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Exposure of Madin-Darby Canine Kidney (MDCK) Cells to Oxalate and Calcium Oxalate Crystals Activates Nicotinamide Adenine Dinucleotide Phosphate (NADPH)-Oxidase

Aslam Khan, Karen Byer, and Saeed R. Khan

Department of Pharmacy, Shaheed Benazir Bhutto University, Sheringal, Dir Upper, Khyber Pakhtunkhwa, Pakistan; and the Department of Pathology, Immunology and Laboratory Medicine, University of Florida College of Medicine, Gainesville, FL

Abstract

OBJECTIVE—To investigate nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity in Madin-Darby canine kidney (MDCK) cells and the production of reactive oxygen species on exposure to oxalate (Ox) or calcium oxalate (CaOx) crystals.

METHODS—Monolayers of confluent Madin-Darby canine kidney cells were exposed to 100, 300, 500 μmol , 1 mmol Ox or 33, 66, 132 $\mu\text{g}/\text{cm}^2$ CaOx crystals for 15 minutes, 30 minutes, 1 hour, 2 hours, or 3 hours. After specified periods of exposure to Ox and CaOx crystals, lactate dehydrogenase release, trypan blue exclusion, activation of NADPH oxidase, and superoxide production were determined using standard procedures. The production of Nox4, a membrane associated subunit of the NADPH oxidase enzyme, was determined by western blot analysis.

RESULTS—Exposure to Ox and CaOx crystals leads to time- and concentration-dependent activation of NADPH oxidase. Western blot analysis showed an increase in the production of Nox4. The production of superoxide also changed in a time- and concentration-dependent manner, with maximum increases after 30-minute exposure to the highest concentrations of Ox and CaOx crystals. Longer exposures did not change the results or resulted in decreased activities. Exposure to higher concentrations also caused increased lactate dehydrogenase release and trypan blue exclusion indicating cell damage.

CONCLUSION—Results indicate that cells of the distal tubular origin are equipped with NADPH oxidase that is activated by exposures to Ox and CaOx crystals. Higher concentrations of both lead to cell injury, most probably through the increased reactive oxygen species production by the exposed cells.

Oxalate (Ox) is a naturally occurring substance endogenously produced and obtained from dietary sources and excreted at the rate of 10–40 mg/24. Urinary Ox excretion is increased in a variety of diseases, including idiopathic hyperoxaluria, primary hyperoxaluria, enteric hyperoxaluria, and pyridoxine deficiency, which might lead to oxalosis, cardiomyopathy, cardiac conductance disorders, calcium oxalate (CaOx) nephrolithiasis, and renal failure.¹ Primary hyperoxaluria is caused by mutations in *AGXT*, *GRHPR*, and *HOGA1* genes, causing deficiency of alanine-glyoxylate aminotransferase, glyoxylate reductase/hydroxypyruvate reductase enzymes, or 4-hydroxy-2-oxoglutarate aldolase, respectively, which lead to increased production and urinary excretion of Ox.² Enteric hyperoxaluria can

be a result of ileal disease,¹ chronic inflammatory bowel disease, fat malabsorption, steatorrhea, sprue, colitis or Crohn's disease or after ileal resection in jejunoileal bypass surgery, and certain bariatric surgeries for obesity.

Both Ox and CaOx crystals are toxic to cells,³⁻⁵ and cell injury is most likely caused by the production of reactive oxygen species (ROS)⁶⁻⁸ with the involvement of both mitochondria⁹⁻¹² and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.^{9,13} Ox or CaOx crystal induced cellular responses can be inhibited by antioxidants and inhibitors of NADPH oxidase, which is a major source of ROS in the kidneys,¹⁴ particularly in the presence of angiotensin II.¹⁵

Activation of NADPH oxidase in renal cells exposed to Ox and/or CaOx crystals has so far been investigated either in vivo in animal models^{16,17} or in vitro in cells of mostly proximal tubular origin.¹⁸⁻²⁰ Because epithelial cells of distal tubules and collecting ducts are most likely exposed to higher concentrations of Ox and CaOx crystals,²¹ this study was performed to determine the activation of NADPH oxidase in the distal tubular cells as represented by Madin-Darby canine kidney (MDCK) cell line. We exposed the cells to various concentrations of Ox or CaOx crystals for up to 3 hours and determined NADPH oxidase activity and production of its membrane subunit Nox4, as it is the most common isoform to be expressed in the renal epithelial cells.²² Cell viability was established by determining the release of lactate dehydrogenase (LDH) into the medium and trypan blue exclusion by the exposed cells. Nitroblue tetrazolium assay was carried out to detect intracellular superoxide (SOD).

