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Oxalate-induced ceramide accumulation in Madin-Darby canine kidney and LLC-PK1 cells

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Oxalate-induced ceramide accumulation in Madin-Darby canine kidney and LLC-PK1 cells.

Background. Oxalate exposure produces oxidant stress in renal epithelial cells leading to death of some cells and adaptation of others. The pathways involved in these diverse actions remain unclear, but appear to involve activation of phospholipase A_2 (PLA_2) and redistribution of membrane phospholipids. The present studies examined the possibility that oxalate actions may also involve increased accumulation of ceramide, a lipid-signaling molecule implicated in a variety of pathways, including those leading to apoptotic cell death.

Methods. Ceramide accumulation was examined in renal epithelial cells from pig kidney (LLC-PK1 cells) and from dog kidney [Madin-Darby canine kidney (MDCK cells)] using the diacylglycerol kinase assay. Sphingomyelin degradation was assessed by monitoring the disappearance of ^{3}H-sphingomyelin from cells that had been prelabeled with [^{3}H]-choline. The effects of oxalate were compared with those of other oxidants (peroxide, xanthine/xanthine oxidase), other organic acids (formate and citrate), and a known activator of sphingomyelinase in these cells [tumor necrosis factor-α (TNF-α)]. Separate studies determined whether oxalate-induced accumulation of ceramide could be blocked by pretreatment with antioxidants [Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin (Mn TMPyP, a superoxide dismutase mimetic) or N-acetylcysteine (NAC; an antioxidant)], with an inhibitor of ceramide synthase [fumonisin B1 (FB1)] or with an inhibitor of PLA_2 [arachidonyl trifluoromethylketone ($AACOCF_3$)].

Results. Oxalate exposure produced a significant time- and concentration-dependent increase in cellular ceramide. A reciprocal decrease in ^{3}H-sphingomyelin was observed under these conditions. Increases in cellular ceramide levels were also observed after treatment with other oxidants (hydrogen peroxide, and xanthine/xanthine oxidase), activators of sphingomyelinase (TNF-α), exogenous sphingomyelinase, or arachidonic acid. Formate produced similar (albeit smaller) effects, and citrate did not. The oxidant-induced increases in ceramide were attenuated by pretreatment with NAC (a glutathione precursor) and MnTMPyP (a superoxide dismutase mimetic), suggesting a role for cellular redox states. The oxalate-induced increase in ceramide was also attenuated by pretreatment with $AACOCF_3$, suggesting a role for PLA_2. Pretreatment with FB1 produced a small but statistically insignificant attenuation of the response to oxalate.

Conclusions. Oxalate exposure produces a marked accumulation of ceramide in renal epithelial cells by a process that is redox sensitive and mediated in part by activation of PLA_2. Since cellular sphingomyelin decreased as ceramide increased, it seems likely that oxalate actions are mediated, at least in part, by an increase in sphingomyelinase activity, although alterations in ceramide synthase are also possible. Further study is required to define the steps involved in oxalate actions and to determine the extent to which ceramide signaling mediates oxalate actions.

Key words: renal epithelial cells, lipid signals, oxidant stress, PLA_2, apoptosis.

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Studies in our own [1–6] and in other laboratories [7–11] have shown that exposure to high levels of oxalate produces oxidant stress in renal epithelial cells, causing frank toxicity in some cells and adaptation in others. This finding has been of considerable interest to renal pathologists in that cellular injury promotes the attachment of crystals to renal epithelial cells [12–14]. Thus, high levels of oxalate in the urine could promote kidney stone disease in several ways: by favoring the formation of calcium oxalate crystals, by providing cellular debris for crystal nucleation, and by promoting crystal adherence to renal cells.

Many or all of the cellular actions of oxalate appear to be initiated by alterations in cellular membranes. Oxalate exposure increases membrane lipid peroxidation [9–11], increases membrane permeability to vital dyes and to cytosolic enzymes [1–4, 7, 8], promotes a redistribution of phosphatidylserine to the membrane surface (L.C. Cao, unpublished observations), and activates phospholipase A_2 (PLA_2) [15]. Any or all of these changes may contribute to the observed cytotoxicity of oxalate. The possible linkage(s) between the observed membrane alterations and cellular adaptation is less clear. Oxalate treatment clearly induces a variety of adaptive responses that range from an induction of immediate early genes (for example, c-*myc*, *egr*-1), initiation of DNA synthesis and cellular proliferation [1, 6], and an induction of genes associated with remodeling and repair (for example, os-

teopontin and clusterin; I. Jonassen, personal communication). However, studies to date (data not published) have failed to detect an activation of signaling molecules (mitogen-activated protein kinase or JunK) that have been implicated as mediators of other forms of oxidant stress [16]. Given the evidence that oxalate affects cell membrane properties, it seemed likely that oxalate actions may be mediated by lipid-signaling molecules. Thus, the present studies assessed the effects of oxalate on the accumulation of ceramide, a lipid metabolite that has been implicated as an intracellular signal for a variety of cellular responses, including cellular differentiation, proliferation, cytotoxicity, and cell death [17]. The results from our current studies provide evidence that oxalate exposure produces rapid and significant increases in ceramide that may mediate some of the observed effects of oxalate on renal cell growth and viability.

