Renal oxidative vulnerability due to changes in mitochondrial-glutathione and energy homeostasis in a rat model of calcium oxalate urolithiasis

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Meimaridou, Eirini, Edgar Lobos, and John S. Hothersall. Renal oxidative vulnerability due to changes in mitochondrial-glutathione and energy homeostasis in a rat model of calcium oxalate urolithiasis. Am J Physiol Renal Physiol 291: F731–F740, 2006. First published May 2, 2006; doi:10.1152/ajprenal.00024.2006.—Calcium oxalate monohydrate (COM) crystals are the commonest component of kidney stones. Oxalate and COM crystals in renal cells are thought to contribute to pathology via prooxidant events. Using an in vivo rat model of crystalluria induced by hyperoxaluria plus hypercalcemia [ethylene glycol (EG) plus 1,25-dihydroxycholecalciferol (DHC)], we measured glutathione and energy homeostasis of kidney mitochondria. Hyperoxaluria or hypercalcemia without crystalluria was also investigated. After 1–3 wk of treatment, kidney cryosections were analyzed by light microscopy. In kidney subcellular fractions, glutathione and antioxidant enzymes were measured. In mitochondria, oxygen consumption and superoxide formation as well as cytochrome c content were measured. EG plus DHC treatment increased formation of renal birefringent crystal. Histology revealed increased renal tubular pathology characterized by obstruction, distension, and interstitial inflammation. Crystalluria at all time points led to oxidative stress manifest as decreased cystosolic and mitochondrial glutathione and increased activity of the antioxidant enzymes glutathione reductase and -peroxidase (mitochondria) and glucose-6-phosphate dehydrogenase (cytosol). These changes were followed by a significant decrease in mitochondrial cytochrome c content at 2–3 wk, suggesting the involvement of apoptosis in the renal pathology. Mitochondrial oxygen consumption was severely impaired in the crystalluria group without increased mitochondrial superoxide formation. Some of these changes were also evident in hyperoxaluria at week 1 but were absent at later times and in all calciuric groups. Our data indicate that impaired electron flow did not cause superoxide formation; however, mitochondrial dysfunction contributes to pathological events when tubular crystal-cell interactions are uncontrolled, as in kidney stones disease.

Crystalluria; hyperoxaluria; mitochondria

KIDNEY STONE FORMATION is a complex process and involves a cascade of events, including crystal nucleation, growth and aggregation, retention within the renal tubules, and migration to the renal papillary surfaces (17). Hyperoxaluria is a major risk factor of human idiopathic calcium oxalate disease and leads to increased calcium oxalate supersaturation and calcium oxalate stone formation (1). Exposure to oxalate has been shown to be toxic to renal epithelial cells, which results in lipid peroxidation mediated by free radicals (42). Oxalate toxicity is mediated, in part, by activation of lipid signaling pathways that produce arachidonic acid, lysophospholipids, and ceramide (40). These lipids, in turn, are able to disrupt mitochondrial function by increasing production of reactive oxygen intermediates (ROI), decreasing mitochondrial membrane potential, and increasing mitochondrial permeability (16).

ROI generated by redox-active and cell-compartmentalized processes are involved in various diseases including cancer (8). ROI mediate damage via modification of lipids, proteins, and DNA, and this can result in cell dysfunction, which then leads to hyperplasia, necrosis, or apoptosis. Mammalian cells rely on a spectrum of ROI-scavenging enzymes to prevent excessive oxidative stress. The balance between the prooxidant generation of ROI and the tissue antioxidant defenses dictates the level of tissue injury. Central to this is the redox balance of glutathione, an intracellular thiol that plays a key role in the detoxification of ROI (28). Glutathione redox equilibrium is maintained by the enzymes glutathione peroxidase (GPx) and glutathione reductase (GR), whereas glucose-6-phosphate dehydrogenase (G6PDH) supplies reducing equivalents in the form of NADPH, a key cofactor in maintaining glutathione in its reduced form.

Oxalate exposure of renal cells results in pathological changes involving reinitiation of DNA synthesis, cell growth, cell injury, and death (16, 21, 29, 39, 40). Furthermore, we have shown that in an in vitro stone model using kidney epithelial cells, exposure to calcium oxalate monohydrate (COM) resulted in an increased oxidative stress manifest as superoxide production (20). In addition, we have identified increased levels of superoxide in response to COM crystals, originating from the mitochondria, which consequently result in depletion of mitochondrial glutathione.