MATERIALS AND METHODS

Cell Culture

Madin-Darby canine kidney epithelial-derived cell line, MDCK, was obtained from American Type Culture Collection (CCL-34; Manassas, VA). Details of cell culture, maintenance, and exposures to Ox and calcium oxalate monohydrate (COM) crystals can be seen in our earlier publications.^{19,23} The cells were exposed to potassium oxalate (Ox, 0.1, 0.3, or 0.5 mmol; Fisher, Norcross, GA; Cat # P-273), COM (33, 66.7, or 132 $\mu\text{g}/\text{cm}^2$; BDH Limited, Poole, England; Cat # 27,609). The cells were exposed for 15 minutes, 30 minutes, 1 hour, 2 hours, or 3 hours for studies, except for the quantification of the protein by western blot analyses. In that case, on the basis of our earlier studies,¹⁹ cells were incubated with the additives for 24 or 48 hours. They were collected after incubation of additives for the presence of intracellular superoxide, trypan blue exclusion (TBE for cell viability), and NADPH Oxidase. Media were retained for the detection of LDH (LDH for cell viability). Control cultures were untreated cells.

NADPH Oxidase

The assay used to determine NADPH oxidase activity was derived from Rao, et al.²⁴ Briefly, after incubation with additives, cells were washed with ice cold Dulbecco's Phosphate-Buffered Saline (DPBS). Cells were scraped off in DPBS with a rubber policeman and collected into 15-mL conical tubes. Samples were centrifuged at $750 \times g$ at 4°C for 10 minutes. Supernatant was aspirated and resuspended in 2 mL of working reagent A (potassium phosphate solution with 50 μL of 10 $\mu\text{g}/\text{mL}$ Aprotinin, 25 $\mu\text{g}/\text{mL}$ leupeptin, and 1 mM PMSF). Cell suspension was homogenized on ice with a cell homogenizer at 20 strokes. An aliquot of homogenate was added to working reagent B (50 mM DPBS (Mediatech), 1 mM EGTA, 150 mM sucrose in distilled, deionized water) in 12×75 mm polystyrene tubes. Tubes were read using a luminometer. Photoemission expressed in terms of relative light units was measured at time zero and every minute for 5 minutes. Final

concentration was calculated as follows: relative light units/mg of total protein = O.D./mg of total protein at each time interval.

Amount of Nox4, a membrane component of NADPH oxidase in the distal tubule, was determined by western blot analyses using standard techniques.^{17,19} Rabbit anti-NOX4/NADH antibody (Cat#:bs-1091R, Bioss Inc.) was used to identify the protein. Semiquantitative evaluation of protein levels was performed with computer-assisted densitometric scanning.

Nitroblue Tetrazolium Assay – Intracellular Superoxide Dismutase Detection

MDCK cells were exposed to the serum free media (0.2 mL) supplemented with 1 mg/mL NBT. Ox or COM was added to the serum free media and incubated at 37°C for the specified times. At specified times, the supernatant NBT solution was aspirated from the wells, and the wells were thoroughly washed with 75% methanol to halt the reaction. The wells were then washed 4 times with 100% methanol to remove unreduced NBT dye and allowed to air-dry. The reduced formazan precipitate remained visible as purple granules on the bottom of the wells. After air-drying, 70 µL of 2M potassium hydroxide was added to each well to lyse the cells. The formazan was then solubilized by the addition of 82 µL of dimethyl sulfoxide to the KOH (at a ratio of 1:1.17 volume per volume). The content of the wells was then mixed by pipetting to complete solubilization. The O.D.₆₅₅ of the solution was read on the ELISA reader (BioRad 3550 microplate reader; BioRad). The blank for this experiment was consisted of wells with no cells that were incubated with NBT solution and subjected to the same processing (fixing, washing, and solubilization steps), which remained colorless.

Cell Viability – LDH

Media was aliquoted to designated wells of a 96-well plate (Fisher Scientific, Norcross, GA Cat # 21-377-205). The CytoTox 96 Non-Radioactive Cytotoxicity assay kit (Fisher Scientific, Norcross, GA, Promega Cat # PR-G1780) was used to determine LDH percent release. Substrate (supplied with kit) was added to all samples, positive control (MDCK cells lysed with lysis solution-supplied with kit), and blanks (acclimation media). The plate was incubated at room temperature for 30 minutes in the dark. Stop solution (supplied with kit) was added to all samples, positive control, and blanks. Optical density absorbency was read at 490 nm on a Bio-Rad 3550 microplate reader (Bio-Rad, Hercules, CA).

