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Oxalate toxicity in renal cells

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Abstract Exposure to oxalate, a constituent of the most common form of kidney stones, generates toxic responses in renal epithelial cells, including altered membrane surface properties and cellular lipids, changes in gene expression, disruption of mitochondrial function, formation of reactive oxygen species and decreased cell viability. Oxalate exposure activates phospholipase A2 (PLA2), which increases two lipid signaling molecules, arachidonic acid and lysophosphatidylcholine (Lyso-PC). PLA2 inhibition blocks, whereas exogenous Lyso-PC or arachidonic acid reproduce many of the effects of oxalate on mitochondrial function, gene expression and cell viability, suggesting that PLA2 activation plays a role in mediating oxalate toxicity. Oxalate exposure also elicits potentially adaptive or protective changes that increase expression of proteins that may prevent crystal formation or attachment. Additional adaptive responses may facilitate removal and replacement of dead or damaged cells. The presence of different inflammatory cells and molecules in the kidneys of rats with hyperoxaluria and in stone patients suggests that inflammatory responses play roles in stone disease. Renal epithelial cells can synthesize a variety of cytokines, chemoattractants and other molecules with the potential to interface with inflammatory cells; moreover, oxalate exposure increases the synthesis of these molecules. The present studies demonstrate that oxalate exposure upregulates cyclooxygenase-2, which catalyzes the rate-limiting step in the synthesis of prostanoids, compounds derived from arachidonic acid that can modify crystal binding and may also influence inflammation. In addition, renal cell oxalate exposure promotes rapid degradation of IκBα, an endogenous inhibitor of the NF-κB transcription factor. A similar response is observed following renal cell exposure to lipopolysaccharide (LPS), a bacterial cell wall component that activates toll-like receptor 4 (TLR4). While TLRs are primarily associated with immune cells, they are also found on many other cell types, including renal epithelial cells, suggesting that TLR signaling could directly impact renal function. Prior exposure of renal epithelial cells to oxalate in vitro produces endotoxin tolerance, i.e. a loss of responsiveness to LPS and conversely, prior exposure to LPS elicits a similar heterologous desensitization to oxalate. Renal cell desensitization to oxalate stimulation may have profound effects on the outcome of renal stone disease by impairing protective responses.

Keywords Kidney · Oxalate · Phospholipase A2 · Lysophosphatidylcholine · Toll-like receptors · Cyclooxygenase

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Introduction

Overview of oxalate toxicity

Kidney stone diseases represent a group of disorders that give rise to the deposition of renal calculi. Multiple risk factors are associated with nephrolithiasis; these include genetic susceptibility, metabolic perturbations (including hyperoxaluria), anatomical anomalies, bacterial infections and various dietary and environmental factors [1]. Nonetheless, with few exceptions, it still unclear how such risk factors translate into the development of kidney stone disease.

Although urine is generally supersaturated with respect to calcium and oxalate, the ionic constituents of the most prevalent form of kidney stones, the normal urine transit time is rapid enough (3–4 min) to make it unlikely that individual crystals would normally grow to a size sufficient to be trapped within the tubular lumen [2, 3]. In addition, numerous urinary molecules have been posited to play varying roles in preventing stone formation [recently reviewed in 3, 4]. Nonetheless, kidney stones do form in a significant fraction of the population [5] and much research has been devoted to elucidating the processes that tip the balance from benign microscopic urinary crystals to kidney stones.

In most idiopathic stone formers, an initial deposition of calcium phosphate on the basement membrane of the thin loops of Henle appears to form a nidus for subsequent calcium oxalate stone formation [6], although in stone disease associated with bowel resection, crystal adhesion to tubular epithelial cells may contribute to stone formation. Many investigators have suggested that underlying renal cell injury increases the likelihood of crystal attachment to renal epithelial cells, both in vivo and in renal cell cultures [4, 7–11]. Fragments of injured cells may also contribute to stone formation by providing nidi for crystal nucleation and by promoting crystal agglomeration [12, also see 4 for a recent review]. Although the mechanism(s) underlying the role for renal cell injury in stone disease have not been fully resolved, many laboratories have explored the idea that exposure to oxalate (in ionic or crystal form) elicits cell damage and thus, may contribute to the development of stones. Several recent reviews discuss this concept in greater detail [4, 14–16]. Here, we briefly review evidence that supports the notion that oxalate exposure can be toxic to renal cells and focus on more recent studies that suggest that oxalate exposure may induce changes in renal cell function that could potentially activate inflammatory responses.

Oxalate, a dicarboxylate byproduct of metabolism, is freely filtered at the glomerulus, where its concentration is normally ~1–5 μM [17]; individuals with chronic renal disease may have filtered oxalate loads that are 10–20 times higher [18]. Tubular oxalate concentrations rise in response to fluid reabsorption and oxalate secretion and can also increase acutely following oxalate-rich meals [19]. At the cortical collecting duct, oxalate concentrations may reach $\geq$300–500 μM, with even higher concentrations observed in patients with primary hyperoxaluria or following bowel resection [17]. Many laboratories have demonstrated that exposure to these oxalate concentrations can elicit a range of toxic responses at the level of renal cells.

Early evidence for renal oxalate toxicity was suggested indirectly by studies analyzing the urinary excretion of gamma-glutamyl transpeptidase, angiotensin I converting enzyme, beta-galactosidase and N-acetyl-beta-glucosaminidase in stone-forming and healthy individuals; higher than normal urinary enzyme values suggested the presence of tubular damage in patients with stones [20]. Rat models of stone disease have been used to study such damage in greater detail [4, 7, 8], revealing brush border membrane disruption, an increase in the incidence of inclusion bodies in tubular cells, release of cellular enzymes, epithelial erosion and the formation of plaques at the tips of the renal papillae. Exposing cultured renal epithelial cells to elevated oxalate concentrations elicited similar toxic effects in vitro: compromised cell integrity, release of cellular enzymes and necrotic and apoptotic cell death [see 4, 14–16 for recent reviews]. Debris from damaged cells may promote crystal nucleation and agglomeration, and may also become a part of the organic matrix that is located in all kidney stones [4]. In addition, non-lethal injury leads to changes in membrane surface properties that facilitate crystal binding to cells in vitro [8–11, 21, 22], thereby promoting crystal retention and potentially favoring the growth of stones.

