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Oxalate toxicity in LLC-PK$_1$ cells: Role of free radicals

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Oxalate toxicity in LLC-PK$_1$ cells: Role of free radicals. Oxalate, the most common constituent of kidney stones, is an end product of metabolism that is excreted by the kidney. During excretion, oxalate is transported by a variety of transport systems and accumulates in renal tubular cells. This process has been considered benign; however, recent studies on LLC-PK$_1$ cells suggested that high concentrations of oxalate are toxic, inducing morphological alterations, increases in membrane permeability to vital dyes and loss of cells from the monolayer cultures. The present studies examined the basis for oxalate toxicity, focusing on the possibility that oxalate exposure might increase the production/availability of free radicals in LLC-PK$_1$ cells. Free radical production was monitored in two ways, by monitoring the reduction of nitroblue tetrazolium to a blue reaction product and by following the conversion of dihydrorhodamine 123 (DHR) to its fluorescent derivative, rhodamine 123. Such studies demonstrated that oxalate induces a concentration-dependent increase in dye conversion by a process that is sensitive to free radical scavengers. Specifically, addition of catalase or superoxide dismutase blocked the oxalate-induced changes in dye fluorescence/absorbance. Addition of these free radical scavengers also prevented the oxalate-induced loss of membrane integrity in LLC-PK$_1$ cells. Thus it seems likely that free radicals are responsible for oxalate toxicity. The levels of oxalate that induced toxicity in LLC-PK$_1$ cells (350 μM) was only slightly higher than would be expected to occur in the renal cortex. These considerations suggest that hyperoxaluria may contribute to the progression of renal injury in several forms of renal disease.

Oxalate, the most common constituent of kidney stones, is a simple dicarboxylic acid that is produced as a byproduct of a number of metabolic pathways [1]. This byproduct cannot be processed further in man and is excreted by the kidney. Studies of renal oxalate handling have shown that oxalate is freely filtered at the glomerulus and undergoes both reabsorption and secretion in the proximal tubules [2], with secretion predominating under normal physiological conditions [3, 4]. Removal of oxalate in the kidney is facilitated by a variety of transport systems at the apical and basolateral surfaces of both proximal [5–7] and distal [8] tubular cells; in the course of vectorial transport, oxalate accumulates intracellularly [9]. This accumulation has been considered benign, despite the fact that oxalate can alter the activity of a number of enzymes (lactate dehydrogenase, malate dehydrogenase, pyruvate kinase, etc., see [10]). Indeed, the only pathologies that have been attributed to oxalate (oxalosis, hyperoxaluria, calcium oxalate stone disease) stem from the tendency of this molecule to form calcium oxalate crystals [11].

Recent studies in our laboratory have suggested that oxalate may induce additional effects on renal cellular function, however. Studies using using LLC-PK$_1$ cells, a line of renal epithelial cells with characteristics of proximal tubular cells [12–14], revealed that oxalate exerts biphasic, concentration-dependent effects on the viability and growth of renal epithelial cells, acting as a mitogen at low concentrations of oxalate [15] and acting as a toxin at high concentrations [16]. These findings are reminiscent of those seen in response to oxidant stress in other systems [17, 18]. Thus the present studies examined the possibility that oxalate may induce a form of oxidant stress in renal epithelial cells. These studies demonstrated that oxalate exposure increases the production/availability of free radicals in LLC-PK$_1$ cells and suggested that this increase in free radical production is responsible for oxalate toxicity. The results from these studies suggest possible explanations as to how hyperoxaluria may promote stone disease. They also suggest a role for oxalate in other forms of renal disease in which serum oxalate levels are elevated. Preliminary reports of these findings have been presented [19, 20].

Methods

Cell cultures

LLC-PK$_1$ cells (CRL 1392, American Type Culture Collection) were used between passages 205 and 240. Aliquots of 1.5 million cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, glucose and antibiotics at 37°C, under 5% CO_2. Cells were used three to four days after plating.