Cell Viability – Trypan Blue Exclusion

Cells were grown in an 8-well chamber slide (Fisher Scientific, Norcross, GA Cat # 12-565-8) to 70% confluence. The growth media was removed and replaced with media without sodium pyruvate and serum containing with or without 3 mg/mL of sodium citrate (Sigma- Aldrich, St. Louis, MO, Cat # C-7254) for 8–12 hours. Media was removed after the 8–13 hours and replaced with fresh media without sodium pyruvate and serum containing citrate or no citrate. Cells were then exposed to potassium oxalate at 100, 300, and 500 µmol, or COM at 33, 66.7, and 132 µg/cm² for 15 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours. After incubation with Ox or COM, media was removed and 300 µL of DPBS (Fisher Scientific, Norcross, GA, Cat # MT21031CV) with 20 µL of 4% Trypan blue solution (Sigma, St. Louis, MO Cat # T-8154) was aliquoted to each well. Trypan blue solution was incubated at room temperature with the monolayer for 10 minutes. The solution was removed, and 300 µL of DPBS was placed in each well. Total number of cells (those that excluded dye and those that did not) was counted. Five microscopic fields in each well were counted to determine total percent of cells that did not excluded the trypan blue solution.

Statistical Analysis

Statistical comparisons between the groups were made using one/two-way analysis of variance with posthoc Dunnett's/Bonferroni Post-test, using Graph Pad Prism and Graph Pad InStat3 (GraphPad Software, San Diego, CA).

RESULTS

Exposure to Ox

As expected, cells responded in a time- and concentration-dependent manner on an exposure to both Ox and CaOx crystals. In general, there was a significant increase in the NADPH oxidase enzyme activity over time with exposure to all concentrations of Ox (Fig. 1A). Exposure to 0.1 or 0.3 mmol for 15 minutes, however, did not cause an increase in the activity over that of control but the exposure to 0.5 mmol for 15 minutes produced a significant increase. One hour exposure resulted in consistent and significant increase in enzyme activity with increasing concentrations of Ox. Production of Nox4 also increased significantly on exposure to Ox (Fig. 2A).

Superoxide production increased over time in response to the exposure to 0.1 or 0.3 mmol of Ox (Fig. 3A). There was a significant increase in SOD on exposure to 0.5 mmol of Ox for up to 30 minutes and then it decreased, still significantly higher than control and after exposures to 0.1 or 0.3 mmol of Ox.

Exposure to Ox resulted in cell injury as determined by Trypan Blue Staining (Fig. 4A) and release of LDH into the medium (Fig. 4C). Significantly higher numbers of cells stained with the dye after an exposure to various concentrations of Ox (Fig. 4A). Higher concentrations resulted in significantly more stained cells, and their number increased up to 2 hours of exposures. There were similar time- and concentration-dependent increases in LDH release into the medium (Fig. 4C).

Exposure to CaOx Crystals

Exposure of MDCK cells to CaOx crystals also produced significant increase in NADPH oxidase activity compared with the controls (Fig. 1B). CaOx crystals at 33 and 66 $\mu\text{g}/\text{cm}^2$ produced significantly higher increases for all periods. However, exposure to 132 $\mu\text{g}/\text{cm}^2$ CaOx crystals produced exceedingly high level of activity just after 15 minutes. After that, the activity started to decrease with time and after 3 hours was even lower than the control levels. The amount of Nox4 increased with increasing crystal concentrations reaching the highest amount on exposure to 66 $\mu\text{g}/\text{cm}^2$ of crystals (Fig. 2B).

The production of SOD also increased on exposure to CaOx crystals (Fig. 3B) and followed a pattern similar to the NADPH oxidase activity. The SOD activity was higher after an exposure of 15 minute to all 3 concentrations of CaOx crystals. After exposure for longer duration, the SOD activity increased in response to 33 and 66 $\mu\text{g}/\text{cm}^2$ of crystals but showed no change or decreased in response to 132 $\mu\text{g}/\text{cm}^2$ concentration of the crystals, likely a result of exhaustion of enzymatic resources.