Whether it is free oxalate or COM that is responsible for cytotoxicity is a controversial issue. COM rather than free oxalate has been shown to be responsible for cell toxicity (11), and we have recently shown that mitochondrial superoxide formation, although requiring free oxalate, is potentiated by the presence and binding of crystals (26). Furthermore, mitochondrial dysfunction was recently shown to be a primary event in cultured renal epithelial cells exposed to COM (2). However, it has also been reported that free oxalate is cytotoxic to kidney cell lines (19).

The aim of the present study was to examine whether this oxidative stress, which occurs as a result of the interaction between COM and tubular epithelial cells in culture, also occurs in an animal model of crystalluria arising from hyperoxaluria and hypercalcemia. To achieve this, we have used a rat COM crystalluria model comprising low (0.5%) ethylene glycol (EG) feeding, which raises urinary oxalate (22), and 1,25-

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dihydroxycholecalciferol, the active metabolite of vitamin D₃, to raise calcium levels in tubular fluid (13). To investigate whether the interaction of oxalate crystals with renal epithelial cells resulted in mitochondrial dysfunction leading to apoptotic events, some key mitochondrial parameters were investigated, specifically cytochrome c release from the mitochondrial inner membrane, a necessary first step for initiation of apoptosis. Changes in biochemical parameters (antioxidant status, mitochondrial oxygen consumption, superoxide formation), resulting from COM crystal-tubular epithelial cell interactions, were also assessed.

**METHODS**

**Materials.** 1,25-Dihydroxycholecalciferol (DHC), lucigenin, EG, protease inhibitor cocktail, potassium superoxide, GR, and all substrates and cofactors were purchased from Sigma (Poole, UK). The anti-cytochrome c mouse monoclonal antibody was obtained from R&D Systems (Oxon, UK). The anti-prohibitin mouse monoclonal antibody was from Labvision (Westinghouse). Goat anti-mouse horse-radish peroxidase-labeled antibody was obtained from Vector Laboratories (Burlingame, CA). pH was measured using a Radiometer PHN 84 meter fitted with a WVR Gelpas combination electrode.

**Animals and treatments.** All animal work was carried out according to United Kingdom Home Office-approved guidelines, following University College London animal committee ethical approval. This rat model of calcium oxalate nephro lithiasis employed EG to induce hyperoxaluria and DHC dissolved in propylene glycol to initiate hypercalciuria and is a modification of that used by Halabe et al. (12).

Male Sprague-Dawley rats (250 –280 g) were maintained under constant conditions and were divided into four groups (control, EG, DHC, and EG+DHC). Each group was then subdivided into three groups, each with six animals, with the above treatments for 1, 2, and 3 wk. Control animals received water, whereas in the EG and EG+DHC experimental groups 0.5% EG was administered daily in the drinking water. Fluid intake was monitored in all groups. DHC (50 ng/100 g body wt) was administered intraperitoneally every other day to the EG+DHC (starting 24 h after first exposure to EG) and DHC groups. At various times throughout the treatment (3 h to 21 days), urine was collected onto microscope slides prewarmed to 37°C during animal handling and examined immediately under a light microscope. After 7, 14, and 21 days of treatment, animals were anesthetized with halothane, and urine was taken directly by needle from the bladder. The anesthetized animals were then killed by cervical dislocation, and the kidneys were removed.

**Tissue sampling.** The right kidney was excised and immediately frozen in liquid nitrogen and stored at −80°C until use. The left kidney was washed in saline at 4°C, trimmed of adipose and connective tissue, weighed, and homogenized in a Potter/Elvehjem homogenizer (10% wt/vol) in buffer containing 0.25 M sucrose, 5 mM HEPES, 1 mM EDTA, and 0.1% BSA, pH 7.2, with a protease inhibitor cocktail [1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A, and 14 μM E-64]. The whole homogenate was centrifuged at 1,000 g for 5 min to remove the nuclear fraction and cell debris. The supernatant was then fractionated by centrifugation at 9.5 × 10³ g for 20 min to isolate mitochondria, and a further 30 min at 100 × 10³ g to separate the cytosolic and membrane components. Mitochondria, after one wash in original homogenization media, were resuspended (12–15 mg protein/ml) in the above buffer. Contamination from the cytosol (lactate dehydrogenase measured spectrophotometrically at 340 nm by NADH oxidation), microsomes (glucose-6-phosphatase measured by the blue phosphomolybdic complex at 700 nm), and peroxisomes (urate oxidase measured by oxidation of urate at 292 nm) was all <7.5%.

