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
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), antimoncy A, Triton X-100, Trypan Blue solution, triethanolamine (TEA), 5-sulfosalicylic acid (5-SA), 2-methyl-5-vinylpyridine (MVP), DTNB (Ellman's Reagent) and diethylethermethoxy) phenylhydrazone), antimycin A, Triton X-100, Trypan Blue solution, triethanolamine (TEA), 5-sulfosalicylic acid (5-SA), 2-methyl-5-vinylpyridine (MVP), DTNB (Ellman's Reagent) and diethylthioacetate (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% CO2 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. 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 were collected from healthy subjects (n = 10; 32.0 ± 3.3 years of age)

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 Neuffer [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 phosphatase 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 TBS-T for 1 h. After blocking, membranes were incubated with primary antibodies 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 described 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 ESI (Eschwege, Germany).
Eurofins MWG Operon (Ebersberg, Germany) and used to detect the specific genes listed: Human GAPDH forward 5′-CTCCTGTGGCAGGTCTCC-3′ reverse 5′-TGGAATTTGCCATGGGTGGA-3′ and Human MnSOD forward 5′-GTTGGGGTGCTTGTGGTC-3′ and reverse 5′-GATTAGGTATACATGGTA-3′. GAPDH was used as an internal control and the ΔΔ Ct Method [25,26] was used to quantify relative mRNA expression. Results were expressed as fold change over untreated controls.

2.1.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism (La Jolla, CA, USA). All data reported as mean ± SEM and n = 3 or more determinations. Data were analyzed by one-way analysis of variance (ANOVA) or paired t-tests. A p < 0.05 was considered statistically significant.

3. Results

3.1. Oxalate decreases cell viability in THP-1 cells

The Trypan Blue exclusion assay was utilized to assess cell viability in THP-1 cells treated with increasing concentrations of either CaOx, CaP, or cystine crystals or NaOx for 24 h. As shown in Fig. 1A, lower concentrations of CaOx crystals (insoluble oxalate) did not change cell viability. However, there was a significant decrease in cell viability at higher doses. In particular, 500 μg/ml of CaOx crystals significantly decreased cell viability to 85%. This was further exacerbated to 68% viability when cells were treated with 1000 μg/ml of CaOx crystals. However, treatment with CaP, cystine crystals or NaOx (soluble oxalate) did not alter THP-1 cell viability at any of the concentrations tested (Fig. 1B–D).

3.2. Oxalate alters mitochondrial function in THP-1 cells

To determine whether oxalate negatively impacts monocyte mitochondrial function, THP-1 cells were treated with various concentrations of CaOx, CaP, and cystine crystals or NaOx for 24 h prior to assessing mitochondrial function and glycolysis. As shown in Fig. 2A, treating THP-1 cells with increasing concentrations of CaOx crystals decreased mitochondrial function in a dose-dependent fashion compared to THP-1 cells not exposed to crystals. In particular, basal OCR significantly declined in cells treated with 100 μg/ml or higher concentrations of CaOx crystals (Fig. 2B). ATP-linked OCR showed a progressive decline in a dose dependent manner with CaOx crystal

Fig. 1. The effect of (A) calcium oxalate (CaOx) crystals, (B) calcium phosphate (CaP) crystals, (C) cystine crystals, and (D) sodium oxalate (NaOx) on THP-1 cell viability. Results are means ± SEM; n = 3–5 individual experiments. *p < 0.05 compared to untreated monocytes.
Both proton leak and maximal OCR decreased when cells were treated with the highest concentrations of CaOx crystals (500 and 1000 µg/ml) (Fig. 2B). However, reserve capacity and non-mitochondrial OCR was not affected. Interestingly, both CaP and cystine crystals did not alter mitochondrial function in THP-1 cells at any of the concentrations examined (Supplementary Fig. 1). However, NaOx caused a dose dependent decrease in mitochondrial function (Fig. 3A). Basal OCR significantly decreased when cells were treated with 0.5 mM or higher concentrations of NaOx (Fig. 3B). ATP-linked OCR was inhibited only at 0.5 and 2 mM concentrations. Maximal OCR was significantly decreased at higher concentrations (1.5 and 2 mM NaOx) (Fig. 3B). The remaining mitochondrial parameters were not affected in NaOx treated cells. The oligo-sensitive ECAR was not different in any of the cells treated with CaOx, CaP, or cystine crystals or NaOx (data not shown).