DMEM, penicillin and streptomycin were obtained from GIBCO (Grand Island, NY, USA), fetal bovine serum from Hyclone Laboratories Inc. (Logan, UT, USA). Formic acid, oxalic acid, malonic acid, succinic acid, citric acid and urate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nitroblue tetrazolium, catalase (from bovine liver, 3100 U/mg), superoxide dismutase (from bovine kidney, 4950 U/mg), and hydrogen peroxide (30%) were also purchased from Sigma. Dihydrorhodamine 123 (DHR 123), ethidium homodimer-1 and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR, USA). Stock solutions of DHR 123 (10 mM) were prepared in dry dimethyl formamide and stored in air tight vials at −20°C. Stock solutions of ethidium homodimer-1 and Hoechst 33342 were made up as required in incubation buffer. All chemicals were of the highest grade available. Experiments were conducted in

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either DMEM (buffered with either bicarbonate or HEPES) supplemented with 0.25% serum or mammalian Kreb's Ringer (MKR) buffer containing: NaCl 122 mM, KCl 3 mM, $MgCl_2$ 2.25 mM, HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) 24 mM, $NaHCO_3$ 15 mM, $CaCl_2$, 1.8 mM, dextrose 10 mM, pH 7.2. Where indicated, sodium oxalate was added at a concentration of 0.1, 0.2, 0.4, 1.0, 2.0 or 4.0 mM (total), which increased the free oxalate levels to 0.03, 0.07, 0.14, 0.35, 0.75 or 1.6 mM, and increased the relative supersaturation level (RSS) for calcium oxalate to 2.6, 5.2, 10.3, 24.9, 47.2 or 84.3, respectively. Estimates of free oxalate and RSS were obtained using the EQUIL program [21]. Note, however, that these estimates of free oxalate and RSS do not include possible effects of amino acids in DMEM since the program does not take these molecules into account.

Monitoring free radical production

Spectrophotometry. To obtain evidence that oxalate increases the production of free radicals in renal epithelial cells, we monitored oxalate-induced changes in the reduction of nitroblue tetrazolium (NBT) as described previously [22]. For these studies confluent cultures of LLC-PK1 cells in 6 well plates were exposed for varying periods (0 to 120 min) to DMEM containing 25 μg/ml NBT, a dye that reacts with superoxide [23, 24]. Where indicated oxalate (0 to 4 mM total, 0 to 1.6 mM free), superoxide dismutase (500 U/ml) and/or catalase (300 U/ml) were also added. At predetermined intervals (60, 90, 120 or 240 min) the medium was removed and the reaction was halted by the addition of 70% methanol. The monolayers were washed with four changes of 100% methanol to remove nonreduced NBT, air dried and solubilized with a mixture of 2 M potassium hydroxide and dimethyl sulfoxide (1:1.167). Samples were then centrifuged four minutes at 15,000 rpm in a Beckman microfuge and read at 700 nm (density OD_{700}) using a Beckman DU-7 spectrophotometer against a blank cuvette containing KOH and DMSO. Optical densities in treated samples were then normalized to those in untreated samples in which the optical densitites averaged 0.025 ± 0.005 (means ± SEM from 20 separate monolayers from 3 separate platings).

Spectrofluorimetry. In other experiments, free radical production was monitored on-line using dihyrorhodamine 123, a nonfluorescent compound that is converted to a fluorescent product, rhodamine 123, by reactive oxygen molecules [25–27]. For such studies confluent cultures of LLC-PK_1 cells (on rectangular 12 × 25 mm coverslips) were removed from their culture medium, washed once with MKR and transferred to a standard quartz cuvette containing MKR with 1 to 10 μM DHR. (Preliminary studies indicated the importance of short loading times with low dye concentrations since responses are damped when cells accumulate excess dye). After two minutes exposure to dye-containing solution, the coverslip was removed, the cuvette was rinsed 3 × with MKR and refilled with MKR or DMEM (results were the same in both buffers; thus results were pooled below). Where indicated, superoxide dismutase, catalase or citrate were added along with oxalate. The coverslip was then placed diagonally across the cuvette, and the fluorescent signal at 540 nm was detected at right angles to the excitation source (λ = 510 nm). Baseline fluorescence was recorded for a minimum of 10 minutes, then the effects of various additives (30 μl additions) were monitored for an additional 10 minutes. Further additions were made at 10 minutes intervals. The final addition was always 30 μl of 0.8 M hydrogen peroxide (a positive control to demonstrate that cells retained sufficient dye to provide a robust response). All experiments were conducted at 22 ± 1°C, fluorescence intensity was recorded each second and the rate of change in fluorescence was analyzed by linear regression analysis of the last five minutes of the 10 minutes interval. Each coverslip culture was received a single treatment; data for each experimental condition reflect means from nine or more monolayers. Because there was considerable variation among experiments even under basal conditions, each coverslip served as its own control, and comparisons employed paired *t*-tests with a confidence level of 0.025.