Renal cell cultures have been used to demonstrate that oxalate exposure alters a number of cellular functions. For instance, oxalate exposure promotes a rapid redistribution of phosphatidylserine (PS) to the surface of renal cells [11, 21]. PS may serve as an attachment site for calcium oxalate crystals; it may also signal macrophages to engulf and remove damaged cells [23]. Oxalate exposure also activates one or more isoforms of phospholipase A2 (PLA2), enzymes that hydrolyze the acyl group from the sn-2 position of phospholipids to liberate arachidonic acid and assorted lysophospholipids (e.g. Lyso-PC) [24–27]. These bioactive lipids alter mitochondrial function [27], induce changes in gene expression [14, 15, 26] and activate caspases [27] that may participate in apoptotic cell death. Oxalate exposure also promotes free radical production and lipid peroxidation in cultured kidney cells, and antioxidants block oxalate-induced cell death and oxalate-induced changes in gene expression, consistent with a role for oxidant stress in oxalate toxicity [4, 14–16, 28, 29].

Reactive oxygen species may be produced in renal cells in response to oxalate exposure by several cellular mechanisms. Mitochondria are a major site of oxidant formation in mammalian cells [30]; in cultured renal cells, oxalate exposure perturbs mitochondrial function [27, 29] and elicits mitochondrial membrane depolarization [27], an event often preceding ROS production in other cell types [30]. The oxalate effect is mimicked in cultured renal cells by arachidonic acid and Lyso-PC and is blocked by PLA2 inhibitors [27]; indeed, isolated kidney mitochondria respond directly to these lipid signaling molecules by increasing their production of ROS and lipid peroxides [27]. Recent studies indicate that exposure to calcium oxalate monohydrate crystals induces the mitochondrial permeability transition in isolated kidney mitochondria [31], again consistent with disruption of mitochondrial function. Oxalate-induced activation of NADPH oxidase [4] may be another source of reactive oxygen molecules in renal cells. Whether oxidant stress plays any role in nephrolithiasis in vivo is still unresolved, but oxidant stress does occur in other

acute and chronic renal diseases [32] and may also accompany stone disease [33], as has been suggested by observations of elevated urinary lipid peroxides in patients with calcium oxalate nephrolithiasis [34, 35]. Moreover, experimental conditions that increase urinary oxalate loads in rats also increase urinary lipid peroxides and decrease renal antioxidants [33], consistent with a role for oxidant stress during hyperoxaluric conditions.

Although oxalate exposure elicits a range of toxic effects, potentially adaptive responses have also been observed. For instance, oxalate exposure elicits limited proliferation in cultured renal cells [reviewed in 4, 14, 15]; this process may facilitate replacement of damaged/dead cells. Other potentially adaptive responses include changes in gene expression and synthesis of molecules that may inhibit crystal attachment or stone formation [4, 14–16]. For instance, oxalate exposure increases expression of osteopontin (OPN) in renal cells, both in vivo and in cultured cells [4, 14–16, 36–38]. This urinary macromolecule inhibits nucleation, growth, aggregation and cellular attachment of calcium oxalate crystals [39]. As described below, OPN also plays significant roles in inflammation. Oxalate-induced expression of this and other urinary macromolecules, including prothrombin, bikunin, fibronectin, and matrix gla protein [4, 14–16], may serve adaptive functions by limiting crystal nucleation, growth and deposition.

Oxalate and immune/inflammatory systems

The pathophysiology of stone disease is likely to extend beyond the changes that have been observed in renal epithelial cells. There is increasing evidence for the involvement of inflammatory and immune responses in both experimental stone disease models, and in kidneys from patients with acute and chronic oxalosis [see 16 for a recent review]. Histological evidence indicates an increased infiltration of immune cells in kidneys of rats rendered transiently hyperoxaluric by ingestion of low levels of ethylene glycol and ammonium chloride [40, 41]. In particular, there are increased numbers of cells expressing the leukocyte common antigen (CD45), the ED1 antigen (specific for monocytes and tissue macrophages), and cells expressing MHC-II [40, 41]. Crystals are often detected in these immune cells, raising the possibility that macrophages and multinucleate giant cells play a role in crystal disposition [41].

It is likely that the signal for immune cell infiltration in hyperoxaluric rat kidneys is derived from renal epithelial cells, which may secrete cytokines, lipids or other proteins that promote immune cell migration and activation. Experimental hyperoxaluria (induced in rats by ethylene glycol) alters renal expression of many genes associated with immune/inflammatory systems, including cytokines and other immune-related genes (CD24, MHC-II and lipocortin 1 (annexin I), and interferons [42, 43]. As described above, OPN is also dramatically increased by oxalate exposure in kidney stone disease models [4, 14–16, 36–39, 42, 43]. Beyond its role as an inhibitor of crystallization, OPN is a well-characterized modulator of immune function, capable of recruiting immune cells to the site of injury [44] and stimulating cytokine production [39]. Other molecules such as prothrombin and inter-alpha-trypsin inhibitor (induced in kidney cells by oxalate exposure) may play roles not only as inhibitors of crystallization, but also in inflammation-mediated tissue repair [4, 14–16, 45].

