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Oxalate induces mitochondrial dysfunction and disrupts redox homeostasis in a human monocyte derived cell line

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ABSTRACT

Monocytes/macrophages are thought to be recruited to the renal interstitium during calcium oxalate (CaOx) kidney stone disease for crystal clearance. Mitochondria play an important role in monocyte function during the immune response. We recently determined that monocytes in patients with CaOx kidney stones have decreased mitochondrial function compared to healthy subjects. The objective of this study was to determine whether oxalate, a major constituent found in CaOx kidney stones, alters cell viability, mitochondrial function, and redox homeostasis in THP-1 cells, a human derived monocyte cell line. THP-1 cells were treated with varying concentrations of CaOx crystals (insoluble form) or sodium oxalate (NaOx; soluble form) for 24 h. In addition, the effect of calcium phosphate (CaP) and cystine crystals was tested. CaOx crystals decreased cell viability and induced mitochondrial dysfunction and redox imbalance in THP-1 cells compared to control cells. However, NaOx only caused mitochondrial damage and redox imbalance in THP-1 cells. In contrast, both CaP and cystine crystals did not affect THP-1 cells. Separate experiments showed that elevated oxalate also induced mitochondrial dysfunction in primary monocytes from healthy subjects. These findings suggest that oxalate may play an important role in monocyte mitochondrial dysfunction in CaOx kidney stone disease.

1. Introduction

Kidney stones are one of the most common urological conditions that affects approximately 9% of the population in the United States [1]. The recurrence rate within the first 5 years of having a stone event is between 35% and 50% [2]. Unfortunately, the etiology of stone formation is a complex process, which is not well defined. Thus, there is a crucial need to dissect mechanisms that contribute to stone formation with an aim to identify potential therapeutic targets for intervention. Several lines of evidence have identified lifestyle factors [1,3] and genetics [4] as contributors to stone formation. Kidney stones form by a process of crystallization, growth, and accumulation in the renal epithelium involving mineral and organic substances such as calcium, magnesium, phosphate, and oxalate [5,6].

The most common type of kidney stone is comprised of calcium oxalate (CaOx). Oxalate is derived from dietary sources (e.g. plant and plant-derived foods) and can be synthesized by the body and is excreted in the urine [3,7]. Oxalate also exists in soluble and insoluble forms. CaOx crystals form when the urine becomes supersaturated with calcium and oxalate. Several studies have reported that crystals and oxalate stimulate inflammatory responses, including monocyte chemoattractant protein-1 (MCP-1) release in renal epithelial cells [8–12]. MCP-1 plays an important role in monocyte/macrophage recruitment and activation, and has been shown to be elevated in the urine and renal tissue of patients with kidney stones [13,14].

Monocytes are derived from myeloid progenitor cells and are key players in the innate immune system. They are important for fighting infections and responding to inflammation. Their ability to carry out their physiological functions depends on oxidative phosphorylation/mitochondrial function [15]. Mitochondria are critical for regulating intracellular signaling via formation of reactive oxygen species (ROS) [16]. However, excessive levels of ROS can damage the cell, disrupt mitochondrial function and stimulate a cascade of events leading to further ROS generation and inflammation. Crystals and oxalate have

Abbreviations: ATP, adenosine triphosphate; CaOx, calcium oxalate; CaP, calcium phosphate; ECAR, extracellular acidification rate; GSH, glutathione; GSSG, glutathione disulfide; MnSOD, manganese superoxide dismutase; NaOx, sodium oxalate; OCR, oxygen consumption rate

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been reported to generate ROS in renal cells [17]. In addition, it has been reported that human macrophages exposed to CaOx crystals release inflammatory cytokines and chemokines [18]. Thus, monocytes recruited to sites of inflammation and injury within the kidney may have compromised mitochondria due to the pro-inflammatory and pro-oxidative environment.

We have previously determined that patients with CaOx kidney stones have decreased mitochondrial function in their circulating monocytes compared to healthy subjects [19]. A potential candidate responsible for suppressing mitochondrial function in monocytes is oxalate. We have previously shown that healthy subjects that consume a high dietary oxalate load have elevated urinary oxalate [7]. It is possible that high oxalate levels may stimulate crystal formation and elicit an immune response. How oxalate affects monocytes that respond to these signals has not been elucidated and warrants further investigation. Identifying and defining such processes may provide insights into mechanism of kidney stone formation. Here, we investigated whether elevated oxalate (soluble and insoluble forms) alters cell viability, mitochondrial function, and redox imbalance in monocytes. In addition, we assessed whether other types of kidney stone precursors (i.e. calcium phosphate (CaP) and cystine crystals) would negatively affect mitochondrial function and redox imbalance in monocytes. The results from this study suggest that oxalate may impact monocyte mitochondrial function during CaOx kidney stone disease.

2. Materials and methods

2.1. Reagents

The following reagents were purchased from Sigma-Aldrich (St. Louis, MO): Calcium oxalate (CaOx), calcium phosphate (CaP), cystine, sodium oxalate (NaOx), oligomycin, FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), antimycin A, Triton X-100, Trypan Blue solution, triethanolamine (TEA), 5-sulfosalicylic acid (5-SA), 2-metlyl-5-vinylpyridine (MVP), DTNB (Ellman's Reagent) and diethylenetriamine pentaacetate (DTPA). All other reagents or kits used are noted elsewhere.