Separate studies demonstrated that the rate of dye cleavage was stable for at least an hour under each of these conditions; thus dye loss appeared to be minimal. Other studies demonstrated that the various additives tested (oxalate, catalase, superoxide dismutase, etc.) exerted no direct effects on dye fluoresecence in the absence of cells.

Cell viability assay

Ethidium homodimer-1. To determine whether or not free radicals were responsible for oxalate toxicity, LLC-PK_1 cells were exposed to oxalate for one to four hours in the presence or absence of extracellular free radical scavengers and stained with the vital dye ethidium homodimer-1, a fluorescent vital dye which is excluded from intact cells [28]. For such studies subconfluent cultures in 12 well plates were treated for four hours at 37°C in DMEM with varying concentrations of oxalate (0 to 1 mM total, 0 to 350 μM free). Where indicated, catalase (300 to 500 U/ml, an enzyme that degrades peroxide to H_2O), superoxide dismutase (500 U/ml, an agent that converts superoxide to peroxide and O_2), citrate (1 mM, a naturally occurring compound that limits kidney stone formation *in vivo*), desferoxamine mesylate (50 μM to 20 mM, an iron chelator that blocks iron-dependent free radical generation), or mannitol (10 or 60 mM, a hydroxyl radical scavenger) were added along with 0.35 mM free oxalate. After four hours the solution was decanted and each well was stained for 30 minutes at 37°C with 50 μl of a mixture containing 4 μM ethidium homodimer-1 (which stains dead cells) and 15 nM Hoechst 33342 (which stains all cells). A minimum of six wells with two or three randomly selected fields in each well was then examined using a fluorescence microscope (Nikon). Each field of cells was photographed twice, once with a filter set for ethidium homodimer-1 (excitation λ 525 nm, emission λ 600 nm) and once with a filter set for Hoechst 33342 (excitation λ 340 nm, emission λ 480 nm). The number of dead cells per field was counted and normalized to the control (which averaged 1 to 5 dead cells/field, <1% of the total population). The statistical significance of these data were ascertained using simple paired *t*-tests where appropriate or multiple *t*-tests with a Bonferroni correction.

Results

Two different methods were used to monitor free radical production in LLC-PK_1 cells. One assay utilized nitroblue tetrazolium (NBT), a dye that is converted to a blue reaction in the presence of superoxide [22–24]. The other assay utilized dihydrohodamine 123 (DHR), a nonfluorescent dye that is converted to a fluorescent derivative (rhodamine 123, R 123) by free radicals [25–27].

Studies using NBT indicated that oxalate exposure produces a time and concentration-dependent increase in the accumulation