Exposure of cells to CaOx crystals led to release of LDH (Fig. 4D) into the medium and changes in Trypan blue (Fig. 4B) exclusion indicating injury to the cell membrane. LDH release was higher after exposure to all the concentrations of crystals, exceedingly high after exposures to crystals at 66 and 132 $\mu\text{g}/\text{cm}^2$ levels. It also increased with increase in the length of exposure. Trypan blue exclusion assay also showed significant injury, which was generally time- and concentration-dependent, exposure to 132 $\mu\text{g}/\text{cm}^2$ for 2–3 hours being most injurious.

COMMENT

Ox at high concentrations is injurious to cells through its direct interaction with the cells and by producing CaOx crystals, which are also harmful. The 2 together are even more damaging because they act synergistically. Various studies of renal epithelial cells and Ox/CaOx interaction indicate that cell response is catalyzed by ROS.^{9,25} ROS are free radicals, atoms, or molecules with unpaired electrons, and their metabolites include superoxide anion ($O_2^{\bullet-}$), nitric oxide radical ($NO^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2), which are generated by several pathways. $O_2^{\bullet-}$ anions are produced by NADPH oxidases, xanthine oxidase, lipooxygenase, cyclooxygenase, hemeoxygenase, and as a byproduct of mitochondrial respiratory chain.

Most studies have investigated the involvement of mitochondria in the production of ROS and development of Ox-induced oxidative stress (OS).^{3,10} The investigations of other pathways have only recently started, and both animal model and tissue culture studies indicate that the exposure to Ox/CaOx crystals also activates NADPH oxidase, and blocking of the pathways reduces the production of ROS and cell injury.^{13,17,19,20} Blocking the angiotensin II receptor, which is known to be involved in activation of NADPH oxidase, not only reduced the production of osteopontin but also deposition of CaOx crystals in the ethylene glycol induced hyperoxaluria model of nephrolithiasis.²⁶ Apocynin, an antioxidant and inhibitor of NADPH oxidase activity, reduced the urinary excretion of osteopontin, kidney injury molecule, ROS hydrogen peroxide, and deposition of CaOx crystals in kidneys of hydroxyproline-induced hyperoxaluric rats.¹⁷ Gallatonin, an ingredient in green tea, suppressed CaOx crystal induced NADPH oxidase production, oxidative stress, and CaOx crystal binding to renal epithelial cells in culture. Inhibition of Ox-induced membrane associated protein kinase C and Rac1 activation in LLC-PK1 cells resulted in significant reduction of NADPH oxidase activity, production of ROS, and development of oxidative stress.²⁰

NADPH oxidase consists of 2 transmembrane units, p22^{phox} and gp91^{phox}; 4 cytosolic units, p47^{phox}, p67^{phox}, p40^{phox}, and the small GTPase rac1 or rac2. The 2 transmembrane units, gp91^{phox} and p22^{phox}, associate with a flavin to make cytochrome b₅₅₈. The gp91^{phox} unit is the core catalytic component for the electron transfer activity, whereas p22^{phox} has regulatory and stabilizing functions. Cytosolic units translocate to the membrane and assemble with the cytochrome to activate the enzyme. Several homologues of gp91^{phox} have been recognized. The NADPH oxidase enzyme transfers electrons to molecular O_2 via the flavin-containing subunit. Gp91^{phox} and the homologues Nox1 and Nox4 have been identified as the electron-transferring subunit. Nox4 with 39% sequence identity to gp91^{phox}, often called renal oxidase or renox, has high expression in various segments of the renal tubules and high constitutive activity. We hypothesized that epithelial exposure to Ox and CaOx crystal leads to increased production of various subunits of NADPH oxidase and its subsequent activation. Cells of the human epithelial line HK2 were exposed to Ox or CaOx crystals, which lead to significant increases in the expression of p22^{phox} and p47^{phox} leading to activation of NADPH oxidase. Increased NADPH oxidase activity was associated with increased superoxide production and lipid peroxidation.¹⁹ We recently conducted a study, using global transcriptome analyses, to determine changes in the NADPH oxidase system in the kidneys of rats fed a diet leading to hyperoxaluria and CaOx crystal deposition in the presence or absence of apocynin. Genes encoding both membrane- and cytosolic-NADPH oxidase complex-associated proteins, together with *p21rac* and *Rap1a*, were coordinately upregulated significantly in hyperoxaluric rats. Simultaneously, genes encoding ROS scavenger proteins were downregulated. Hyperoxaluric rats receiving apocynin had a complete reversal in the differential-expression of the NADPH oxidase system genes.²⁷