The renin-angiotensin system has also been implicated in the renal immune cell influx and the tubulointerstitial injury that accompany many kidney pathologies, including kidney stone disease models. In addition to its well-known function in control of blood pressure and intrarenal hemodynamics, angiotensin II promotes tubulointerstitial fibrosis by increasing expression of cytokines and chemokines, promoting infiltration of immune cells, stimulating cellular proliferation, and promoting apoptosis [see 46, 47 for reviews]. A number of studies [48–51] have demonstrated that angiotensin converting enzyme (ACE) inhibitors can block the progression of tubulointerstitial lesions in a rat hyperoxaluria model, suggesting an involvement of angiotensin II in stone disease. Other investigators did not observe any ameliorating effects of ACE inhibition (under slightly different experimental conditions) on immune cell infiltration in hyperoxaluric rat kidneys however [52], suggesting that further work is needed to clarify the role of the renin-angiotensin system in stone disease.

Because of the complexity and diversity of cells within the kidney, it is difficult to determine which cells (and which signals) are responsible for recruitment of immune cells to the hyperoxaluric kidney in vivo. Cell culture experiments have provided important clues, indicating that in response to oxalate exposure, renal epithelial cells can produce a variety of cytokines and chemoattractants with the potential to recruit immune cells. In particular, oxalate exposure (in ionic or crystalline form) increases expression of OPN in renal cell cultures; as described above, OPN is known to recruit immune cells in vivo [44]. Other studies have demonstrated that oxalate (or calcium oxalate crystal) exposure induces expression of monocyte chemoattractant protein-1 (MCP-1) in cultured renal epithelial cells [53]. MCP-1 is a cytokine with potent chemoattractant activity. It is noteworthy that cytokine involvement in stone disease is also suggested by observation of significant elevations in urinary levels of the cytokine IL-6 in stone patients [54]; the source of this IL-6 (renal vs immune cells) has not yet been resolved.

Caspases provide yet another potential link between hyperoxaluria and inflammation: in addition to their well-known role in mediating the execution of apoptosis, caspases also activate proinflammatory cytokines [55]. Recent studies indicate that oxalate exposure increases caspase activity in renal epithelial cells, as detected by labeling with CaspaTag, a carboxyfluorescein derivative of a broad-spectrum caspase inhibitor [27]. More information is needed to identify the caspases that

are activated in renal cells by oxalate exposure and to determine whether these caspases can activate cytokines.

Taken together, data from renal cell culture studies suggest that oxalate exposure can induce a variety of different molecules known to recruit immune cells. The present studies have pursued this idea further, asking if oxalate exposure could stimulate two additional pathways with potential roles in modulating immune and or inflammatory responses. The rationale for studying the influence of oxalate exposure on cyclooxygenase-2 (COX-2), an enzyme that catalyzes the rate-limiting step in prostaglandin synthesis, and on toll-like receptors (TLRs), molecules that play crucial roles in innate immunity, is described below.

Cyclooxygenase

Cyclooxygenase (COX) or prostaglandin G/H synthase (PGHS) is an oxidoreductase that converts arachidonic acid to PGH2, the precursor to prostaglandins (PG), thromboxanes and prostacyclins [56]. Of the two identified COX genes, COX-1 is expressed constitutively whereas COX-2 can be expressed constitutively or be rapidly induced in response to a variety of agents that include proinflammatory cytokines, hormones, growth factors and environmental stressors [56]. In kidney, COX-2 expression is regulated by sodium and water uptake, medullary tonicity, adrenal steroids and various growth factors and chemokines [57]. Prostanoids formed in response to COX-2 activation help to preserve renal blood flow and glomerular filtration, stimulate natriuresis and promote renin secretion during conditions of low-sodium intake. Renal COX-2 may also play roles in renal diseases since it is often upregulated in a variety of renal pathologies [58]. Prostanoids have well-characterized effects on inflammatory and immune cells that are mediated by a family of receptors with differing ligand specificities and different tissue distributions [recently reviewed in 59]. Prostanoid effects may be either proinflammatory or anti-inflammatory, depending upon the prostanoid, the receptors and the cells which contain the receptors.

As indicated above, PLA2 activation is an early response to renal cell oxalate exposure, and a number of the toxic responses to oxalate (alterations in cell viability, gene expression, mitochondrial function and oxidant stress) can be mimicked by exogenous Lyso-PC and/or by exogenous arachidonic acid, lipids that are liberated by PLA2 activation [14, 15, 24–27]. In many cells, PLA2 activation occurs in concert with stimulation of downstream prostaglandin production [60], suggesting that in addition to direct effects of the lipids released by PLA2 activation, downstream conversion of arachidonic acid to other bioactive lipids may also occur. Thus, we asked if exposure to oxalate could regulate renal cell COX-2 gene expression in cultured kidney cells. We also asked if Lyso-PC, one of the lipids generated in response to PLA2 activation (and shown previously to induce immediate early gene expression, [26]) would regulate COX-2 expression in MDCK cells.

Toll-like receptors

Toll-like receptors (TLRs) are a family of at least ten different receptors that are conserved evolutionarily and recognize pathogen-associated molecular patterns (PAMPs) from many bacteria and viruses. Activation of TLRs induces complex intracellular events that upregulate the expression of proinflammatory genes, thereby regulating innate and adaptive immune responses [see 61–63 for recent reviews]. TLR ligands are quite diverse and include microbial components such as endotoxin [also known as lipopolysaccharide (LPS), a component of the membrane of gram-negative bacteria that is heat stable and toxic]; LPS binds to TLR4 and can cause septic shock. Other pathogen-associated TLR ligands (and their receptors) include lipoproteins and peptidoglycans (binding to TLR1, TLR2, TLR6), viral double stranded RNA (binding to TLR3), and bacterial and viral unmethylated cytosine-guanosine dinucleotide (CpG)-DNA (binding to TLR9). Endogenous molecules may also serve as TLR ligands. These molecules may include heat-shock proteins, necrotic cells, endogenous CpG-DNA, reactive oxygen species and extracellular matrix molecules such as heparan sulfate, fibrinogen and hyaluronin [reviewed in 63, 64]. A potential caveat in any discussion of endogenous TLR ligands is the often unrecognized risk that low-level contamination by LPS or other TLR ligands may account for the TLR ligand activity in substances being tested as potential TLR ligands [64].