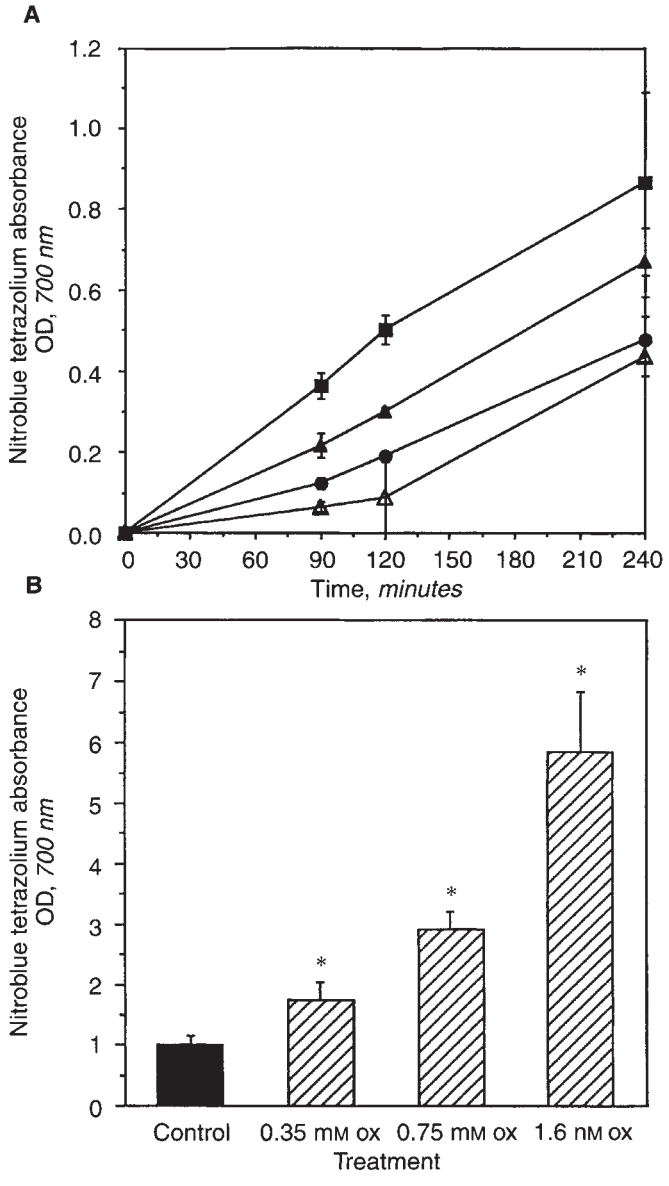

Fig. 1. *Effect of oxalate on free radical production - NBT assay.* **A.** Time dependence. In this representative experiment confluent monolayers of LLC-PK_1 cells were exposed to DMEM containing 25 μg/ml NBT plus varying amounts of oxalate (0 to 4 mM total, 0 to 1.6 mM free). At the times indicated (60, 90 120 or 240 min) the medium was removed and samples were processed as described in the text. The amount of blue reaction product was quantitated in a spectrophotometer by measuring the absorbance at 700 nm (OD_{700}). Data are means ± SD for 6 wells. **B.** Concentration dependence. Confluent monolayers of LLC-PK_1 cells were exposed to DMEM containing 25 μg/ml NBT plus varying amounts of oxalate (0 to 1.6 mM free) for 90 minutes and the extent of NBT reduction was assessed. Data reflect means ± SE from 28 to 44 determinations made on cells from 7 separate platings; analysis employed multiple *t*-tests with a Bonferroni correction to determine significance; hence $^*P < 0.00167$. Symbols are: (△) control; (●) 0.35 mM Ox; (▲) 0.75 mM Ox; (■) 1.6 mM.

of reduced dye (Fig. 1), with significant increases seen after 90 minutes exposure to free oxalate concentrations ≥ 0.35 mM (Fig. 1B, lower concentrations not tested in this assay) [28]. Extracellular addition of superoxide dismutase (SOD, 500 U/ml) signifi-

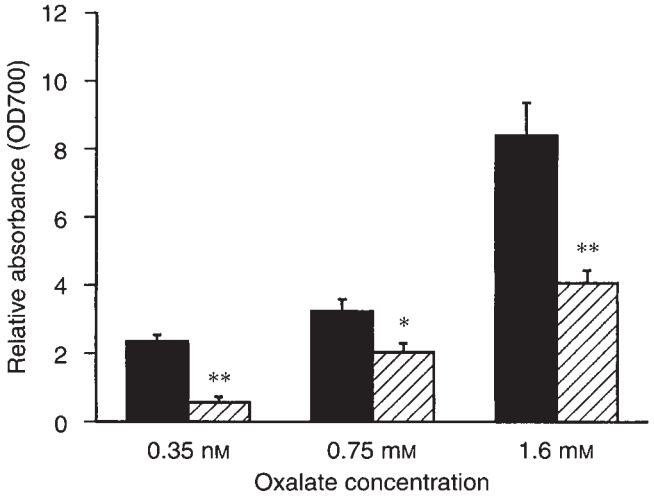

Fig. 2. *Effect of extracellular free radical scavengers on oxalate-induced changes in NBT reduction.* Experiments were performed as in Figure 1B except that half of the samples received 500 U superoxide dismutase (SOD) along with the oxalate. Symbols are: (■) Ox; (▨) Ox + SOD. SOD significantly reduced the effects of oxalate at all of the concentrations tested (data are means ± SE from 9 to 20 determinations made on cells from 3 separate platings; $^*P < 0.05$, $^{**}P < 0.01$ using a Student's *t*-test;).

cantly attenuated the oxalate-induced changes in NBT reduction (Fig. 2), suggesting that this effect of oxalate was mediated by superoxide. Extracellular addition of catalase had no effect on NBT reduction (data not shown).

