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The influence of oxalate on renal epithelial and interstitial cells

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Abstract Most renal stones in humans are composed of calcium oxalate. An increase in urinary oxalate levels has been shown to result in renal epithelial cell injury and crystal retention. However, the underlying mechanisms are unclear. Although the localization of primary stone formation and the associated cells playing the pivotal role in stone formation are still unknown, renal epithelial cells and interstitial cells seem to be involved in this process. The aim of this study was to evaluate the effects of oxalate on distinct renal epithelial and endothelial cells as well as fibroblasts. The first part focused on the toxicity of oxalate on the cells and a potential time- and dose-dependency. In the second part, renal epithelial cells were cultured in a two-compartment model to examine the vulnerability of the tubular or basolateral side to oxalate. LLCPK1, MDCK, renal fibroblast and endothelial cell lines were cultured under standard conditions. In part 1, cells were grown in standard culture flasks until confluent layers were achieved. Sodium oxalate was delivered at final concentrations of 1, 2 and 4 mM to either the apical or basolateral side (plain medium was delivered to the contralateral side). Cell survival was assessed microscopically by trypan blue staining after 1, 2 and 4 h. The influence of oxalate on proliferation and apoptosis induction was also investigated. In the second part, MDCK and LLCPK1 cells were grown in 6-well plates until confluent layers were achieved. Sodium oxalate at the above concentrations was applied, to either the apical or basolateral side and plain medium was delivered to the opposite side. The same protocol was then followed as in part 1. Part 1: sodium oxalate led to a time- and concentration-dependent decline in cell survival that was comparable in LLCPK1 and MDCK. Non-tubular cell lines like fibroblasts and endothelial cells were significantly more vulnerable to oxalate. These observations were reflected by significant impairment to cell proliferation. We could not demonstrate an induction of apoptosis in any cell line. Part 2: both cell lines were more vulnerable to oxalate on the basolateral side. This effect was more pronounced in MDCK cells at high oxalate concentrations (4 mM). Cells are apparently more resistant on the apical (tubular) side. Our results show that sodium oxalate has a negative effect on the growth and survival of renal epithelial cells and, to a greater extent, also fibroblasts and endothelial cells. We could not demonstrate any induction of apoptotic processes which implies a direct induction of cell necrosis. The finding of interstitial calcification and the proximity of tubules, vessels and interstitial cells make involvement of non-tubular renal cells in tissue calcification processes possible. Renal epithelial cells are apparently more vulnerable to oxalate on their basolateral side. Therefore, calcification processes within the interstitium may exert pronounced toxic effects to these cells, leading to inflammation and necrosis. These observations further support the idea of the interstitium as a site of primary stone formation.

Keywords Oxalate · LLCPK1 · MDCK · Fibroblasts · HUVEC · Urinary calculi

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Introduction

High levels of calcium oxalate (CaOx) are not only found in the urine of stone formers, but also in that of healthy humans [1]. CaOx is the main component of

most renal stones. It has been shown that high levels of urinary oxalate cause renal epithelial cell injury and crystal retention [2]. However, the underlying mechanisms and the exact formation sites of stones still remain unclear. Although reports have been made on the remains of renal tubules found within stones, implying an early intratubular crystallization process [3], free crystallization alone is doubtful. Urine remains for only 3–5 min within the renal tubules and is generally undersaturated with CaOx before reaching the collecting ducts [4]. Furthermore, in vitro crystallization studies have disclosed that the intratubular precipitation of calcium phosphate (CaPh) crystals, which are suggested to promote the nucleation of CaOx, takes hours [5]. Several investigators have described an enhanced crystal attachment to injured tubular cells, an effect that could promote fixed stone formation within the renal tubules [6, 7]. As subepithelial plaques are often found in renal papillae, there is the possibility that the renal interstitium is the primary side of stone formation [8]. These observations can also be explained by the secondary deposition of crystals outside of the collecting system. Apart from the side on which the initial crystallization process occurs, CaOx crystals or microliths could be deposited into the renal interstitium by either the endocytosis of crystals and consecutive cell death, or by the overgrowth of epithelial cells [2, 9, 10]. This can lead to edematous swelling of the renal matrix, proliferation of interstial cells and the induction of an inflammatory reaction [7, 10, 11, 12, 13]. Meanwhile, the crystals increase in size and destroy the surrounding cells until the urinary stone penetrates the papillary surface and enters the renal calyx. Although the renal interstitium obviously plays a significant role in the pathogenesis of urinary calculi, only minor attention has been paid to the interaction of CaOx with renal interstitial cells, e.g. fibroblasts or endothelial cells which are located in close proximity. Recently, Evan et al. demonstrated that in CaOx stone patients, precursors of later urinary stones are found near the thin loops of Henle within the interstitium [14]. Therefore, we compared the vulnerability of non-tubular and tubular renal cells to oxalate. Furthermore, oxalate deposited in the renal interstitium could possibly exert more distinct toxic effects on tubular cells from the basolateral side. For this reason, the role of tubular cell polarity was examined with respect to oxalate toxicity using a two-compartment model.