METHODS

Cell culture

Madin-Darby canine kidney (MDCK; CCL 34, passage 53 to 90) and LLC-PK1 cells (CRL 1392, passage 205 to 240) from the American Type Culture Collection (Rockville, MD, USA) were plated on 100 or 60 mm dishes and maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), antibiotics 100 U/mL penicillin-100 μg/mL streptomycin (GIBCO-BRL), and 4.5 g/L D-glucose. Cells were grown to approximately 80% confluence at 37°C under 5% CO_2/95% air, washed twice with serum-free DMEM, and incubated for predetermined periods in media containing the desired stimuli or combinations of stimuli. The cells were then harvested by scraping and were recovered by centrifugation. In some experiments, cells were dispersed further by trypsinization, and assays were carried out in cell suspensions containing equal numbers of cells in each tube (2 to 4 $\times$ 10^6 cells). In other cases, assays were performed using the entire cell population from a given dish. Previous studies have verified that cultures plated at the same time and grown under the same conditions show minimal variability in cell density (<10% difference between dishes, as assessed by cell counting or by determination of cell protein or lipid phosphorus; unpublished observations).

Lipid extraction and ceramide assay

The harvested cells were recovered by centrifugation (300 $\times$ *g*, 10 min), washed twice with ice-cold phosphate-buffered saline (PBS), and extracted with 5 mL of chloroform:methanol:1% perchloric acid (2:2:1, vol/vol/vol). The organic phase was washed twice with 2 mL of 1% perchloric acid:methanol (7:1, vol/vol) and dried under nitrogen. The lipid extracts were stored at −70°C until assayed for ceramide and sphingomyelin content.

Cellular ceramide was quantitated using the diacylglycerol (DAG) kinase assay described previously [18] with minor modifications. In brief, the cell lipids were extracted from cultures as described previously in this article and dried under nitrogen. The dried lipids were dissolved in 20 μL of 7.5% n-octyl-β-glucopyranoside, 5 mmol/L cardiolipin, and 1 mmol/L diethylenetriaminepentaacetic acid (DETAPAC, pH 7.0). The following solutions were then added in order: 50 μL of reaction buffer (100 mmol/L imidazole HCl, pH 6.6), 2 μL of 100 mmol/L dithiothreitol in 1 mmol/L DETAPAC, pH 7.0, 10 μL of DAG kinase from *E. coli* (0.5 μg/μL in 100 mmol/L imidazole HCl, pH 6.6, containing 1 mmol/L DETAPAC; CalBiochem, La Jolla, CA, USA), and 8 μL of water. Note that these conditions provide an excess of added DAG kinase to insure quantitative conversion of ceramide to ceramide 1-phosphate [reviewed in 19]. The reaction was initiated by the addition of 10 μL of 10 mmol/L cold adenosine 5′-triphosphate (ATP) containing [γ-^{32}P] ATP (4.5 μCi for each sample; NEN™ Life Science Products, Inc., Boston, MA, USA) in 20 mmol/L imidazole, pH 6.6, 1 mmol/L DETAPAC and was incubated at room temperature for 40 minutes. The reaction was stopped by the addition of 0.7 mL of 1% (wt/vol) perchloric acid, and the lipids were extracted with 5 mL of chloroform/methanol/1% perchloric acid (2:2:1, vol/vol/vol). The lower chloroform phase was washed twice with 3 mL of 1% perchloric acid/methanol (7:1, vol/vol). A portion of the chloroform phases (1.5 and 0.1 mL, respectively) was aspirated and dried under nitrogen, with the former sample taken for ceramide determination and the latter taken for assay of lipid phosphorus. The ^{32}P-labeled ceramide-1 phosphate was separated from the rest of the lipids by thin layer chromatography (TLC) on silica gel 60 TLC plates (Merck, Darmstadt, Germany) using chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, vol/vol/vol/vol/vol) as the solvent. The plate was dried and subjected to autoradiography overnight. The band of ^{32}P-labeled ceramide-1 phosphate was recovered by scraping and counted in a scintillation counter. The amount of ceramide in the samples was compared with a standard curve generated by adding known amounts of ceramide (0 to 10 μg of C_2-ceramide) to the reaction mixture and processing these standards along with the experimental samples. Data are expressed as a percentage of the ceramide in untreated control cells. Estimates of the content of ceramide in controls are provided in the text.

Sphingomyelin assay

Sphingomyelin levels were measured as described by Dressler, Mathias, and Kolesnick [20]. Two days before the experiment, cells were transferred to DMEM me-

dium containing 10% FBS supplemented with 1 μCi/mL [methyl ^{3}H]-choline chloride (NEN™ Life Science Products, Inc.). At the end of this period, the labeled medium was removed, and the cells [either in 60 mm dishes or cell suspensions (1 to 2 × 10^6 cells per treatment group)] were incubated for an additional 30 minutes in fresh DMEM supplemented with 10% FBS. The cultures were then exposed to various agents for 0, 10, 20, 30, or 60 minutes. At the indicated times, the medium was rapidly removed. The cells were recovered (by scraping or by centrifugation), washed with cold PBS, and sonicated in 400 μL of a chloroform/methanol/concentrated HCl solution (100:100:1, vol/vol/vol) and 100 μL of PBS containing 10 mmol/L ethylenediaminetetraacetic acid (EDTA). After centrifugation for five minutes at 12,000 × *g*, the aqueous phase was removed and re-extracted with 100 μL of chloroform, which was subsequently added to the chloroform phase. The combined chloroform phases were dried under a stream of nitrogen. The extracted dried lipids were incubated in 50 μL of 0.1 mol/L methanolic potassium hydroxide for 60 minutes at 37°C to deacylate glycerophospholipids. The samples then were dried under nitrogen and redissolved in 40 μL of the chloroform phase. This solution was re-extracted with 10 μL of the PBS with 10 mmol/L EDTA and then re-evaporated. The samples were then stored at −70°C under nitrogen until they were analyzed for sphingomyelin. The sphingomyelin was resolved by TLC using chloroform/methanol/acetic acid/water (60:30:8:5, vol/vol/vol/vol) as solvent. Individual lipids were visualized with iodine vapor staining and quantitated by liquid scintillation counting. The results were expressed either as a relative percentage as compared with controls or by lipid phosphorus assay [21].