Additional evidence for oxalate-induced changes in free radical production were provided by studies using DHR 123 in which free radical production was monitored "on-line" as an increase in DHR fluorescence. These studies also demonstrated that oxalate produces concentration-dependent increases in free radical production (Fig. 3a). Significant increases in the rate of dye cleavage were observed at oxalate concentrations ≥ 140 μM free oxalate ($P < 0.025$), with 350 μM free oxalate increasing the rate eightfold. The concentration dependent changes in free radical production in oxalate-treated cells were mirrored by concentration-dependent increases in membrane damage as evidenced by the increased number of cells that took up vital dye (Fig. 3B). Significant increases in the number of dead (permeant) cells were observed at free oxalate concentrations ≥ 350 μM free oxalate.

To examine the apparent link between oxalate-induced increases in free radicals and cell injury, we assessed the effects of various free radical scavengers on free radical production (monitored as an increase in DHR fluorescence) and cell death (detected as an increase in staining by vital dyes). Simultaneous addition of catalase, an enzyme that degrades peroxide, or superoxide dismutase, an enzyme that converts superoxide to peroxide, significantly reduced the effect of oxalate on DHR fluorescence and on cell death (Table 1). Moreover, pretreatment with desferoxamine, an iron chelator, or mannitol, a hydroxyl radical scavenger, significantly reduced oxalate toxicity (Table 1). Treatment with citrate, a metabolite that affords protection from stone formation *in vivo* [29] produced modest effects on free radical production (28% reduction) due perhaps to its ability to inhibit dye peroxidation [30] but afforded no protection from oxalate-inducd changes in membrane integrity (Table 1).

