analysis was performed to confirm such biological relevance. The functional data revealed that the increased alpha-enolase on apical cell surface played a significant role in enhancement of crystal adhesion induced by high-oxalate.

2. Materials & methods

2.1. Cell cultivation and high-oxalate treatment

MDCK distal renal tubular cells were maintained in Eagle's minimal essential medium (MEM) (Gibco; Grand Island, NY) containing 10% heat inactivated fetal bovine serum (FBS) (Gibco), 2 mM glutamine (Sigma; St.Louis, MO) in the presence of 100 U/ml penicillin G and 100 mg/ml streptomycin (Sigma). Cells were maintained in a humidified incubator at 37 °C with 5% CO_2. For oxalate treatment, sodium oxalate solution was added into the mentioned growth MEM medium to make a final concentration of 10 mM (in 5 ml total volume). This concentration was based on the findings reported previously by Schepers, et al. [11]. The cells were incubated with or without oxalate treatment in a humidified incubator at 37 °C with 5% CO_2 for 72 h, and were then harvested.

2.2. Protein extraction and two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

Cellular proteins derived from the controlled and oxalate-treated cells were subjected to 2-D PAGE (n=5 gels derived from 5 independent cultures per group; a total of 10 gels were analyzed). Briefly, the cell monolayers were washed three times with PBS and were gently scraped. Whole cell lysates were obtained using a lysis buffer containing 7 M urea, 2 M thiourea, 40 mg/ml 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS), 120 mM dithiothreitol (DTT), 2% (v/v) ampholytes pH 3–10, and 40 mM Tris-base) at 4 °C for 30 min. Unsolubilized cellular debris and particles were removed by a centrifugation at 13,000×*g* and 4 °C for 5 min. Protein concentrations in the clarified supernatants were determined using Bio-Rad Protein Assay (Bio-Rad Laboratories; Hercules, CA) based on the Bradford method. An equal amount of 150 μg proteins recovered from each sample was loaded for 2-D PAGE detailed in "Supplementary methods". The resolved proteins were stained with SYPRO Ruby fluorescence dye (Invitrogen-Molecular Probes; Eugene, OR) and gel images were captured by using a Typhoon laser scanner (GE Healthcare; Uppsala, Sweden).

2.3. Matching and quantitative analysis of protein spots

Image Master 2D Platinum (GE Healthcare) software (version 6.0) was used for matching and analysis of protein spots visualized in individual gels. Details are provided in "Supplementary methods". Intensity volumes of individual spots were obtained and subjected to statistical analysis. Differentially expressed protein spots were subjected to in-gel tryptic digestion and identification by mass spectrometry.

2.4. Protein identification by quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) and tandem MS (MS/MS)

In-gel tryptic digestion was performed as detailed in "Supplementary methods". 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, UK) (more details are provided in "Supplementary methods"). The MS peptide masses and MS/MS ions were analyzed by the MASCOT search engine (http://www.matrixscience.com) and queried to the NCBI mammalian protein database, assuming that peptides were monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Only 1 missed trypsin cleavage was allowed, and peptide mass tolerances of 100 and 50 ppm were used for MS and MS/MS data, respectively.

2.5. Protein network analysis and subcellular localization

The STRING software [12] was adopted to translate expressional proteomic data into biological relevance. This public database weighs and integrates protein–protein interactions from numerous resources, including experimental repositories, computational prediction and published articles [12]. All the significantly altered proteins identified in oxalate-treated MDCK cells were inputted into STRING 8.3 (http://string-db.org) to retrieve the data of (i) protein function, (ii) subcellular localization and (iii) protein interaction/functional connectivity.