3.3. Oxalate alters manganese superoxide dismutase (MnSOD) and Glutathione levels in THP-1 cells

Manganese superoxide dismutase (MnSOD) is a mitochondrial antioxidant that detoxifies superoxide and reduces oxidative stress within the mitochondria. The effect of CaOx crystals and NaOx treatment (24 h) on MnSOD gene expression and protein levels were determined...
via qRT-PCR and western blotting, respectively (Fig. 4). THP-1 cells exposed to CaOx crystals had increased MnSOD mRNA levels starting at the 100 μg/ml concentration (Fig. 4A). NaOx treatment elicited different responses in THP-1 cells (Fig. 4B). MnSOD protein levels were significantly decreased in a dose dependent fashion starting at 100 μg/ml in cells treated with CaOx crystals (Fig. 4C). NaOx concentrations between 0.1 mM and 1.5 mM did not alter MnSOD protein levels compared to control cells (Fig. 4D). Only 2 mM NaOx significantly increased both MnSOD gene expression and protein levels in THP-1 cells (Fig. 4B and D). MnSOD did not change significantly following treatment with CaP or cystine crystals (Supplementary Fig. 2). The highest concentration of cystine crystals significantly increased MnSOD mRNA levels but not protein expression (Supplementary Fig. 2).

Reduced glutathione (GSH) is an antioxidant that maintains redox balance within the cell. Intracellular GSH and oxidized glutathione (GSSG) levels were determined using modifications of the Tietze assay [22]. As shown in Fig. 4E, low concentrations of CaOx crystals (100 and 200 μg/ml) caused a significant decrease in the GSH/GSSG ratio (a surrogate for redox homeostasis within cells) in THP-1 cells. However, the GSH/GSSG ratio in cells treated with higher concentrations was similar to control cells. Additionally, NaOx concentrations of 0.1, 0.5, and 1.5 mM significantly decreased the GSH/GSSG ratio in THP-1 cells (Fig. 4F).
3.4. Oxalate alters mitochondrial function and glycolysis in primary monocytes

Primary monocytes from healthy subjects were treated with 50 μg/ml CaOx (crystalline oxalate) or 0.1 mM NaOx (soluble oxalate) for 40 min prior to assessing mitochondrial function. As shown in Fig. 5A, CaOx crystals caused a decrease in mitochondrial function in primary monocytes compared to monocytes not treated with CaOx crystals. Specifically, CaOx crystals significantly decreased ATP-linked and maximal OCR, and reserve capacity (Fig. 5B). There was no difference in basal, proton leak, and non-mitochondrial OCR. The oligo-sensitive ECAR was not affected in primary monocytes treated with CaOx crystals (data not shown). Treating primary monocytes with NaOx also affected mitochondrial function as shown in Fig. 6A. However, these responses were different than those observed in CaOx treated monocytes. Basal, ATP-linked, maximal OCR and non-mitochondrial OCR were significantly decreased (Fig. 6B); whereas, proton leak and reserve capacity were not affected. Only 0.1 mM NaOx significantly decreased the oligo-sensitive ECAR in primary monocytes (p = 0.0104) (data not shown).

4. Discussion

Mitochondrial function is important in regulating energy production and redox signaling in monocytes [15]. We have previously reported that monocyte mitochondrial function is impaired in patients with CaOx kidney stone disease [19]. The objective of this study was to identify whether oxalate in its soluble or crystalline form could decrease monocyte mitochondrial function. CaOx crystals, the crystalline form of oxalate, form in the nephron when urine becomes supersaturated with calcium and oxalate. A large source of oxalate is from dietary sources and is linked to stone formation [3]. CaOx crystals have been observed in the renal cortex of experimental animals exposed to lithogenic agents and in individuals with Primary Hyperoxaluria [27,28]. In addition, CaOx crystals stimulate the production of chemokines such as MCP-1 by renal epithelial cells [10,11]. Macrophages are likely to be involved in crystal clearance and if not properly cleared, could lead to increased inflammation, oxidative stress, and tissue injury. CaOx crystals have been shown to induce innate inflammatory pathways in dendritic cells [29] and to induce changes in mitochondrial proteins involved in metabolism in a human monocytic cell line [30]. Thus, we investigated whether CaOx crystals could affect mitochondrial function and redox signaling in monocytes.
function and disturb redox balance in monocytes.