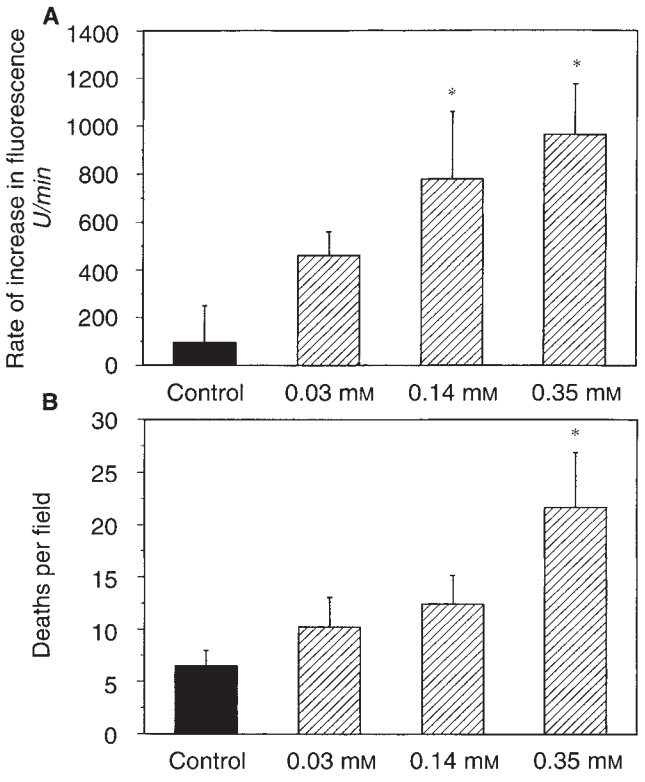

Fig. 3. *Parallel effects of oxalate on free radical production and cell death in LLC-PK$_1$ cells.* **A.** Oxalate-induced changes in free radical production were assessed by monitoring the cleavage of dihydrorhodamine 123 (DHR) to rhodamine 123. Confluent monolayers of LLC-PK$_1$ cells were loaded with DHR 123 for two minutes; the basal rate of DHR cleavage was determined and then varying concentrations of oxalate (30, 140 or 350 μM free oxalate) were added. The rate of change in DHR fluorescence (the slope of the fluorescence trace) was taken as an estimate of the rate of free radical production. Oxalate (▨) produced a concentration-dependent increase in DHR cleavage, with significant increases seen at concentrations $\geq$ 140 μM. Data are means ± SEM from 6 to 10 monolayers; statistical significance was assessed using paired *t*-tests. **B.** Oxalate-induced changes in cell death. LLC-PK$_1$ cells were exposed to varying concentrations of oxalate (30, 140 or 350 μM oxalate) for four hours and then stained with a combination of vital dyes (Hoechst 33342 which is taken up by all cells and ethidium-homodimer 1 which is taken up only by dead cells). Oxalate exposure produced a concentration dependent increase in the average number of dead (ethidium-stained) cells, with significant increases seen at concentrations $\geq$ 0.35 mM. Data are means ± SE from 9 to 25 separate monolayers; analysis employed multiple *t*-tests with a Bonferroni correction to determine significance; hence $^*P < 0.0125$.

The observed increase in free radical production and in cell death appeared to be relatively selective for oxalate. Other mono- and dicarboxylates produced much more modest increases in free radical production (monitored using DHR cleavage fluorescence; Fig. 4A) and minimal effects on cell viability (Fig. 4B). The effects of 1 mM malonate or 1 mM succinate on free radical production were approximately half that seen in response to equimolar addition of oxalate, comparable to those produced by 0.1 mM total oxalate (Fig. 3A). These increases in free radical production, like those produced by 0.1 mM oxalate (Fig. 3B) failed to increase cell death. Only formate, which shares the oxalate transporter in the kidney [6, 8], produced a significant increase in both free radical production and cell death in LLC-PK$_1$ monolayers; and the responses to formate (both with respect to free radical production and cell death) were less than half produced by oxalate. Exposure to citrate had no effect on free radical production (Fig. 4A) or on cell viability (data not shown). Exposure to urate (0.8 to 2 mM), another crystal-forming molecule, produced produced no changes in cellular viability. Effects of urate on DHR cleavage have not been assessed.

Table 1. Effect of free radical scavengers on oxalate-induced changes in cell death and in DHR fluorescence

Treatment	Deaths/field[a]	% Oxalate DHR response[b]
Oxalate	14.6 ± 1.93 (N = 46)	100 (N = 11)
Oxalate + catalase	6.75 ± 1.39 (N = 34)[c]	51.4 ± 19.5 (N = 19)[c]
Oxalate + SOD	5.75 ± 0.92 (N = 18)[c]	43.3 ± 16.6 (N = 14)[c]
Oxalate + DFO	9.53 ± 1.47 (N = 21)[c]	ND
Oxalate + mannitol	2.99 ± 0.98 (N = 9)[c]	63.8 ± 7.0 (N = 3)[c]
Oxalate + citrate	10.45 ± 2.06 (N = 18)	64.2 ± 9.0 (N = 6)[c]

ND is not determined.

[a] Each culture was exposed to 350 μM free oxalate (1 mM total) ± additive for 4 hr and then stained with vital dyes. Stained cells were counted in 2 or 3 random fields from each culture and the average was corrected for spontaneous cell death (that is, that which occurred in untreated control cultures). Where indicated, cultures were exposed to catalase (500 U/ml), superoxide dismutase (SOD, 400 U/ml) or mannitol (10 or 60 mM) for 4 hr. Cultures treated with DFO (desferoxamine mesylate) were preincubated for 1 hr with 20 mM DFO prior to oxalate addition. Data reflect mean ± SEM deaths/field in 18 to 46 cultures.

[b] For each experimental series, the average response to 350 μM free oxalate (ie the average rate of cleavage of DHR) was determined and used to normalize other treatment groups from that series. Where indicated catalase (500 U/ml), superoxide dismutase (400 U/ml) or mannitol (10 mM) was added along with oxalate. Data reflect mean ± SEM % inhibition of oxalate-induced DHR fluorescence from 3 to 14 cultures.