Statistics

To assess the significance of the observed time- and concentration-dependent changes in ceramide and sphingomyelin levels, data were analyzed using an analysis of variance. Experimental treatments were considered to have produced statistically significant effects when the probability of chance occurrence was $P < 0.05$. To determine whether the antioxidants N-acetylcysteine (NAC) or MnTMPyP, the PLA_2 inhibitor arachidonyl trifluoromethylketone ($AACOCF_3$), or the ceramide synthase inhibitor fumonisin B1 (FB1) significantly attenuated responses to oxalate or other oxidants, data were compared with its respective control group (for example, oxalate alone vs. oxalate plus other treatment) using a paired Student's *t*-test. For comparing the effects of multiple treatments with a single control, a Bonferroni correction was also applied [22].

RESULTS

Oxalate-induced ceramide accumulation

Exposure of MDCK cells or LLC-PK1 cells to 0.5 mmol/L oxalate produced a time-dependent increase in ceramide accumulation, with significant increases observed within 15 minutes (Fig. 1A). Basal levels of ceramide averaged 9.2 ± 0.7 and 20.9 ± 3.3 pmol/nmol lipid phosphorus in LLC-PK1 cells and MDCK cells, respectively, and the response to oxalate was greater in MDCK cells than in LLC-PK1 cells. The oxalate-induced accumulation of ceramide also exhibited concentration dependence in both cell lines, with significant increases observed at 0.25 mmol/L oxalate (Fig. 1B). This concentration of oxalate produces significant increases in free radical production and modest (but statistically insignificant) increases in membrane permeability to vital dyes in LLC-PK1 cells [3]. Other organic acids produced variable effects on ceramide production. In MDCK cells, formate also produced a significant increase in ceramide accumulation, although the response to formate required a higher concentration (0.5 vs. 0.25 mmol/L) than the response to oxalate (Fig. 2). In LLC-PK1 cells, a similar pattern was observed, although the response to formate did not reach statistical significance in the limited series performed here ($N = 3$). Formate shared the oxalate transporter in the kidney [23], and produced modest increases in free radical production and in membrane permeability in LLC-PK1 cells [3]. Citrate, a dicarboxylate that reportedly protects, afforded protection from stone disease in vitro [24]; while it produced a modest reduction in oxalate toxicity in LLC-PK1 cells [3], in our study citrate did not increase ceramide accumulation in either cell line (Fig. 2).

Increases in ceramide correlate with decreases in sphingomyelin

Previous studies suggest that multiple pathways can contribute to an increase in ceramide (for example, by increased sphingolipid degradation, by reduced breakdown of ceramide, or by increased synthesis of ceramide) [17]. Thus, we carried out studies to distinguish among these possibilities. The first series of studies examined the effects of oxalate on the breakdown of sphingomyelin in renal epithelial cells. Such studies revealed a significant effect of oxalate on ^{3}H-labeled sphingomyelin levels. Exposure to 0.5 mmol/L oxalate produced a significant (40 to 50%) decline in cellular sphingomyelin within 30 minutes both in MDCK cells and in LLC-PK1 (Fig. 3). Similar effects could be observed after a two-hour exposure to 50 ng/mL tumor necrosis factor-α (TNF-α; data from two experiments not shown), a known activator of sphingomyelinase in many cells [25]. These studies suggest the involvement of sphingomyelinase in oxalate actions, a possibility that is further supported by experi-