TLR activation rapidly stimulates multiple, complex signaling pathways, many of which activate/mobilize members of the NF-κB transcription factor family, and ultimately increase expression of antimicrobial peptides, inflammatory cytokines, and other molecules that initiate adaptive immunity. TLRs are selectively expressed on subsets of leukocytes, but endogenous TLR are expressed in many non-immune cells.

Renal tubular epithelial cells are among the non-immune cells that express TLRs (to date, TLR1, 2, 3, 4, and 6 have been identified in kidney cells. TLR activation has been associated with many different renal pathologies, including bacterial pyelonephritis, sepsis, transplant nephropathy, antigen-induced immune complex glomerulonephritis and lupus nephritis [63]. Although a potential role for TLR in stone disease has been relatively unexplored, renal stones (both infection and non-infection stones) contain appreciable amounts of endotoxin (the ligand for TLR4) [65]. Stone-associated endotoxin has been associated with the sepsis that occasionally occurs after stone manipulation [66]. These links between TLR and renal pathologies prompted us to explore a potential role of TLR in the renal cell responses to oxalate. To this end, we assessed the

expression of several TLRs in cultured human renal epithelial cells, assessed the impact of oxalate exposure on a cellular pathway known to be activated by LPS, and determined whether activation of that pathway by a TLR4 ligand could be modulated by prior oxalate exposure.

Materials and methods

Studies of COX-2 gene expression were performed on canine MDCK cells (obtained from ATCC; Manassas, Va.). MDCK cells (80% confluent) were rendered quiescent by serum starvation for 48 h [26]. To initiate experiments, media were exchanged for serum-free DMEM containing agents of interest. Where indicated, sodium oxalate was added at a concentration of 1 mM (total), equivalent to free oxalate levels of 350 μM, and relative supersaturation levels (RSS) for calcium oxalate of 24.9, determined using the EQUIL program [67]. Additional studies examined the response to 20 μM Lyso-PC for 1, 2 or 4 h. Lyso-PC was dissolved in dimethylsulfoxide just prior to use.

Northern blot analysis of COX-2 mRNA

Total RNA was extracted from cells using RNeasy Mini Kits (Qiagen, Valencia, Calif.) as previously described [26]. RNA was size-fractionated on denaturing 1% agarose gels (10 μg total RNA per lane), transferred by capillary blotting to nitrocellulose (Hybond-C, Amersham Pharmacia Biotech, Piscataway, N.J.), then immobilized by baking at 80°C for 2 h. Blots were prehybridized for 2 h at 42°C, then hybridized overnight to a [^{32}P]dCTP-labeled COX-2 cDNA probe that was prepared by random primer labeling. Blots were washed stringently, and COX-2 mRNA abundance was assessed. The human COX-2 cDNA (prostaglandin G/H synthase 2; PGHS-2) was a gift from Dr. D. DeWitt, Michigan State University and contained the full-length human COX-2 cDNA. This cDNA hybridizes to a 4.5 kb mRNA species in MDCK cells. COX-2 hybridization was assessed by phosphorimage analysis, using a Bio-Rad Molecular Imager (Hercules, Calif.). Quantitative analysis of mRNA abundance was performed using Multi Analyst program (Bio-Rad, Hercules, Calif.). COX-2 mRNA abundance was corrected to the abundance of 18S RNA (assessed by ethidium bromide staining of gels prior to blotting). All experiments were performed at least three times. Prior to data analysis, COX-2 mRNA data were corrected to 18S RNA abundance, then normalized to respective time point controls (i.e., COX-2 mRNA in MDCK cells exposed to media alone for 1, 2 or 4 h). Because variance around the arithmetic means was not normally distributed, normalized hybridization data were logarithmically transformed prior to analysis, then converted back to antilogarithms and plotted on an arithmetic scale. Data were analyzed by one-way ANOVA using SuperAnova statistical software (Abacus Concepts, Berkeley Calif.) and Duncan's post-hoc test; results were considered significant if $P < 0.05$.

Western blot analysis of COX-2 protein

COX-2 protein expression was assessed by Western blot analysis. MDCK cells (not serum-starved) were treated with 350 μM oxalate 0, 2 or 4 h, proteins isolated and fractionated by PAGE, transferred to nitrocellulose and probed with rabbit anti-murine COX-2 polyclonal antiserum (Cayman Chemical, Ann Arbor, Mich.).

TLR studies

Studies of TLR receptors used cultured human renal proximal tubular epithelial cells (RPTEC), a commercially available line of kidney epithelial cells (Cambrex, Baltimore Md.). Cells were weaned from the proprietary medium (REGM Renal Epithelial Cell Medium, Cambrex) to DMEM containing 10% FBS, 100 IU/ml penicillin, 100 μg/mlstreptomycin, 1 mM glutamine and 1× MEM-non-essential amino acids. To determine whether TLR are expressed in human RPTEC cells, cells were dispersed with trypsin, 1×10^6 cells/sample were incubated with fluorescein isothiocyanate (FITC) labeled anti-human Toll-like receptor 2 (anti-TLR2-FITC), phycoerythrin (PE) anti-human Toll-like receptor 4 (anti-TLR4-PE), isotype control antibody labeled with FITC or unstained; antibodies were obtained from eBioscience (San Diego, Calif.). Samples were fixed with 2% paraformaldehyde and analyzed by FACS using a FACSCalibur Flow Cytometry System (Becton Dickinson, Franklin Lakes, N.J.). Data were analyzed using the FlowJo analytic program (TreeStar, San Carlos, Calif.).