2.1.1. Cell culture and viability assessment

THP-1 cells (TIB202), a human monocyte derived cell line, were obtained from the American Type Culture Collection (Manassas, VA). THP-1 cells were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum and 2-mercaptoethanol (0.05 mM) in T-75 flasks. For all experiments, cultured THP-1 cells were treated with CaOx, Cystine or CaP crystals (50, 100, 200, 500, 1000 μg/ml) or NaOx (0.1, 0.5, 1, 1.5, and 2 mM) and incubated at 37 °C in 5% CO_2 for 24 h. Following treatment, cell viability was determined by the Trypan Blue exclusion assay. In brief, cells were treated with 0.4% Trypan Blue (1:1 dilution) and counted using the Countess Automated Cell Counter (Thermo Fisher Scientific Inc., Waltham, MA).

2.1.2. Primary human monocytes

Written informed consent was obtained from all study participants following UAB Institutional Review Board approval. Blood samples were collected from healthy subjects (n = 10; 32.0 ± 3.3 years of age) to isolate monocytes as previously described [20]. In brief, blood was separated on Ficoll-density gradients and the mononuclear cell fraction was collected. Monocytes were isolated from the mononuclear cell layer using $CD14^+$ magnetic antibodies and magnetic bead separation (Miltenyi Biotec Inc., San Diego, CA). Cells were counted using the Bio-Rad TC20 Automated Cell Counter (Bio-Rad, Hercules, CA). Monocytes were subsequently exposed to either CaOx crystals (50 μg/ml) or NaOx (0.1 mM) for 40 min prior to analysis.

2.1.3. Cellular bioenergetics analysis

Mitochondrial function was assessed using the Seahorse XF96e Analyzer (Agilent Technologies, Santa Clara, CA). Following treatment, 150,000 cells per well were seeded on Cell-Tak coated Seahorse plates. Cells were equilibrated in XF media prior to measuring the mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The mitochondrial stress test was implemented as previously described [20]. Oligomycin (0.5 μg/ml), FCCP (0.6 μM), and antimycin A (10 μM) injections defined the following parameters: basal OCR, ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity, and non-mitochondrial OCR [21]. In addition, the oligo-sensitive ECAR was determined.

2.1.4. Determination of GSH and GSSG levels

Reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione levels were determined in THP-1 cells following treatment. Glutathione was determined based on modifications of the Tietze recycling assay [22]. In brief, cells were lysed in lysis buffer containing 0.1% Triton X-100 in PBS buffer, pH 7.4, containing 10 μM DTPA. Cell lysates were treated with triethanolamine (TEA), 2-methyl-5-vinylpyridine (MVP), and 5% 5-sulfosalicylic acid (5-SA) to measure GSSG or 5-SA alone to measure GSH based on adapted methods from Anderson and Neufer [23,24]. Glutathione was determined based on the reduction of DTNB (Ellman's Reagent) at 412 nm in the Diabetes Research Center BioAnalytical Redox Biology Core (DK 079626) using a Synergy-2 Multimode plate reader (Biotek, Winooski, VT). Samples were normalized to cellular protein.

2.1.5. Western blotting

Following treatment, THP-1 cells were lysed in 25 mM HEPES buffer containing 0.1% Triton X-100 with protease and phosphate inhibitors. Protein concentrations were quantified using the Bradford protein assay (Thermo Fisher Scientific). Protein extracts (15 μg) were separated on 12% polyacrylamide precast gels (Bio-Rad) at 180 V for 45 min before being transferred to PVDF membrane using Trans-Blot Turbo (Bio-Rad) 24 V for 7 min. Membranes were blocked with 5% milk solution in 1X Tris buffered saline with 0.001% Tween 20 (1XTBST) for 1 h at room temperature and probed for manganese superoxide dismutase (MnSOD) antibody (Millipore, Billerica, MA) at 1:1000 dilution overnight at 4 °C. The next day, membranes were washed 3 times for 10 min each with 1XTBST. Membranes were then incubated with anti-rabbit secondary horse radish peroxidase-conjugated antibody (Abcam, Cambridge, UK) at 1:10,000 dilution for 1 h at room temperature. The membranes were washed again with 1XTBST and incubated with Luminata Forte Chemiluminescence (Millipore) for the detection of horseradish peroxidase activity. The bands were detected and analyzed using ImageQuant LAS 4000 imager and software (GE Healthcare Life Sciences, Marlborough, MA). Membranes were prepared for re-blotting by using ReBlot Plus Strong Solution (Millipore) for 15 min at room temperature and then blocked with 5% milk in 1XTBST for 1 h at room temperature. After washing with 1XTBST, membranes were subsequently incubated with GAPDH antibody (1:5000) overnight at 4 °C. The membranes were washed with 1XTBST and incubated with HRP anti-rabbit secondary antibody (Abcam) (1:10,000) for 1 h at room temperature prior to imaging as detailed above. Densitometry analysis was performed using the Image-J software package. Protein levels were normalized using GAPDH as a loading control.

2.1.6. Quantitative real time (qRT-PCR) analysis

To assess gene expression, total RNA from cells was isolated using Maxwell 16 LEV simplyRNA Cells kit (Promega; Madison, WI). Genomic DNA contamination was removed and cDNA was synthesized from half of a microgram of RNA using QuantiTect Reverse Transcription Kit (QIAGEN; Hilden, Germany). PowerUP SYBR Green Master Mix (ThermoFisher Scientific, Grand Island, NY) was used for quantitative RT-PCR along with primers for human MnSOD and GAPDH. Reactions were performed in duplicates and specificity was monitored using melting curve analysis after cycling. Primers were purchased from