**Western blot analysis.** Mitochondria were isolated by differential centrifugation as described above. Mitochondrial protein (30 μg) was suspended in Laemmli buffer, heated at 95°C for 5 min, resolved by electrophoresis using 12.5% SDS-polyacrylamide gels, and electro-transferred to nitrocellulose as described (24). Blots were incubated with the anti-cytochrome c antibodies at 4°C overnight in Tris-buffered saline containing 0.1% Tween 20 and 0.5% BSA, followed by horseradish peroxidase-conjugated anti-mouse secondary antibody for 1 h at room temperature. The signal was visualized by autoradiography using ECL Western Detection Reagent (Amersham Pharmacia Biotech UK, Little Chalfont, UK), followed by exposure to X-Omat AR film (Eastman Kodak). Blots were incubated with anti-prohibitin antibodies, a mitochondrial marker, to determine equal loading of samples (34).

**Calcium and crystalluria.** The presence of urinary crystals was assessed by light microscopy on glass slides of freshly passed urine. Formation of stones was determined in cryosections examined by polarized light to determine the appearance and size of birefringent particles. Urine calcium was measured using an ion-selective electrode.

**Kidney histology.** Analysis of renal histopathological changes and the location of the birefringent crystals were performed in 5-μm-thick hematoxylin- and eosin (H&E)-stained sections using plain and polarized light by an experienced pathologist.

**Superoxide: lucigenin-dependent chemiluminescence assay.** Superoxide production was measured by chemiluminescence (CL), using lucigenin (10 μM), at a subrecycling concentration (20). Mitochondrial samples (200 μg protein) were added in 35-mm dishes to 1 ml of respiration buffer (70 mM sucrose, 220 mM mannitol, 1 mM EDTA, 2.5 mM KH₂PO₄, 1 mM MgCl₂, and 2 mM HEPES, pH 7.4). The complex I substrates malate and pyruvate at 5 and 2 mM were added, respectively, and CL was measured in a photon-counting device comprising a gallium arsenate photomultiplier tube (Hamamatsu R943) thermoelectrically cooled to −20°C. CL emission from samples in dishes maintained at 37°C in a thermostatic light sealed chamber was reflected and focused onto the photomultiplier tube. Initial CL was determined (200 measurements at 1-s intervals). Potassium superoxide in crown ether/diethyl sulfoxide was used as a standard for CL calibration.

**Total glutathione measurements.** Whole homogenates of fresh kidney were prepared as described above and immediately processed for mitochondrial and cytoplasmic total glutathione (GSH+GSSG) measurements. Whole homogenate (1 ml) was incubated with 20 μl of digitinon (10 mg/ml) for 20 s with mixing and centrifuged at 14,000 g for 20 s. The resultant cytosolic fraction (supernatant) was added to 50 μl of 5% metaphosphoric acid (MPA). The unsuspended pellet was washed with 200 μl of MOPS/mannitol followed by 5-s centrifugation at 14 × 10³ g. This pellet (mitochondrial fraction) was finally resuspended in 250 μl of MOPS (10 mM)/mannitol (300 mM)/EDTA (1 mM) buffer containing 0.5% MPA. Total glutathione was then measured by the method of Tietze (43). In brief, whole homogenate, cytosolic, and mitochondrial aliquots (20 μl) were added to 180 μl of assay solution (0.1 M KH₂- and K₂H-PO₄, pH 7.2, 240 μM NADPH, 76 μM DTNB) and GR (∼1.0 mg/ml). The rate of DTNB reduction was measured over 5 min at 412 nm in a Wallac Victor plate reader. The rate of reaction using standards of oxidized glutathione in 0.5% MPA was used to calculate total glutathione (reduced plus oxidized) expressed as picomoles per milligram of tissue.