TLR4 signaling was assessed indirectly by Western blotting for IκBα degradation. TLR receptor activation initiates a complex series of intermediary steps that converge on degradation of IκB, an endogenous inhibitor of the transcription factor NF-κB [61]. Degradation of IκBα enables NF-κB to mobilize to the nucleus, where it promotes selected gene transcription. RPTEC cells were grown to 90% confluence treated with LPS (10 μg/ml), a known activator of TLR4 with oxalate (500 μM total or 175 μM free, prepared in endotoxin-free water) or with Lyso-PC (20 μM) for 0–90 min, cells were lysed and proteins (20 μg /lane) were fractionated on a 4–12% gradient SDS gel. Proteins were transferred to Immobilon membranes (Millipore, Billerica Mass.), hybridized with anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, Calif.; 1:2000 dilution), then hybridized to goat anti-rabbit HRP (Santa Cruz Biotechnology; 1:2000 dilution). Bands were visualized with ECL Plus chemiluminescence (Amersham Biosciences). To determine whether cross-tolerance could be observed in renal

epithelial cells, RPTEC cells were pretreated with LPS, oxalate, or Lyso-PC for 120 min, then stimulated with an alternative activator for 0–90 min; IκBα Western blotting was performed as described above. Following hybridization with anti-IκBα, blots were stripped and rehybridized with an anti-actin antibody (Santa Cruz Biotechnology) to assess protein loading.

Results

Cyclooxygenase

Oxalate exposure induced COX-2 mRNA in MDCK cells. As shown in the Northern blot in Fig. 1A, exposure to 350 μM free oxalate for 4 h increased the abundance of COX-2 mRNA. A time course response to oxalate exposure is shown in Fig. 1B. Modest increases in COX-2 mRNA were noted within 1 h and significant increases in COX-2 mRNA were evident at 2 h; further increases in COX-2 mRNA were seen at 4 h. As shown in Western blot (Fig. 1C), COX-2 protein was also induced in MDCK cells in response to oxalate exposure. Immunoreactive COX-2 protein was increased at 2 h and showed some decline at 4 h although it was still elevated above control (time zero) levels. Exposure to Lyso-PC 20 μM also increased COX-2 mRNA abundance in MDCK cells, showing a similar time course to the induction of COX-2 by oxalate (Fig. 1D).

Toll-like receptors

As shown by the representative scans in Fig. 2, RPTEC cells expressed both TLR2 and TLR4, as indicated by FACS analysis of cells stained with antibodies to TLR2 (left panel) and to TLR4 (right panel).

To investigate potential TLR signaling pathways, RPTEC cells were stimulated with unpurified LPS (which stimulates both TLR4 and TLR 2), with oxalate or with Lyso-PC, and IκBα degradation was assessed via Western blot analysis. Results from a typical experiment are shown in Fig. 3. The left panels examine the initial or "normal" response to each agent. Exposure to LPS (Fig. 3A) or to oxalate (Fig. 3B) each induced IκBα degradation in RPTEC cells. The LPS response occurred more rapidly (maximal at 15–30 min) than did the re-