[c] $P < 0.025$ by paired analysis

Discussion

The present studies provide compelling evidence that exposure to oxalate increases the production or availability of free radicals in renal epithelial cells. This was demonstrated using two separate asssays: (1) the reduction of nitroblue tetrazolium to a blue reaction product, a process that is mediated by superoxide [22–24], (2) the cleavage of DHR 123 to a fluorescent derivative, a process that is also mediated by reactive oxygen molecules [25–27]. The oxalate-induced increase in free radical production was mirrored by an increase in the number of damaged cells in the monolayers. Indeed, these two effects of oxalate, increased free radical production and cell death, appeared to be linked in that both responses showed a similar concentration dependency and a similar sensitivity to free radical scavengers.

Of the various mono- and dicarboxylates examined in the present studies, oxalate was the most potent at increasing free radical production and cell death. Formate, which shares the oxalate transporter on the apical surface of renal brush border membranes [6, 8] also increased DHR cleavage and increased the incidence of dead cells in the LLC-PK$_1$ cultures; however, the effects on both parameters was less than half that produced by comparable levels of oxalate. Other structurally related molecules (malonate, succinate, citrate) produced modest increase in free radical production with no effect on cell viability.

The precise sequence of events by which oxalate increases free radical production/availability remains unknown. The effects are presumably secondary to oxalate entry into the cells since oxalate

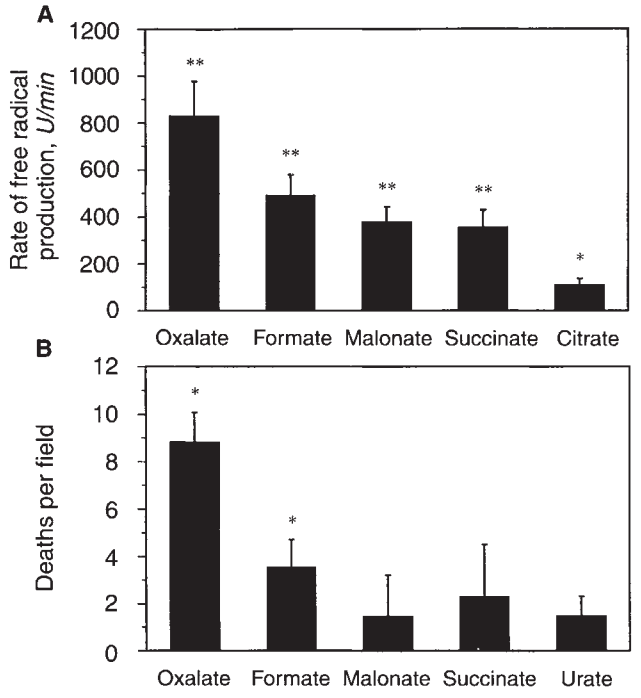

Fig. 4. *Specificity of oxalate-induced changes in free radical production and cell death.* **A.** Free radical production. The rate of cleavage of DHR was assessed as described above after the addition of structurally related mono- and dicarboxylic acids (1 mM total in all cases). Note that the oxalate-induced changes in free radical production were considerably higher than those produced by other compounds. Data are means ± SEM from 4 to 10 monolayers for each analogue; $P < 0.01$ for all additives except citrate. **B.** Cell death. Cells were exposed to various additives (added at 1 mM) for four hours and stained with vital dyes. Of the compounds tested, only oxalate and formate induced significant increases in the number of dead cells Data are means ± SEM from 6 (malonate, succinate, urate) or 29 (formate) monolayers.