CONCLUSION

The concentration of Ox is higher in the later segments of the nephron with greater possibility of CaOx crystallization. In addition, results of human studies indicate that many types of stones form in association with the deposition of crystals in the ducts of Bellini.^{28,29} Crystal deposition is associated with tubular and interstitial inflammation. ROS play a significant role in renal inflammation and fibrosis. As a result, a number of studies have investigated the interaction between cells of the collecting duct and distal tubular origin and Ox and/or CaOx crystals to determine the mechanisms involved. Most of the studies, which used cells of the collecting duct or distal tubular origin, have only examined the mitochondrial involvement.^{10,30} Because NADPH oxidase is also a significant contributor to renal inflammation, we decided to investigate whether cells of distal nephron origin respond to an exposure to Ox or CaOx crystals by the activation of NADPH oxidase and production of superoxide.

Previous studies have shown that all components of both phagocytic and nonphagocytic NADPH oxidase are present in all sections of the nephrons. The activities of both oxidases are increased during various disease states.³¹ Results of the present study confirm the observations showing the occurrence and activation of NADPH oxidase and production of superoxide by the cells of the distal tubular epithelium. The exposure of MDCK cells to both agents, Ox and CaOx crystals, provoked similar responses. There were increases in NADPH oxidase and SOD in response to both the Ox and CaOx crystal exposures. Both agents caused cell injuries after longer exposures to higher concentrations. However, CaOx crystals appeared to be more injurious to the cells as determined by LDH release and Trypan blue exclusion assay. This might be a result of the physical damage caused by the crystals in addition to what happens through the production of ROS.

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Figure 1.

Activation of NADPH oxidase by exposure to oxalate (Ox) or calcium oxalate monohydrate (COM) crystals. **(A)** Exposures to 0.1, 0.3, or 0.5 mmol Ox for 15, 30 minutes or 1, 2, 3 hours produced time- and concentration-dependent increase in activation of NADPH oxidase. **(B)** Exposures to 33, 66.7, or 132 $\mu\text{g}/\text{cm}^2$ COM crystals for 15, 30 minutes or 1, 2, 3 hours also produced time- and concentration-dependent increase in the activation of NADPH oxidase.

Figure 2. Production of Nox4 subunit of NADPH oxidase by MDCK cells on exposure to oxalate (Ox) or calcium oxalate monohydrate (COM) crystals. **(A)** Exposure to Ox for 24 hours produced concentration-dependent increase in Nox4 as determined by western blot analyses. Results are shown for exposures to 0.3, 0.5, or 1 mmol Ox. **(B)** Exposures to COM crystals for 48 hours produced significant increase in Nox4 production. Results are shown for exposures to 33, 66.7, or 132 µg/cm² COM crystals.

Figure 3.

Activity of intracellular superoxide dismutase in cells exposed to oxalate (Ox) or calcium oxalate monohydrate (COM) crystals, as determined by Nitroblue tetrazolium Assay (NBT). **(A)** Exposures to 0.1, 0.3, or 0.5 mmol Ox for 15, 30 minutes or 1, 2, 3 hours produced concentration-dependent increase in SOD activity. **(B)** Exposures to 33, 66.7, or 132 $\mu\text{g}/\text{cm}^2$ COM crystals for 15, 30 minutes or 1, 2, 3 hours produced mostly concentration-dependent increases in SOD activity.

Figure 4.

Cellular injury on exposure to oxalate (Ox) or calcium oxalate monohydrate (COM) crystals as determined by trypan blue assay (A,B) and release of lactate dehydrogenase (LDH) into the medium (C,D). **(A)** Exposures to 0.1, 0.3, or 0.5 mmol Ox for 15, 30 minutes or 1, 2, 3 hours produced time- and concentration-dependent changes in cell viability. There were significant increases in nonviable cells on exposures to higher concentrations of Ox for longer periods. **(B)** Exposures to 33, 66.7, or 132 $\mu\text{g}/\text{cm}^2$ COM crystals for 15, 30 minutes or 1, 2, 3 hours also produced time- and concentration-dependent increases in nonviable cells. **(C)** Exposures to 0.1, 0.3, or 0.5 mmol Ox for 15, 30 minutes or 1, 2, 3 hours produced mostly concentration-dependent increase in LDH release. **(D)** Exposures to 33, 66.7, or 132 $\mu\text{g}/\text{cm}^2$ COM crystals for 15, 30 minutes or 1, 2, 3 hours also produced mostly concentration-dependent increases in LDH release.