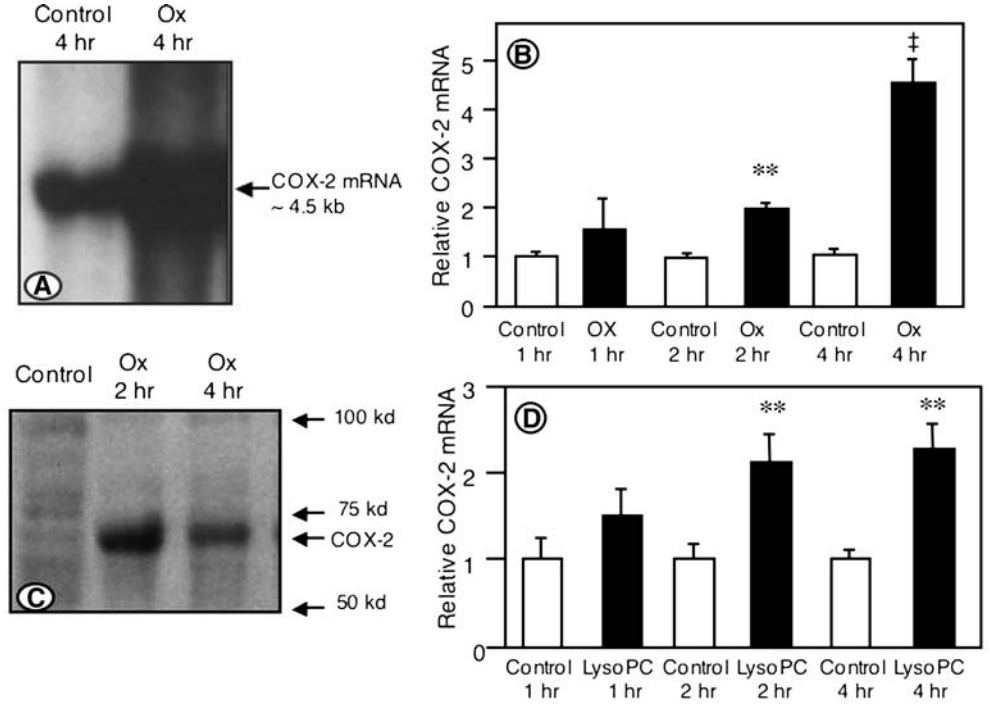

Fig. 1 Oxalate stimulates COX 2 mRNA in MDCK cells. **A** MDCK cells were treated with media (*Control*) or with 350 μM free oxalate (*Ox*) for 4 h; COX-2 mRNA was assessed by Northern blot. Oxalate induced an increase in COX-2 mRNA at 4 h. **B** Time course of COX-2 mRNA induction by oxalate. MDCK cells were treated with media (*white bars*) or 350 μM free oxalate (*black bars*) for 1, 2 or 4 h, and COX-2 RNA abundance was determined by Northern blot using a human COX-2 probe. Data were corrected for 18S RNA abundance, then normalized to media controls for each time point. Each *bar* depicts the mean ± SEM relative COX-2 mRNA abundance for 4–12 independent experiments. ** $P < 0.01$ vs respective time point control; ‡ $P < 0.01$ vs the same treatment at 2 h. **C** MDCK cells were treated with oxalate (350 μM free) for 0 2 or 4 h; proteins fractionated by PAGE and COX-2 expression were assessed by Western blot. Blots were incubated for 1 h with a rabbit anti-murine COX-2 polyclonal antiserum, washed, then incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence. Oxalate exposure induced expression of COX-2 protein within 2 h. **D** Lyso-PC induction of COX-2 mRNA. MDCK cells were treated with media or with 20 μM Lyso-PC for 1, 2 or 4 h; COX-2 mRNA abundance was assessed by Northern blot as above. Each *bar* depicts mean ± SEM COX-2 mRNA abundance (normalized to 18S RNA) for 3–5 independent experiments. ** $P < 0.01$ vs control

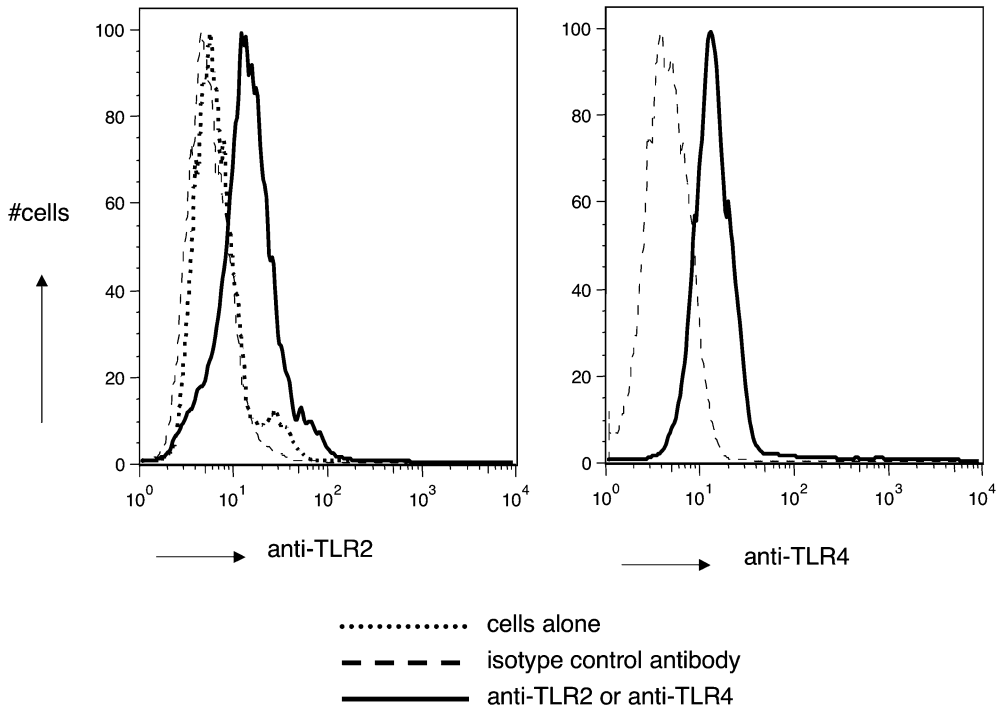

Fig. 2 Fluorescence activated cell sorting (FACS) was used to identify the presence of TLR receptors in RPTEC cells. *Left panel* TLR2 receptors were assessed after staining 10^6 RPTEC cells with anti-TLR2-FITC (*solid line*). An isotope control FITC-labeled antibody was used as a control (*dashed lines*). *Dotted lines* represent unstained cells. *Right panel* TLR4 receptors assessed by staining 10^6 RPTEC cells with anti-TLR4-PE. The PE-labeled isotype control profile (not shown) was identical to that for unstained cells

sponse to oxalate (which was maximal at 30–60 min); the LPS response also appeared to be more robust under these experimental conditions, as evidenced by a greater depletion of IκBα. In both LPS-treated and oxalate-treated RPTEC cells, IκBα protein recovered to starting levels by 90 min post-stimulation. In contrast to the response seen following LPS and oxalate exposures, treatment with Lyso-PC had no effect on this pathway.

Since both LPS and oxalate signal through NF-κB, we hypothesized that prior exposure to LPS could cause subsequent loss of cellular response (tolerance) to other stimuli, such as oxalate, thereby impeding protective cell responses. This concept of tolerance has been well-described in immune cells [61, 62]. In macrophages and monocytes, an initial exposure to LPS (a major structural component of Gram-negative bacteria) elicits a robust production of various inflammatory cytokines that are essential for defending against bacterial pathogens—indeed the response may be so robust as to produce septic shock. This response is followed by a hyporesponsive state, in which subsequent exposure to LPS fails to elicit a cellular response. This desensitization, known as LPS (or endotoxin) tolerance, is manifested by a decline in the production of inflammatory cytokines and involves alterations in the complex signaling cascades utilized by TLR receptors [61, 62]. Tolerance potentially serves a protective role by upregulating feedback inhibitors of inflammation, thereby limiting potentially damaging inflammatory responses. It is noteworthy that LPS-tolerance can be also be induced by other TLR ligands (whose effects are mediated by TLR receptors other than TLR4, the receptor for LPS), this process is called cross-tolerance. Given the finding that oxalate mimics certain aspects of LPS actions in renal epithelial cells, we wondered if oxalate exposure would produce a loss of responsiveness to LPS and, conversely, if LPS would induce tolerance to oxalate? In our assay system, evidence for tolerance would be a failure to degrade IκBα.