**Antioxidant enzyme activities in the kidney fractions.** The activity of antioxidant enzymes (GPx, GR, and G6PDH) was assayed spectrophotometrically (6). GPx was determined by the oxidation of NADPH to NADP⁺ during a reaction initiated by the addition of cumene hydroperoxide linked to the reduction of oxidized glutathione with excess added GR. GR was determined by directly measuring the rate of NADPH oxidation in the presence of oxidized glutathione. G6PDH activity was determined by measuring the rate of NADP⁺ reduction to NADPH in the presence of glucose-6-phosphate. In these
assays, the change in absorbance at 340 nm was continuously monitored in a Cary 1 spectrophotometer and activity expressed as units per gram tissue. Measurements were run in double-beam mode, samples being present in both beams and the substrate omitted from the rear beam.

Mitochondrial oxygen consumption rates. Oxygen consumption in freshly isolated mitochondria (1–1.5 mg protein/ml) in 1.5 ml of respiration buffer was assayed using a Clark electrode device (Rank Brothers). Malate plus pyruvate at 5 and 2 mM (complex I) or succinate at 5 mM (complex II) was used to measure state IV respiration (ADP limited), and ADP (100 μM) was added to measure state III respiration (no substrate/cofactor limitation).

Urinary oxalate and calcium. The oxalate concentration of urine was measured in supernatants prepared from samples centrifuged at 10,000 g for 1 min by ion chromatography with suppressed conductivity detection on a IonPac AS4A anion chromatographic column using a Dionex high-performance pump. Urinary calcium was measured in the same sample using an ion-selective electrode. Oxalic acid and calcium chloride were used as standards.

Statistics. All values shown are means ± SD from 4–6 animals unless otherwise stated. Student’s t-test was used to determine whether there was a significant difference between two groups (P < 0.05). When multiple means were compared, significance (P < 0.05) was determined by ANOVA, followed by Fisher’s protected least significant difference test.

RESULTS

Early urine crystal formation was checked by light microscopy. Freshly passed urine collected 24 h after the start of EG feeding was clear of crystals, and throughout the study urine remained either free of crystals or exhibited only slight crystal presence. Three hours after administration of DHC in EG-fed animals, crystal formation was observed (data not shown), whereas in animals treated only with DHC, crystal formation was either not detected or slight crystal nucleation was observed. This situation was maintained throughout the 3 wk of treatment.

The concentration of urinary free oxalate measured by conductivity after ion chromatography, ionized calcium concentrations, and urine pH are shown in Table 1. The crystalluria group (EG + DHC) had significantly lower oxalate levels than the EG alone group. This is a result of a proportion of the free oxalate being excreted as crystals, which are removed from the urine before analysis. Kidney crystal and stone formation was analyzed by polarization and light microscopy. The H&E-stained cryosections were analyzed by polarization microscopy (all data are summarized in Table 2). Kidneys from the control group showed no signs of crystal formation and hence no birefringence during the 3-wk treatment period. In contrast, polarization microscopy revealed the presence of birefringent, small crystals in the distal tubules by week 1 of EG + DHC treatment, with increased size, brightness, and birefringence of the crystals at weeks 2 and 3 of treatment. In the kidneys from animals treated with either EG and DHC alone, no crystals were observed at week 1, whereas at weeks 2 and 3 in some sections small crystals were observed, but these were more isolated than with 1-wk EG + DHC (images not shown).

To examine renal histopathology, H&E-stained sections of kidney were examined under light microscopy. Compared with control kidneys, where tubules were compact and at a higher density (Fig. 1A), histological examination of the EG + DHC group even after 1 wk of treatment revealed major changes. Crystals were observed, both in the lumen and within the tubular cells (Fig. 1B, yellow arrows) as were distended distal tubules (Fig. 1B, black arrows). Renal crystal formation was increased 2 wk after EG + DHC treatment (Fig. 1C, yellow arrows), as was tubular distension (Fig. 1C, black arrows). We also observed the establishment of interstitial inflammation with focal infiltrates of inflammatory cells consisting largely of polymorphonuclear leukocytes and lymphocytes in H&E staining at 2 wk (Fig. 1D, black arrows). These changes were even more prominent by week 3 of treatment, with the kidney containing larger crystals (Fig. 1E, yellow arrows) and widely distended tubules (Fig. 1E, black arrows). Furthermore, by week 3 the EG + DHC-treated animals showed a loss of renal epithelial cells (Fig. 1F, black arrows). In addition, signs of interstitial fibrosis were demonstrated by the increased presence of collagen and elastic fibers, as confirmed with van Giesson’s staining (not shown). Histology of kidney tissue of the EG- or DHC-treated groups showed an absence of crystals and no morphological differences compared with controls (Fig. 1, G and H, respectively).