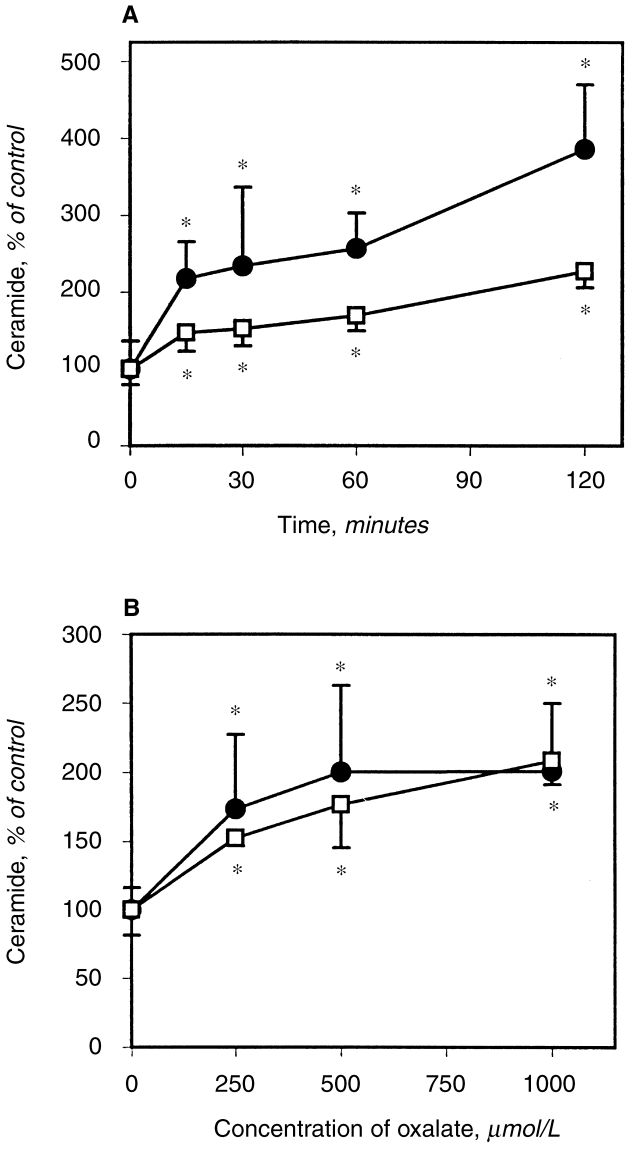

Fig. 1. Time course (*A*) and concentration dependence (*B*) for oxalate-induced ceramide accumulation in MDCK (●) and in LLC-PK1 (□) cells. (A) Cell suspensions were prepared as described in the text and exposed to 0 or 0.5 mmol/L oxalate for the times indicated. Ceramide levels were determined using the diacylglycerol kinase assay [18] and data were expressed relative to levels in untreated controls. The time course for oxalate-induced changes was similar in MDCK cells (●) and in LLC-PK1 cells (□), with significant increases seen within 15 minutes in both cell types; however, the magnitude of the oxalate response was greater in MDCK cells (fourfold vs. twofold increase after 2 hours exposure to 0.5 mmol/L oxalate). Data reflect mean ± SEM from three independent experiments performed in duplicate ($N = 6$). (B) Cells were prepared as described in the text and exposed to oxalate (0, 250, 500, or 1000 μmol/L) for 1 hour. Ceramide levels were determined and expressed as a percentage of levels in untreated controls. After 1 hour of exposure, oxalate produced significant increases in ceramide in both MDCK cells and LLC-PK1 cells at concentrations ≥ 250 μmol/L. Data reflect the mean ± SEM from three independent experiments performed in duplicate ($N = 6$) for each cell line.

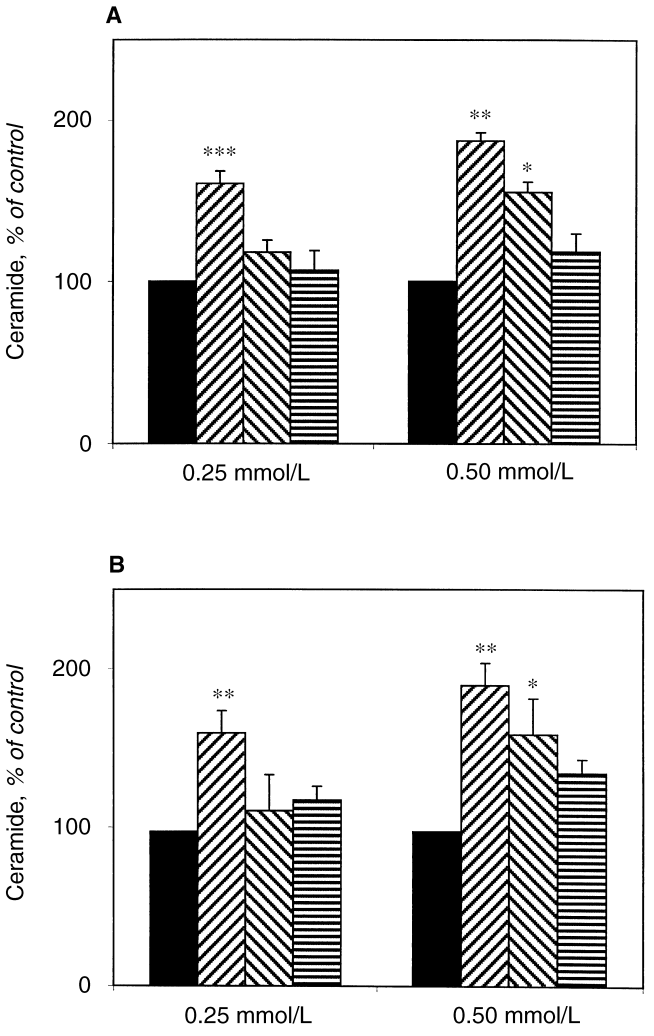

Fig. 2. Effect of other organic acids on ceramide accumulation in renal epithelial cells. Suspensions of MDCK cells (*A*) and LLC-PK1 cells (*B*) were prepared as described in the **Methods** section, and were exposed to 0.25 or 0.5 mmol/L oxalate (▨), formate (▧), or citrate (▤) for one hour (■, control). Lipids were extracted, and ceramide levels were determined using the DAG assay. Note that oxalate exposure produced a significant increase in ceramide levels at both 0.25 and 0.5 mmol/L in both cell lines. Formate produced a significant increase in ceramide only at 0.5 mmol/L and only in MDCK cells. Citrate produced no significant effect at either concentration and in either cell line. Data are mean ± SEM ($N = 426$ for MDCK cells and $N = 325$ for LLC-PK1 cells). The asterisk denotes significance relative to untreated controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

ments in which ceramide accumulation and sphingomyelin degradation were followed in the same batch of cells on the same day. The latter studies showed that the increase in ceramide was paralleled by a decrease in ^{3}H-labeled sphingomyelin in oxalate-treated MDCK and LLC-PK1 cells (Fig. 4), although the percentage changes in the two parameters were somewhat different.

Other studies examined the possibility that oxalate exposure may also stimulate ceramide synthesis in renal cells. LLC-PK1 cells and MDCK cells were pretreated