We treated THP-1 cells, a human derived monocyte cell line, with various crystals (CaOx, CaP, and cystine) known to be precursors to stone formation. The concentrations used in this study are consistent with others who have studied the physiological relevance of oxalate in cell culture models [8,30]. We determined that only CaOx crystals (insoluble oxalate) significantly decreased cell viability; whereas, the other crystal types did not affect cell viability. These findings imply oxalate may be a significant contributor to this pathology. Therefore, we exposed THP-1 cells to NaOx, the soluble form of oxalate. NaOx was used as a surrogate for soluble oxalate and was included to further understand the influence of oxalate on monocytes. We determined that NaOx did not alter monocyte cell viability. However, the impact of soluble oxalate on mitochondrial function and redox status was significant.

It is well known that oxidative stress and inflammation can trigger mitochondrial damage and cell death. It is critical for mitochondrial function to be intact in monocytes/macrophages in order to regulate inflammation [31]. Oxalate has been reported to disrupt mitochondrial proteins and to increase oxidative stress in renal proximal tubular cells [9,17,32]. Consistent with these findings, we determined for the first time to our knowledge that oxalate (CaOx crystals and NaOx) negatively affects mitochondrial function and changes redox status in monocytes. In particular, CaOx crystals disrupted basal, ATP-linked, proton leak and maximal OCR in THP-1 cells in a dose dependent fashion. These findings suggest that oxalate may interfere with
substrate availability or directly at the electron transport level by damaging mitochondrial proteins. NaOx also disrupted monocyte mitochondrial function but not to the same extent as CaOx crystals. In particular, NaOx caused a significant decrease in basal, ATP-linked, and maximal OCR in monocytes. It is logical to conclude from the data that elevated oxalate levels could independently impact monocyte health and function in human subjects. Monocytes exposed to oxalate mediated injury in the circulation could be sub-optimal prior to entering tissues and differentiating into macrophages. We propose that cells previously exposed to oxalate in the circulation are further injured once they enter the kidney and are exposed to CaOx crystals.

A recent study by Zhang et al. demonstrated that oxalate disrupts mitochondrial membrane potential and induces 3,4-Methylenedioxyamphetamine (MDA) release in a rat renal proximal tubule cell line and this is prevented with a mitochondrial targeted antioxidant [33]. We evaluated MnSOD and glutathione to determine whether oxalate could affect these antioxidant proteins. We determined that MnSOD gene expression was significantly elevated after treatment with two concentrations (100 and 500 µg/ml) of CaOx. However, MnSOD protein levels were significantly decreased in THP-1 cells treated with CaOx crystals in a dose dependent manner suggesting these cells are exposed to a highly oxidative environment and that MnSOD could be post-translationally modified. In contrast, increasing concentrations of NaOx did not modify MnSOD gene expression or protein levels. However, the highest concentration of NaOx (2 mM) significantly elevated MnSOD mRNA and protein levels. Interestingly,
these alterations in MnSOD levels are oxalate specific and both CaP and cystine crystals did not significantly change MnSOD protein levels. These data imply oxalate may stimulate a pro-oxidative environment in monocytes. To further understand this phenomenon, we examined the GSH/GSSG ratio, a surrogate for redox status within cells, in THP-1 cells. We found both CaOx crystals and NaOx caused THP-1 cells to be more oxidized than control, untreated cells, suggesting oxalate causes a more oxidative state in monocytes.

To test whether oxalate would have any effect on human monocytes, primary monocytes from healthy subjects were exposed to low doses of CaOx crystals and NaOx. Consistent with our cytoxic findings, primary monocytes from healthy subjects were exposed to low oxalate. The long term effect on human monocytes differentiated into macrophages stimulate inflammatory responses following CaOx crystal exposure and that these cells may play an important role in crystal clearance [18]. It is possible that macrophage differentiation and crystal clearance could be disrupted or cell death may occur in cases where monocytes are exposed to elevated levels of oxalate. The long term effect of some of these events could compromise the immune system over time in patients with kidney stones and/or predispose them to recurring stones. One potential source for such an event is the consumption of oxalate-rich foods. It is likely that oxalate-rich meals that induce CaOx crystalluria could cause inflammation and monocyte mitochondrial dysfunction in patients and would be accentuated in patients with hypercalciuria and/or hyperoxaluria. We have previously determined that oxalate levels are increased in the urine and circulation of human subjects following a dietary oxalate load [7,34].

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