Animal body weight did not change between treatment time and regime (data not shown). The kidney weight (g) of crystalluric animals compared with controls was significantly increased by the end of week 1: control 1.05 ± 0.14, EG 0.94 ± 0.11, EG + DHC 1.35 ± 0.22 (P < 0.05), and DHC 1.07 ± 0.11. Neither hyperoxaluric nor hypercalciuric animals’ kidney weights were significantly changed. By week 3, significant hypertrophy was also observed only in the crystalluria group: control 1.09 ± 0.08, EG 1.20 ± 0.14, EG + DHC 1.65 ± 0.29 (P < 0.01), and DHC 1.18 ± 0.13. This hypertrophy in the

Table 1. Urinary oxalate and calcium concentrations

<table>
<thead>
<tr>
<th>Urinary Oxalate, mM</th>
<th>Urinary Ionized Calcium, μM</th>
<th>Urinary pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 1</td>
<td>Week 2</td>
</tr>
<tr>
<td>Control</td>
<td>0.91 ± 0.11</td>
<td>1.12 ± 0.16</td>
</tr>
<tr>
<td>EG</td>
<td>6.3 ± 0.75</td>
<td>7.03 ± 0.66</td>
</tr>
<tr>
<td>DHC</td>
<td>1.1 ± 0.19</td>
<td>0.89 ± 0.14</td>
</tr>
<tr>
<td>EG + DHC</td>
<td>4.2 ± 0.60</td>
<td>4.6 ± 0.78</td>
</tr>
</tbody>
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Values are means ± SD. EG, ethylene glycol; DHC, 1,25-dihydroxycholecalciferol.

Table 2. Renal crystal occurrence

<table>
<thead>
<tr>
<th>Week</th>
<th>Control</th>
<th>0.5% EG</th>
<th>DHC</th>
<th>DHC + EG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>++</td>
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<tr>
<td>2</td>
<td>–</td>
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–, Absent; +, small and infrequent crystals; ++, medium-sized and moderate crystal occurrence; ++++, large and frequent crystal occurrence.
crystalluria group was not accompanied by increases in kidney tissue, as total kidney protein was unchanged (data not shown). Fluid intake was significantly increased by 70% in the EG/DHC-treated animals but not in the groups treated with EG and DHC alone: control 32.8 ± 8, EG 40 ± 9, EG/DHC 56.0 ± 13 (P < 0.05), and DHC 39 ± 7 (ml·rat⁻¹·day⁻¹). These changes in the crystalluria group were accompanied by an observed distension of renal tubules and urolithiasis of the bladder, resulting in urine retention.

Based on densitometric measurements, cytochrome c protein abundance in the mitochondria was significantly decreased by >25% in the EG+DHC-treated group at weeks 2 and 3 compared with control animals (P < 0.05, Fig. 2). In contrast, cytochrome c content during the first week of EG+DHC-treatment was unchanged from that of control animals (Fig. 2). Incubation with the secondary antibody or with control rabbit IgG resulted in no detectable staining of any 13-kDa band (data not shown), demonstrating complete specificity. Thus kidney stone formation in this in vivo model was linked to a significant decrease in renal mitochondrial cytochrome c content.

Total glutathione from kidney whole homogenate (Fig. 3A) and cytosolic fractions (Fig. 3B) is shown. Throughout the 3-wk course of treatment in the EG+DHC group, there was a significant decrease. There was also a significant decrease in whole homogenate and cytosolic glutathione at week 1 in the EG group; however, no significant change in mitochondrial glutathione was observed, and total glutathione was restored to control levels in subsequent weeks. The differences in whole