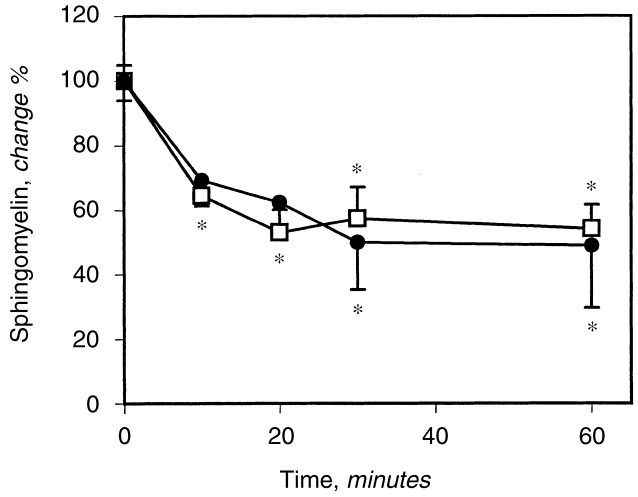

Fig. 3. Oxalate-induced decline in ^{3}H sphingomyelin levels in MDCK (●) and LLC-PK1 (□) cells. Renal epithelial cells were labeled with [^{3}H]-choline for 48 hours and exposed to oxalate (0 or 0.5 mmol/L) for 0, 10, 20, 30, or 60 minutes. Lipids were extracted, chromatographed, and visualized as described in the **Methods** section, and the amount of ^{3}H sphingomyelin was then assessed. Data are means ± SEM from three independent experiments performed in duplicate (N = 6) for each cell line. Note that exposure to 0.5 mmol/L oxalate produced a time-dependent decrease in ^{3}H sphingomyelin levels in both MDCK and LLC-PK1 cells, with significant declines seen by 10 minutes in both cell lines. This effect was large, showing an approximately 40% decline at 60 minutes, comparable to responses obtained with 50 μg/mL TNF-α (data not shown).

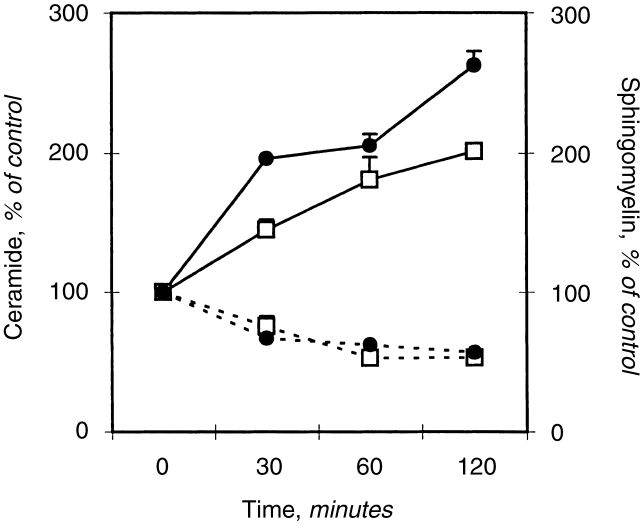

Fig. 4. Simultaneous measurement of oxalate actions on ceramide and on sphingomyelin levels in renal cells. Large batches of MDCK (●) and LLC-PK1 cells (□) were prepared and used as described in Figures 1 and 2 such that oxalate's actions on ceramide and on sphingomyelin content could be examined in the same experiment. Data presented are means ± SEM from three experiments with duplicate measurements in each experiment. Note the reciprocal relationship between the increase in ceramide accumulation and the decrease in ^{3}H sphingomyelin content in both cell lines.

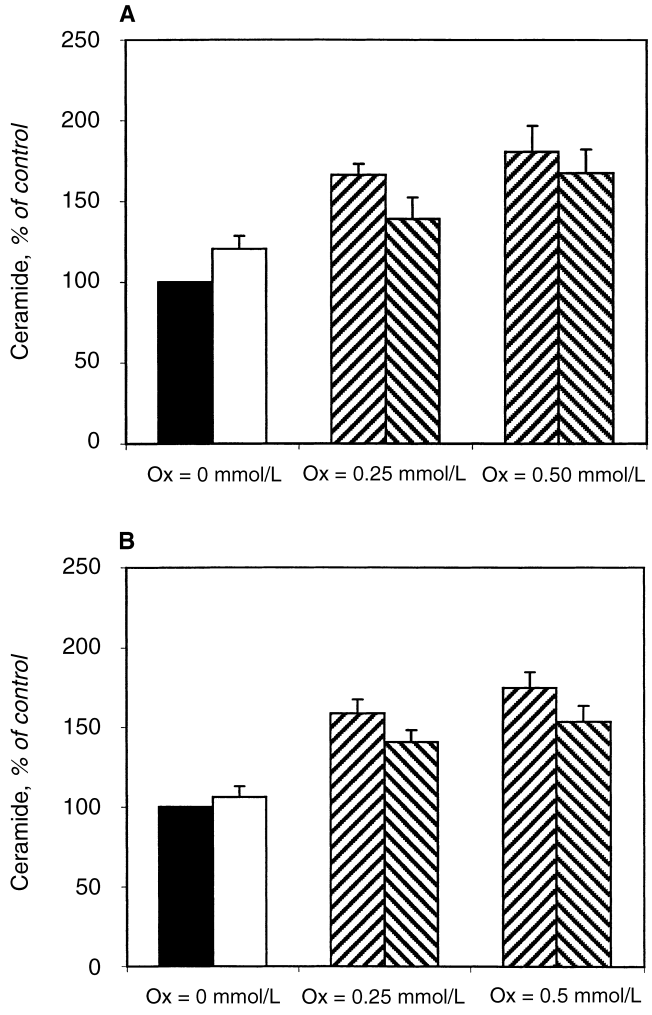

Fig. 5. Pretreatment with fumonisin B1 (FB1), an inhibitor of ceramide synthase, failed to attenuate oxalate actions on ceramide accumulation. LLC-PK1 (*B*) and MDCK (*A*) cell suspensions were prepared as described in the **Methods** section. Symbols are: (■), untreated controls; (□) controls pretreated with FB1 (50 μmol/L) for 1 hour; (▨) oxalate treated; (▧) pretreated with FB1 (50 μmol/L) for 1 hour prior to a 1-hour exposure to oxalate (0.25 or 0.5 mmol/L). Note that ceramide accumulation was slightly reduced in fumonisin-treated cells, although the effects were not statistically significant. FB1 treatment alone had no significant effect on ceramide accumulation in two experiments. Data are mean ± SEM with N = 6 for MDCK cells and N = 3 for LLC-PK1 cells.