As shown in Fig. 3D, 2 h pretreatment with LPS abrogated the oxalate-induced IκBα breakdown. Similarly, prior (2 h) exposure to oxalate inhibited the LPS response (Fig. 3E). Previous Lyso-PC exposure did not inhibit the LPS response (Fig. 3F) further confirming that Lyso-PC does not activate this pathway.

Discussion

The present studies demonstrate that oxalate exposure increases the expression of COX-2 mRNA in MDCK cells. In kidney, COX-2 expression is normally regulated by various physiological stimuli including sodium and water uptake, medullary tonicity, adrenal steroids and various growth factors and chemokines [58]. Prostanoids formed in response to COX-2 activation preserve renal blood flow and glomerular filtration, stimulate natriuresis and regulate renin secretion. COX-2 expression can be upregulated in various renal diseases including diabetic/reflux nephropathies [58]. COX-2 can also be upregulated in cultured renal cells by a diverse variety of agonists and stressors including mechanical stress [68], phorbol esters [69] and hyperosmolarity [70]. The present studies suggest that COX-2 expression is also increased in cultured kidney cells in response to the stress of oxalate exposure. Lyso-PC, a lipid signaling molecule produced following PLA2 activation, mimics the effects of oxalate

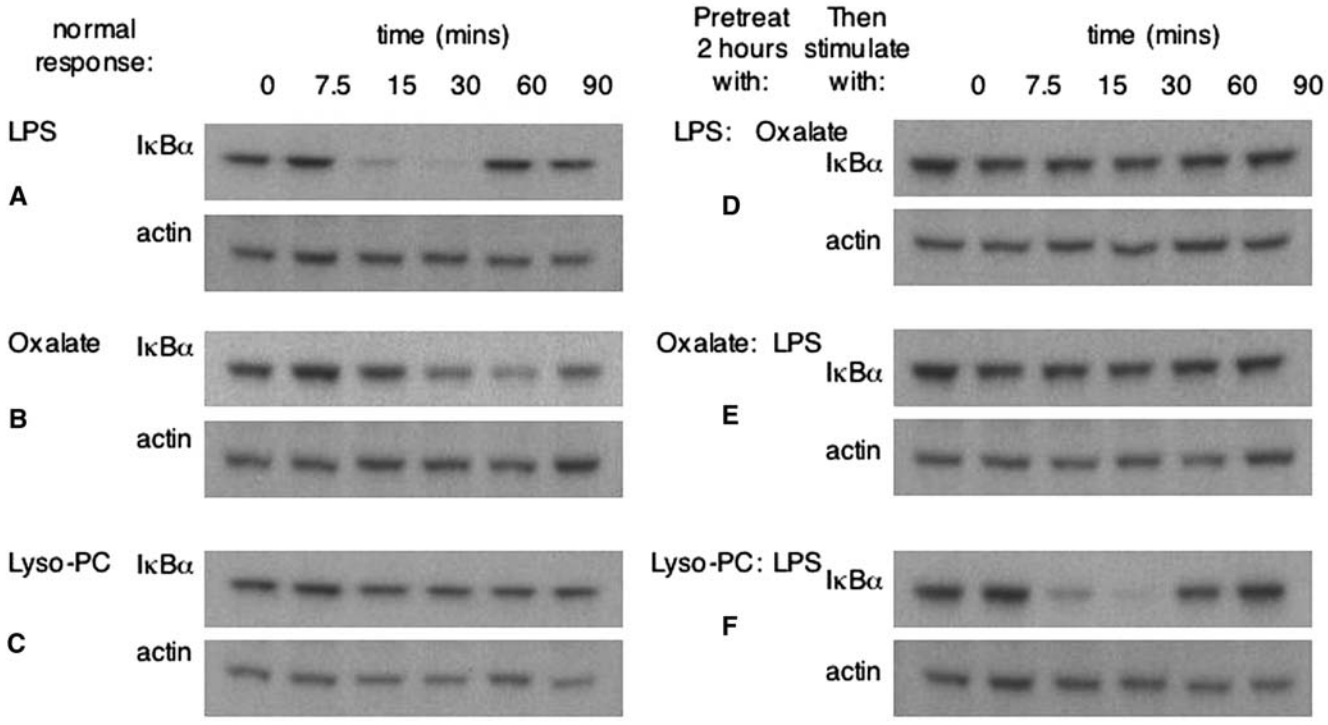

Fig. 3 IκBα degradation in RPTEC cells. **A**–**C**) IκBα degradation in response to initial treatment with LPS, oxalate or Lyso-PC. Cells were treated with LPS (10 μg/ml) oxalate (175 μM free) or Lyso-PC (20 μM) for 0–90 min. Cells were harvested at the indicated time points, protein was isolated and then analyzed by Western blot. Blots were probed with an IκB antibody which recognizes both alpha and beta forms of IκB; only the alpha form is shown here. Blots were stripped and rehybridized to an actin antibody to monitor protein loading. LPS (**A**) and oxalate (**B**) but not Lyso-PC (**C**) induced breakdown of IκBα. **D**–**F** Assessment of tolerance in RPTEC cells. Cells were pretreated with LPS (**D**) oxalate (**E**) or Lyso-PC (**F**) for 2 h, then cells were challenged with a heterologous agent for 0–90 min. LPS pretreatment induced tolerance to oxalate, as determined by a lack of IκBα degradation (**D**). Similarly, oxalate pretreatment rendered RPTEC cells tolerant to a subsequent LPS challenge (**E**). Pretreatment with Lyso-PC was without effect: LPS initiated normal IκBa degradation despite Lyso-PC pretreatment (**F**), again indicating that Lyso-PC does not activate this pathway

on COX-2 mRNA expression [71, and unpublished observations]; the precise mechanism by which Lyso-PC induces COX-2 in renal cells awaits further study.