Fig. 1. Photomicrographs of control, hyperoxaluric, calciuric, and crystalluric hematoxylin- and eosin (H&E)-stained kidney sections. Kidneys from control (A), ethylene glycol (EG) + 1,25-dihydroxycholecalciferol (DHC)-treated rats at 1 (B), 2 (C and D), and 3 wk (E–H) at both low and high magnification are shown in representative photomicrographs demonstrating crystals (yellow arrows), distended tubules (black arrows; B, C, and E), and influx of inflammatory cells (black arrows; D). Kidney morphology of the 3-wk hyperoxaluric (G) and calciuric (H) animals is comparable with controls. Scale bars = 100 μm.
cell and cytosolic glutathione between the EG and EG+DHC groups after 1 wk of treatment are not significant. Total mitochondrial glutathione levels, during the 3-wk treatment, were monitored to investigate changes occurring in crystalluric kidneys during stone formation (Fig. 3C). There was a marked and significant decrease in total mitochondrial glutathione by week 1 in the EG+DHC group, which was exacerbated at weeks 2 and 3.

We further examined the enzymatic activities of GPx, GR, as well as the redox supply (pentose phosphate pathway) enzyme G6PDH. Both of the glutathione cycle enzymes were significantly increased in the mitochondrial fraction from the EG+DHC group after week 1 of treatment (Fig. 4, A and B). However, in subsequent weeks GPx and GR activities were restored to control levels, and the GPx change at 1 wk appears not to be related to crystalluria as it is also present in the calcucria group. The activity of both of these enzymes was unchanged in the cytosolic fraction (data not shown). G6PDH was markedly increased in the EG+DHC group throughout the whole 3-wk treatment (Fig. 4C).

Mitochondrial oxygen consumption was measured to investigate whether the electron transport chain was a potential source of the observed oxidative stress. Oxygen consumption in kidney mitochondria of the hyperoxaluria (EG treatment) or hypercalciumuria (DHC treatment), in the presence of malate and pyruvate (complex I substrates), was not significantly changed compared with control. However, oxygen consumption in the crystalluria (EG+DHC group) was severely compromised. Representative traces of oxygen consumption during state IV (substrate only) and state III (added ADP) are shown (Fig. 5A). The oxygen consumption rate during state III respiration was decreased by 60% at week 1 and by 80% after weeks 2 and 3 (Fig. 5B). Using succinate, a complex II substrate, a smaller diminution (50%) of the state III oxygen consumption rate was observed (data not shown). The observed differences in the EG+DHC group were significant (P < 0.005) and more apparent during state III rather than state IV respiration, and more pronounced with complex I rather than complex II substrates.

Mitochondrial superoxide production measured as lucigenin CL was markedly (>90%) decreased throughout the whole course of treatment in the EG+DHC group (Fig. 5C). At week 1 both the hyperoxaluria and the hypercalciumuria groups also showed decreased lucigenin CL; however, this was much lower (<40%) than in the crystalluria animals and was reversed by weeks 2 and 3 of treatment.

DISCUSSION

Previous studies have shown that 0.5% EG causes hyperoxaluria (22), which was confirmed in our studies, and that at the dose of DHC we employed there is a greater than fivefold increase in urinary calcium (hypercalciumuria) (7, 13). In addition, the serum calcium increase with DHC is independent of oral or intraperitoneal delivery routes (9). In this model of hyperoxaluria (EG treatment), hypercalciumuria (DHC treatment), and crystalluria (EG+DHC, hyperoxaluria plus hypercalciumuria), calcium oxalate kidney stone formation occurred after 1 wk and only in the crystalluria group. Crystal formation became more pronounced in subsequent weeks. Neither the control, EG-, nor DHC alone-treated groups exhibited renal pathology comparable to the EG+DHC-treated animals, whose kidneys showed signs of hypertrophy, renal tubular damage consisting of tubular obstruction due to crystal formation, dilatation, atrophy, interstitial inflammation, and loss of tubular epithelial cells. The observed hypertrophy of the crystalluria animals could be a result of either fluid retention or crystal accumulation, as there was no change in kidney protein content. This is despite an increase in renal workload demonstrated by the increased fluid intake and distented bladders.