with FB1 (an inhibitor of ceramide synthase) [26] and were then exposed to 0.25 or 0.5 mmol/L oxalate for one hour. Ceramide accumulation in cells treated with FB1 + oxalate was compared with that in cells treated with oxalate alone. Such studies revealed only minimal effects of FB1 treatment, however, in that the oxalate response of FB1-treated cells was similar to that in untreated cells (FB1 did produce a small reduction in the oxalate response, but the effect was statistically insignificant and was observed at the lower oxalate concentration; Fig. 5). These findings suggest that increased ceramide synthesis

Table 1. Antioxidants attenuate oxidant-induced increases in ceramide accumulation in renal epithelial cells

	LLC-PK1		MDCK	
Treatment	*% ceramide*	*N*	*% ceramide*	*N*
Ox	187.3 ± 14.0.6[b]	4	178.9 ± 7.1[b]	7
Ox + NAC	110.3 ± 18.2[a]	3	113.3 ± 18.3[a]	3
Ox + MnTMPyP	128.4 ± 11.7[a]	5	128.6 ± 4.56[a]	4
H_2O_2	205.3 ± 35.2[b]	4	254.2 ± 37.2[b]	6
H_2O_2 + NAC	140.5 ± 25.2	3	150.7 ± 28.2	3
X/XO	213.6 ± 22.7[b]	4	305.1 ± 41.6[b]	5
X/XO + NAC	102.1 ± 6.1[a]	4	137.0 ± 15.1[a]	3

LLC-PK1 and MDCK cells were exposed to oxidant (oxalate, 0.5 mmol/L for 1 hour; H_2O_2, 0.5 mmol/L for 0.5 hour or X/XO 1 mmol/L xanthine/50 mU xanthine oxidase/mL for 1 hour) and assayed for ceramide as described in the text. Where indicated cells were preincubated with antioxidants (NAC 10 mmol/L or MnTMPyP 0.5 mmol/L) for 1 hour. Abbreviations are in the Appendix. Data are expressed as a percentage of the ceramide levels in untreated controls. Neither NAC or MnTMPyP alone had any effect.

[a] $P < 0.05$ for oxidant + NAC (or MnTMPyP) vs. oxidant alone

[b] $P < 0.05$ for oxidant vs. untreated controls

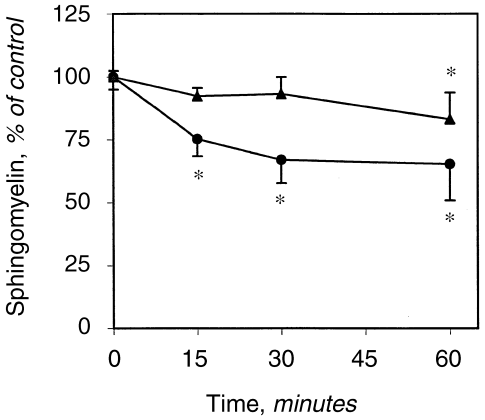

Fig. 6. N-acetylcysteine (NAC) attenuates oxalate-induced ceramide accumulation in MDCK cells by decreasing sphingomyelin hydrolysis. MDCK cells were allowed to incorporate [^{3}H]-choline for 48 hours and were then washed and incubated for an additional hour in DMEM with (▲) or without (●) 10 mmol/L NAC. The cells were then exposed to 0.5 mmol/L oxalate for varying times (0, 30, or 60 minutes) and processed for sphingomyelin analysis (discussed in the **Methods** section). In these studies, exposure to oxalate alone produced a marked decline in sphingomyelin with time. This decline was markedly attenuated by pretreatment with NAC. Data are means from four wells from three separate platings.

may contribute to the oxalate-induced increases in ceramide accumulation, but other responses (for example, activation of SMase) likely play a greater role.

Oxalate-induced ceramide generation is redox-sensitive and blocked by antioxidants

Oxalate-induced oxidative injury of renal epithelial cells has been demonstrated in our previous investigations [3], as well as in those of others [9–11]. Since recent evidence showed that the sphingomyelin-ceramide pathway is redox-sensitive in other cell types [25, 27], we examined the role of oxidant stress in oxalate-induced ceramide generation. The responses to known oxidants and to TNF-α, an agent that activates ceramide by a redox-sensitive pathway [27, 28], were also examined. The effects of two antioxidants—NAC, a known antioxidant and efficient thiol source for glutathione [29], and MnTMPyP, a superoxide dismutase mimetic [30]—on the response to oxalate were also evaluated. These studies showed that ceramide accumulation in renal epithelial cells resembles the accumulation seen in other cell types in that exposure to various oxidants [hydrogen peroxide (500 μL), xanthine/xanthine oxidase (1 mmol/L xanthine plus 50 mU/mL xanthine oxidase)] increases ceramide levels. Moreover, preincubation of the cells with NAC (10 mmol/L for 1 h) significantly reduces the ceramide response. Similar effects were observed after pretreatment of the cells with the superoxide dismutase (SOD) mimetic, MnTMPyP (0.5 mmol/L for 1 hour, tested only against oxalate). The data are summarized in Table 1.

Other studies confirmed that the time-dependent increase in ceramide was paralleled by a time-dependent decrease in ^{3}H-labeled sphingomyelin by a redox-sensitive process. As can be seen in Figure 6, treatment with oxalate led to a decline in ^{3}H-labeled sphingomyelin levels in the MDCK cells; pretreatment with 10 mmol/L NAC for one hour blocked the oxalate-induced decrease in ^{3}H-sphingomyelin.