Potential roles for prostaglandins in stone disease have been relatively unexplored. Prostaglandins play various roles as both proinflammatory and anti-inflammatory molecules via direct interaction with immune cells, and could potentially interact with immune cells recruited to the kidney [58]. An additional role for prostaglandins in stone disease has been suggested by several studies from Lieske's group [10, 72] that demonstrate a direct role for prostaglandins in altering the binding and internalization of calcium oxalate monohydrate (COM) crystals in cultured renal cells. Exposure to exogenous PGE-1 or PGE-2 dramatically decreased the binding of COM crystals to MDCK cells in a process that depended upon intracellular cyclic AMP [10, 72]. Processes that prevent crystal adhesion may defend the kidney against crystal retention and stone formation. The present studies suggest that the induction of COX-2 by oxalate in renal cells (and the downstream formation of prostaglandins) could represent one such adaptive mechanism. Whether COX-2 expression or prostanoid formation play any roles in the course of stone disease will require careful studies in animal models and patients.

The present studies demonstrate the presence of TLR2 and TLR4 on cultured human proximal tubular epithelial cells and add to the growing body of evidence indicating that renal epithelial cells can play immune roles. Renal tubular epithelial cells can function as antigen-presenting cells, and the processing of native antigen and the presentation of peptides bound to MHC class I and II products on tubular epithelial cells increases their ability to interact with T cell antigen receptor; this process may be enhanced by locally produced cytokines [73]. Recent work supports a role for tubular epithelial cells in the process of innate immunity. For instance, TLR2 and TLR4 are constitutively expressed in murine proximal and distal tubular renal epithelial cells in vivo and are upregulated during renal inflammation [74]. The strategic epithelial localization of TLRs renders these cells well-poised to detect and react with bacteria that find their way into the tubular lumen. A major defensive role of TLRs in immune cells is to produce cytokines, and this also appears to be the case in tubular epithelial cells following TLR activation [75]. It is noteworthy that cytokines are also produced in the course of non-pathogen mediated renal injury (such as ischemia-reperfusion injury). These cytokines mediate leukocyte recruitment during renal inflammation [63, 76] and infiltrating leu-

kocytes may exacerbate renal damage by releasing additional inflammatory factors. While down-regulation of the chemokine signal will allow resolution of the inflammatory reaction, ongoing or repeated renal injury would maintain cytokine production and the inflammatory process, promoting renal disease progression. Whether such cytokine production depends upon TLR activation in sterile (i.e., non pathogen-mediated) renal inflammation, and if so, whether endogenous TLR ligands play a role, will require further study.

The present studies demonstrate that oxalate and LPS (a TLR4 agonist) each induced the degradation of IκBα in human proximal tubular cells in vitro. These responses are transient; within 90 min IκBα levels were restored to control levels. It is noteworthy that following subsequent exposures to heterologous agents, little IκBα breakdown was observed, consistent with the development of tolerance The predicted consequence of such tolerance would be a decreased activation of NF-κB and a reduced expression of genes that are dependent on NF-κB activation, although this prediction will need to be assessed more directly. It is perhaps significant, however, that endotoxin has been detected in the matrix of many kidney stones, even non-infection stones [65], a finding that suggests that the kidney may often be 'primed' for the development of tolerance. In any case, the present studies indicate that oxalate, directly or indirectly, affects toll-like receptors and their intracellular target, IκBα, in cultured renal cells and thereby alters the responsiveness of the cells to LPS, the TLR4 ligand. Since these effects were observed in cultured renal epithelial cells, they clearly do not depend on the presence of immune cells such as macrophages or T cells. Although it is clearly premature to extrapolate these in vitro studies to the potential influences of oxalate or LPS on kidney TLR receptors in vivo, they raise the possibility that tolerance could lead to a dampening of the normal compensatory responses that would occur with oxalate exposure alone, e.g., this tolerance may inhibit the normal upregulation of genes that confer protection from oxalate toxicity. This will warrant further study.

Surprisingly, exposure of renal cells to Lyso-PC, which mediates many other actions of oxalate [14, 15, 21, 24–27], had no effect on IκBα degradation, a finding that suggests that the actions of oxalate on the TLR pathway may be upstream of the activation of the PLA2 activation or, alternatively, that they are mediated by an additional action of oxalate. Such an action may be a direct effect of oxalate or could be secondary to the oxalate-induced production of other potential activators of TLR, such as necrotic cells, reactive oxygen species, heparan sulfate or other extracellular matrix constituents [63, 64].

Although there is still some disagreement about the significance of oxalate toxicity in the progression of calcium oxalate kidney stone disease, in vitro studies of cultured renal cells suggest that short-term exposure to hyperoxaluric conditions (minutes to hours) elicits a number of alterations in renal cell lipid metabolism, mitochondrial function, gene expression and effectors of inflammatory responses. Whether oxalate exposure initiates comparable changes in vivo, and if so, how renal (and immune) cells respond to these changes, whether these responses persist over longer-term exposure to oxalate and whether (and how) these responses play a role in the course of stone disease will require further study.

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