The appearance of calcium oxalate crystals in the kidney by the end of week 1 is similar to other reported studies of rat nephrolithiasis, where higher EG treatments (0.75%) were used without simultaneously raising the urinary calcium (without vitamin D or DHC). Under these circumstances, intrarenal crystal appearance was apparent within 1 wk (15). In the present study, calcium oxalate crystals began to aggregate in the tubular lumen, causing widening of the interstitial space.
However, histological studies of kidneys from animals fed with only 0.5% EG did not show any morphological differences. Our findings also differ from those of de Water et al. (5), who showed, using vitamin D3 and 0.5% EG, that some of the rats showed calcium oxalate deposits in the renal cortex or medulla after 4 wk. When 0.75% EG was used, the amount of kidney-associated crystals was proportionally higher. This disparity is most probably a result of differences in the potential of DHC, the active metabolite of vitamin D3, to raise urinary calcium. In addition, Lee et al. (22) have shown the presence of kidney stones in animals fed 0.5% EG alone; however, this study was conducted over a longer single time period of 4 wk. This demonstrates the ability to achieve crystalluria and kidney stone formation at lower doses of EG.

We demonstrate here using a specific glutathione assay that mitochondria prepared by rapid digitonin permeabilization and centrifugation show marked decreases during crystalluria. Changes in total glutathione levels in both mitochondria and the cytosol indicated considerable oxidative stress imposed on the kidneys of animals exhibiting crystalluria, which was absent from the other treatment groups. This indicates the importance of crystalline oxalate rather than the free ion in the pathological process. Muthukumar and Selvam (33) attributed a loss of mitochondrial integrity to similar findings with total thiols measured using DTNB reduction, extrapolated to express glutathione. The decrease apparent at week 1 in the EG-treated group may have resulted either from the initial hyperoxaluric stress or from circulating levels of EG and/or its metabolic products (including oxalate). The reversal of this phenomenon at weeks 2 and 3 indicated that this is a transient event and is certainly not related to crystalluria, which was absent from this group.

Further substantiation of oxidative stress in crystalluric animals is provided by the changes in glutathione cycle enzymes, which are upregulated in the mitochondrial compartment, and the increased level of cytosolic G6PDH. The latter is important in supplying redox equivalents, in the form of NADPH, for thiol maintenance and is a marker routinely observed to be induced under oxidative stress conditions (36, 38). Most of the previous literature usually describes this enzyme as unchanged.

![Fig. 3. Total glutathione concentration in kidney subcellular fractions at 1, 2, and 3 wk of hyperoxaluria, calciuria, and crystalluria. The whole homogenate (A) was used, after digitonin lysis and rapid separation by centrifugation, for cytosolic (B) and mitochondrial (C) preparation. Total glutathione was measured as described. Values are means ± SD expressed as pmol oxidized glutathione/mg kidney wt. Open bars, week 1; filled bars, week 2; and shaded bars, week 3. *, †, ‡: P < 0.01 vs. control for week 1, 2, and 3, respectively.](http://ajprenal.physiology.org/)

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or decreased. However, this is the first time this enzyme has been investigated in a model employing DHC. Vitamin D3 and its analogs have been shown to both upregulate and activate (via a calcium-dependent mechanism) G6PDH, although at the doses used here no changes were observed in animals treated with DHC alone. It is plausible that with the combination of DHC plus oxidative stress (kidney crystals), some increase in the activity of G6PDH becomes evident.

The formation of crystalluria and kidney stones in this animal model correlated with a significant decrease in cytochrome c content in the mitochondria of EG+DHC-treated animals at weeks 2 and 3. This suggests that it is released into the cytosol, an event known to precede both apoptosis and necrosis and requires both a decrease in the association of cytochrome c with cardiolipin together with the permeabilization of the outer mitochondrial membrane with Bax (35). Our result is in agreement with recently published data demonstrating apoptosis and the induction of apoptosis-related genes including Bax in renal epithelial cells of stone-forming rats (31). Many death signals including oxidative stress cause irreversible dysfunction of mitochondria, leading to the release of several mitochondrial intermembrane space proteins such as cytochrome c into the cytosol, where it initiates caspase activation and apoptosis (37). This also conforms to our observation of decreased cellular glutathione concentrations in crystalluric animals, indicative of oxidative stress. In particular, decreased mitochondrial glutathione, which apart from its role in the regulation of mitochondrial function (27), is also associated with a decreased tolerance of these organs to damaging insults (4) and has recently been shown to be critical to cell survival during hypoxia (23). Clearly, severe mitochondrial disruption in the form of diminished oxygen consumption is apparent in this model. In addition, oxidant stress, which results in an increased oxidized/reduced glutathione ratio and is accompanied by protein sulfhydryl oxidation, promotes further cytochrome c-linked events, such as the opening of the permeability transition pore (32) and impairment of the mitochondrial-nuclear signaling pathway (10). Feeding with EG alone at the doses used in this study did not result in loss of cytochrome c, and hence, by inference, did not lead to apoptosis. Further studies will be needed to ascertain the location and induction of apoptosis markers in this animal model.