Oxalate-induced ceramide generation is linked to the PLA_2 pathway

Studies in other cell types have suggested that PLA_2 plays an important role in the processes leading to increased ceramide accumulation and cell death [31]. Furthermore, our own studies suggested a role for PLA_2 in oxalate toxicity [15]. Thus, current studies assessed the possible role of PLA_2 and/or its product, arachidonic acid (AA), in the oxalate-induced change in ceramide accumulation. Such studies assessed the effect of exogenous AA and mellitin (an activator of $cPLA_2$) on ceramide generation in the renal epithelial cells and determined whether $AACOCF_3$, an inhibitor of PLA_2, would block the oxalate response. In these studies, exposure to 25 μmol/L AA or 5 μg/mL mellitin caused a twofold to sevenfold increase in ceramide, both in MDCK cells and in LLC-PK1 cells (Table 2). Moreover, a 15-minute preincubation with 10 μmol/L $AACOCF_3$ significantly reduced ceramide accumulation in response to oxalate (Fig. 7). In preliminary studies, this pretreatment also attenuated oxalate-induced changes in sphingomyelin degradation (data not shown).

DISCUSSION

This study presents the novel finding that oxalate exposure directly alters sphingomyelin metabolism, resulting in a marked accumulation of ceramide in renal

Table 2. Agonist-induced accumulation of ceramide in renal epithelial cells

	LLC-PK1		MDCK	
Stimuli	*% ceramide*	*N*	*% ceramide*	*N*
Arachidonic acid	200.3 ± 22.3[a]	5	234.6 ± 42.5[a]	11
Melittin	718.8	2	275.7 ± 30.4[a]	8
TNF-α	229.5 ± 24.4[a]	7	232.0 ± 37.4[a]	10
Sphingomyelinase	298.6	2	527.5	2

LLC-PK1 and MDCK cells were exposed to various agents, [arachidonic acid (25 μmol/L for 60 minutes), melittin (2 μmol/L for 60 minutes), TNF-α (50 ng/mL for 30 minutes) or sphingomyelinase (200 mU/mL for 30 minutes)] and assayed for ceramide as described in the text. Data are expressed as a percentage of the ceramide levels in untreated controls.

[a] $P < 0.05$ for a given agent vs. untreated controls

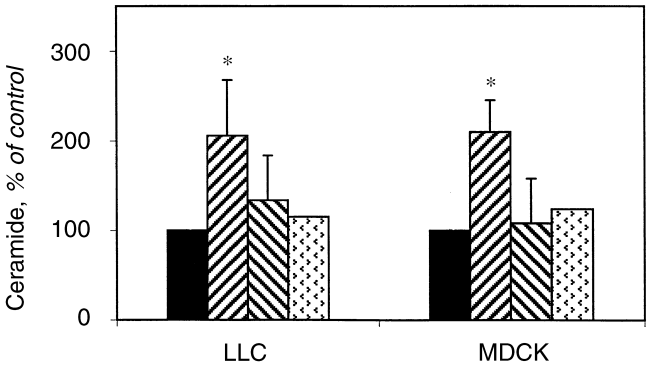

Fig. 7. Role of phospholipase A_2 (PLA_2) in oxalate induced ceramide generation. MDCK and LLC-PK1 cells were grown to approximately 85% confluence in 60 mm cell culture dishes and were washed twice with serum-free DMEM. Cells were preincubated for 15 minutes in the absence or presence of 10 μmol/L $AACOCF_3$ (a selective blocker for PLA_2), and then exposed to 0 or 0.5 mmol/L oxalate for one hour. Cells were then collected and lipid contents were analyzed as described in the text. Symbols are: (■) control; (▨) oxalate; (▧) oxalate + $AACOCF_3$; (▩) $AACOCF_3$. Ceramide levels are expressed as mean ± SEM from three independent experiments performed in duplicate for each cell line ($N = 6$). Note that the PLA_2 inhibitor, $AACOCF_3$ completely blocked the oxalate-induced increase in ceramide in both cell lines. Treatment with $AACOCF_3$ alone had no effect on ceramide levels ($N = 2$). *$P < 0.05$ relative to the untreated controls.

epithelial cells. This finding is of significance given the recent evidence indicating that ceramide triggers a number of signaling cascades that lead to diverse biological responses ranging from differentiation and proliferation to apoptosis and necrosis [17, 32], and our own evidence that oxalate triggers a similar range of responses in renal cells [1–6]. Thus, it is possible that ceramide accumulation plays a central signaling role in oxalate actions on renal cells, including its effects on cellular viability.

Studies in other systems have demonstrated that several processes can contribute to ceramide accumulation: increased degradation of sphingomyelin (catalyzed by acidic or neutral sphingomyelinases), decreased breakdown of ceramide (catalyzed by acidic or neutral ceramidase), increased de novo synthesis of ceramide (catalyzed by ceramide synthase), or increased degradation of dihydroceramide (catalyzed by dihydroceramide desaturase) [17, 33]. More than one of these pathways may contribute to oxalate actions in our cells. However, several lines of evidence suggest a major role for sphingomyelin degradation in the oxalate-induced accumulation of ceramide. For one, oxalate exposure produced a fall in sphingomyelin levels coincident with the increase in ceramide accumulation. Second, the addition of AA, a known activator of cytosolic Mg^{2+}-independent N-SMase [31], also increased ceramide levels. Third, the addition of exogenous N-SMase or TNF-α, a known activator of membrane Mg^{2+}-dependent N-SMase [34], produced changes in ceramide that were comparable to those of oxalate, both with respect to magnitude and time course. Fourth, pretreatment with FB1, an agent that reportedly inhibits ceramide synthase in renal cells [26], failed to block the oxalate-induced change in ceramide accumulation. Taken together, these findings suggest that the oxalate-induced change in ceramide is due, at least in part, to the activation of one or more sphingomyelinases in renal epithelial cells, although other pathways may also play a role.