added in solution had no effect on DHR fluorescence. (The requirement for oxalate uptake could not be tested directly, however; since LLC-PK_1 cells are adversely affected by treatments that inhibit oxalate transport such at exposure to stilbene derivatives and/or to bicarbonate free buffers, data not shown). Once inside the cells, oxalate could increase free radical production in a number of ways: (1) by acting as a precursor in the generation of a reactive metabolite, (2) by altering mitochondrial function, (3) by modifying the activities of various cytosolic enzymes that generate reducing equivalents, (4) by inhibiting endogenous free radical scavenging enzymes. Of these possibilities, several are inconsistent with available data. For example, it seems unlikely that free radicals are produced as a byproduct of oxalate metabolism since animal cells lack the requisite enzymatic machinery for oxalate degradation. Similarly, it seems unlikely that the increase in free radicals reflects direct effects on mitochondrial metabolism since our previous studies on isolated mitochondria revealed no oxalate-induced changes in respiration, ATP production, or transmembrane calcium fluxes [31]. Oxalate could alter mitochondrial metabolism indirectly, however, by altering cytosolic enzymes that supply substrates to mitochondria. Such a possibility is supported by *in vitro* evidence for inhibitory effects of oxalate on a number of cytosolic enzymes including lactate dehydrogenase, pyruvate kinase and malonate dehydrogenase [10]. Alternatively or in addition, oxalate may increase the availability of free radicals by inhibiting enzymes responsible for their degradation. This possibility was suggested by preliminary studies indicating that oxalate can inhibit peroxidase activity *in vitro* (Koul, unpublished results). Whatever the initiating event, exposure to oxalate appears to increase the levels of superoxide within cells. Subsequent reactions within the LLC-PK_1 cells lead to the production of other reactive species including peroxide and hydroxyl radicals by a process that is dependent at least in part on iron. Support for this scenario includes the finding that extracellular addition of superoxide dismutase (which converts superoxide to peroxide and oxygen), catalase (which degrades peroxide), desferoxamine (which chelates iron) and mannitol (which scavenges hydroxyl radicals) all reduced oxalate toxicity.

Other potential mechanisms for oxalate toxicity (such as free radical-independent pathways) were also considered. Possible effects of oxalate on intracellular calcium or intracellular pH were excluded in previous studies [16, 32]. A role for calcium oxalate crystals in oxalate action could not be excluded, however. Birefringent calcium oxalate crystals were observed in our oxalate containing buffers, and evidence from other studies has suggested that calcium oxalate crystals can induce damage to cellular membranes [33–35]. Moreover, recent studies on endothelial cells have demonstrated that the endocytosis of crystals can increase free radical production [22]. Thus effects mediated by crystals may have contributed to the observed responses in LLC-PK_1 cells. No obvious link between crystal adherence and damage was detected in the present studies, however. Some damaged cells were associated with crystals, most were not. Further studies will be required to define the actions of calcium oxalate crystals on renal epithelial cells.

Oxalate toxicity was only observed at free oxalate concentrations ≥ 350 μM, somewhat higher than might be expected to occur within the proximal tubules. Oxalate levels in normal individuals average only 5 to 10 μM in the glomerular filtrate [36] rising ~10-fold by the end of the proximal tubule as fluid is reabsorbed. Levels of oxalate in this region may be increased further by net secretion of oxalate [2, 4], but the final concentration of oxalate that is achieved has not been determined due to limitations with current oxalate assays. Current estimates of regional oxalate levels have been made by assuming oxalate exchange mimics that of other metabolites (ignoring net oxalate secretion). Alternatively estimates have been derived from measurements on lyophilized samples [37]. Thus it is not clear whether oxalate would ever reach toxic levels in the proximal tubules of normal kidneys; however, toxic levels might well occcur in individuals with chronically elevated oxalate levels.

Taken together these results indicate that oxalate can no longer be considered an inert metabolic end product. Our studies suggest that even modest increases in urinary oxalate levels may have serious consequences for renal function and contribute to the progression/severity of several forms of renal disease. For example, oxalate-induced damage to proximal tubular cells could promote stone formation by providing cellular debris for crystal nucleation and aggregation and by enhancing crystal binding to other tubular cells. This scenario is supported by several lines of evidence: (1) tubular damage (enzymuria and membranuria) has been observed both in clinical [38] and experimental [39, 40] stone disease; (2) conditions that promote tubular damage (treatment

with nephrotoxic doses of gentamicin) increase the incidence of plaques and stones in experimental animals [41]; (3) conditions that disrupt renal epithelial polarity *in vitro* (disruption of tight junctions with Ca^{2+} chelators) promote crystal binding [42]; (4) addition of membrane fractions from renal tubular cells promotes the growth of calcium oxalate crystals in artificial urine [43]. These considerations provide a compelling argument for a role of oxalate toxicity in the pathogenesis of calcium oxalate stone disease. Oxalate toxicity may also contribute to the progression of end-stage renal disease [44, 45]. Hyperoxalemia is a common feature in end-stage renal disease and a number of investigators have suggested the importance of reducing the oxalate burden in these individuals [44, 45]. Clearly, additional investigations as to the clinical significance of hyperoxaluria and hyperoxalemia and methods for reducing oxalate toxicity *in vivo* are warranted.

Acknowledgments

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