We had previously observed that mitochondrial-derived superoxide was the source of oxidative stress in renal epithelial cells treated with COM in vitro (20). Mitochondrial oxygen consumption and superoxide formation were investigated in this model to determine whether the electron transport chain was compromised by the observed loss of cytochrome c and whether mitochondria were also the source of oxidative stress in vivo. Oxygen consumption in kidney mitochondria of EG or DHC groups in the presence of malate and pyruvate was not significantly changed compared with control, indicating that hyperoxaluria or hypercalciuria alone was ineffective in changing mitochondrial function, which was in accordance with the cytochrome c data. By contrast, in the nephrolithic kidney (EG+DHC group), the rate at which oxygen was consumed was severely decreased. A restriction in electron flow, which was apparent mainly during state III respiration with complex I substrates, is normally associated with an increased incidence of electron leakage, i.e., superoxide formation. This is a consequence of the decreased flow of electrons and results from an increased concentration of ubiquinol of complex III during the Q cycle (30, 44). However, and in contrast to COM treatment in Madin-Darby canine kidney cells (20), this did not appear to be the case in this model, as superoxide formation was decreased to a greater extent than oxygen consumption. This indicates that the redox state of ubiquinone, i.e., ratios of oxidized ubiquinone to ubiquinol (Q-QH), remained oxidized due to impaired mitochondrial reductive capacity (complex I and/or II). The minimal rate of superoxide production, in contrast to the cell model, may be explained by the loss of intramembrane cytochrome c (absolute requirement for energy transduction), being of sufficient severity to restrict electron transfer and hence leakage. Contrary to this, at week 1 in the crystalluria group, when cytochrome c is unchanged from the control level, the electron flow has already significantly decreased. Similar changes in state III respiration have been observed in vitro in isolated rat kidney mitochondria (25). COM produced a dose-dependent cyclosporine A-independent decrease in state III respiration as well as a cyclo...
soporine-dependent increase in mitochondrial swelling. However, potassium oxalate did not mimic COM, suggesting that it is not the free oxalate ion that leads to cell damage. This is in contrast to other studies that have shown that free oxalate is potentially injurious to cells (18).

There is also the possibility that with in vivo crystalluria, the changes in renal mitochondria are of such severity that the accumulated loss of electron transfer intermediates compromises oxygen consumption and electron leakage. This accumulation is the result of synergy between diminished cofactor supply via substrate reductases (33), lowered cytochrome c levels, and upregulation of mitochondrial uncoupling protein-3 (3).

Other sources of ROI production could be responsible for the observed oxidative stress. These include NAD(P)H oxidase associated with the plasma membrane and lipoxygenase (45). Although we found no evidence for these in COM-treated Madin-Darby canine kidney cells (20), they cannot be discounted in this model.

We believe it is unlikely that physical obstruction of tubules is the cause of the changes we observed, as at week 1 when only limited pathological changes have occurred and crystal deposition is light, changes to some mitochondrial and oxidative markers are already apparent.

In summary, in this in vivo hyperoxaluria and crystalluria model of kidney stones, we observed that the nephrolithic kidney underwent oxidative stress. Specifically, in EG+DHC-treated animals, this oxidative insult was manifest by a decrease in mitochondrial total GSH concentration, as well as increased activity of G6PDH, both important in maintaining cell redox. Severe kidney damage at the mitochondrial level was a key observation, indicated by the diminished oxygen consumption and decrease in mitochondrial cytochrome c. The above findings, observed only in animals with crystalluria, suggest that damage-related events result directly from the occurrence of COM crystals in kidney tubules. Our observations could have clinical implications in treatment options using whole cell and mitochondrial antioxidant therapies, especially in view of the recent finding that total antioxidant status is frequently deficient in stone-forming individuals (41). This could be of particular importance in conditions mimicked by this model and of particular significance to primary and enteric hyperoxaluria and the acute situations in idiopathic stone formers.

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