The type(s) of sphingomyelinase involved in oxalate actions is as yet unclear. Studies in other cell types have demonstrated that there are at least seven isoforms of SMase that differ with respect to their pH optima (the neutral or N-SMases are active at neutral pH, and the acidic or A-SMases are active at acidic pH), ionic dependence (some exhibit a Mg^{2+} dependency, and others do not) and intracellular location (some are membrane bound, some are cytosolic, and others are confined to acidic organelles) [35]. Initial studies pointed to the membrane N-SMase as the mediator of injury-dependent increases in ceramide [20]. However, more recent studies implicated both the N-SMase and the A-SMase in this response [17, 31]. Indeed, recent studies showed that stimuli such as TNF-α activate both N-SMase and A-SMase via different pathways [one involving the c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) cascade of protein phosphorylations, and another involving TNF-α receptor activation of a cascade of cysteine proteases (caspases)] [reviewed in 35]. Thus, further studies are required to determine the precise manner by which oxalate activates SMase in renal cells and to identify the type(s) of SMase that might be involved.

The results from the present studies and from those reported previously suggest an involvement of PLA_2 in the generation of ceramide [17, 31]. Activation of PLA_2 leads to increased ceramide formation in a number of cell types, including kidney cells [31] and oxalate treatment activates PLA_2 in renal epithelial cells [15]. Moreover, the present study showed that the addition of mellitin, a known activator of PLA_2, and AA, a product of PLA_2 activity, stimulated ceramide generation in MDCK and LLC-PK1 cells. In addition, pretreatment with $AACOCF_3$, a selective inhibitor of cytosolic PLA_2, attenuated oxa-

late-induced ceramide generation and sphingomyelin degradation in renal epithelial cells. These findings suggest that the PLA_2/AA pathway is responsible, at least in part, for the oxalate-induced ceramide generation, although there is clearly "cross-talk" between the two lipid signaling pathways. For example, other studies in our laboratory have shown that agents better known for their effects on ceramide generation (C_2-ceramide, N-SMase, and TNF-α) produce an apparent activation of PLA_2 and a release of AA from MDCK cells (Y. Kohjimoto, personal communication). Further study is thus required to determine whether activation of the ceramide and PLA_2 pathways are causally linked or whether the two pathways are both activated by some common action of oxalate such as lipid peroxidation.

The present studies also suggest an involvement of oxidant stress in oxalate-induced ceramide generation. This finding was perhaps to be expected given the evidence from our own studies [3, 4] and those of others [9–11] that oxalate exposure induces oxidant injury to renal epithelial cells, and the evidence in other various cell types, including kidney, linking oxidant stress with ceramide toxicity [17, 26, 34]. However, the present studies provide the first evidence, to our knowledge, that oxalate exposure mimics the effects of other oxidants (peroxide, xanthine/xanthine oxidase) with respect to ceramide accumulation in MDCK and in LLC-PK1 cells. Moreover, these studies confirmed previous reports suggesting that ceramide accumulation can be modulated by the availability of reduced glutathione sulfhydryl (GSH) [25, 36]. Preincubation of cells with NAC, an antioxidant that confers protection by increasing cellular glutathione levels [29], blocked both the oxalate (and other oxidant)-induced increase in ceramide accumulation and the oxalate-induced decrease in sphingomyelin levels in renal cells. GSH serves as the major defense against oxidant injury in mitochondria [37], and disruption of mitochondrial transport of GSH is reportedly responsible for ceramide toxicity in hepatocytes from ethanol-fed rats [38]. These findings suggest that mitochondria (and thiol status) may play a central role in oxalate toxicity, and thus, it may be significant that animals with experimental stone disease show a decrease in GSH and in total thiols and an increase in oxalate binding to kidney mitochondria [38, 39]. A role for kidney mitochondria is also supported by the finding that MnTMPyP reduced oxalate effects on ceramide and on toxicity [6], since mitochondria are the major source of superoxide generation within cells [40].

In summary, the present studies demonstrate that oxalate exposure leads to increased accumulation of ceramide by a process that is redox-sensitive and dependent, at least in part, on the activation of PLA_2. The exact sequence of events remains unclear, but may involve a direct interaction between the oxalate anion and the membrane surface (for example, membrane peroxidation/injury) that leads to an activation of PLA_2. The activation of this enzyme in turn generates byproducts [AA, the precursor for a variety of active metabolites including the prostaglandins, leukotrienes and epoxides, as well as lysolipids (which also have biological activity)] that may activate sphingomyelinase(s) and generate ceramide. The accumulation of ceramide may then initiate a series of events, perhaps involving altered mitochondrial function, that leads to cellular toxicity (perhaps via a caspase cascade) or that lead to cellular adaptation/proliferation (perhaps via a stress kinase cascade) [reviewed in 17, 32, 35]. Further studies are required to assess the steps involved in oxalate actions and the role of ceramide in these responses.

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APPENDIX

Abbreviations used in this article are: AA, arachidonic acid; $AACOCF_3$, arachidonyl trifluoromethylketone; DAG, diacylglycerol; DETAPAC, diethylenetriamine-pentaacetic acid; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FB1, fumonisin B1; FBS, fetal bovine serum; GSH, glutathione sulfhydryl; LLC-PK1, immortalized porcine cell line; MDCK, Madin-Darby canine kidney; Mn TMPyP, Mn (III) tetrakis (l-methyl-4-pyridyl) porphyrin; NAC, N-acetylcysteine; PLA_2, phospholipase A_2; SOD, superoxide dismutase; TLC, thin layer chromatography; TNF-α, tumor necrosis factor-α.

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