Methods

Cell culture

Two renal epithelial cell lines were used: LLCPK1 from porcine kidneys, which represented proximal tubular cells, and Madin-Darby canine kindney (MDCK) cells, which represented distal tubules and collecting ducts, both supplied by the European Collection of Cell Cultures (ECACC, UK). Renal fibroblast cell lines were also supplied by ECACC. LLCPK1 and MDCK cell lines were cultured in DMEM containing 10% FCS and 4 mM glutamine. The renal fibroblasts were cultured in RPMI containing 10% FCS and 4 mM glutamine. The endothelial cells (human umbilical vein endothelial cells, HUVEC) were cultured in medium containing 10% FCS at 37°C in a 5% CO_2 air atmosphere incubator. Cells were subcultured by dissociation with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid. The pH value was adjusted to 7.4. Cells were grown to confluence and weaned from serum to defined DMEM/F12. Cells were further weaned from defined DMEM/F12 media to acclimatization media, which is the same as the defined DMEM/F12 media, but without sodium pyruvate.

Oxalate toxicity experiments

Sodium oxalate was added in final concentrations of 1, 2 and 4 mM to the cell medium. In controls, the medium was changed without the addition of oxalate. Every single experiment was repeated ten times. Cell survival was assessed microscopically by trypan blue staining 1, 2, 4 and 24 h after oxalate delivery using a Neubauer counting chamber. Cell proliferation was determined by the MTT assay, which is widely used to measure the cell proliferation rate and to screen for anticancer drugs. The cells were grown for 1,2, 4 and 24 h after oxalate exposure. The assay is based on a reduction of the tetrazolium salt, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by actively growing cells to produce a blue formazan product (Cell titer96 Aqueous One Solution Proliferation Assay, Promega, Germany). In this one step assay, MTS tetrazolium was added to the cells and incubated for 2 h at 37°C, followed by absorption determination at 490 nm. The quantity of absorption correlates with the number of proliferating cells. Apoptosis was detected by the determination of phosphatedylserine (PS), which is translocated from the inner side of the plasma membrane to the outer layer of the cell during cell death. After trypsinisation, FITC-labeled annexin V, a calcium-dependent phospholipid-binding protein with high affinity for PS, was added to discriminate healthy from dying cells. Propridium iodide, a DNA stain, was used simultaneously to distinguish apoptotic from necrotic cells. We used a commercially available kit (Annexin-V-FLUOS staining kit, Roche Diagnostics, Germany) that has been described extensively [16]. The cells were then analyzed by flow cytometry (FACScalibur, Becton Dickinson), gating on F4/80 negative tubule cells to distinguish between macrophages and tubule cells. Apoptosis-associated fluorescence was measured using a log scale. Cells with high propidium iodide content were necrotic and were deleted from the analysis. As a positive control, cisplatin (Medac, Germany, 10^{-4} M) was added instead of oxalate to the cells.

Cell polarity experiments

MDCK and LLCPK1 cells were grown in 6-well plates (35 mm, 0.4 μm filter, Corning, N.Y., USA) until confluency and transferred into a two-compartment model. In the two-compartment model, the cells were grown as a monolayer on a membrane insert. This membrane insert was placed into a well of a regular 6-well plate. The medium was then added into the insert and the well. This model allowed defined oxalate application either to the upper (apical) or the lower (basolateral) side of the confluent cell layer. Sodium oxalate was then added in final concentrations of 1, 2 or 4 mM to the cell medium to one of the anatomic sides, whilst regular medium was delivered to the other side. Cell survival, cell proliferation and the induction of apoptosis were investigated.

Calculation of supersaturation

Free oxalate concentrations were determined with the EQUIL2 program as previously described [17] (kindly provided by the Department of Pathology, University of Florida, College of Medicine, Gainesville, Fla., USA). The concentrations of the following ions within the medium were calculated by standard laboratory procedures: sodium, chloride, potassium, phosphate, calcium, oxalate, magnesium and uric acid.

Statistical calculation

Statistical analyses were performed with the SAS software package. The following procedures were used: FREQ, MEANS, GLM (for multivariate analysis), Mann-Whitney U-test. Data are expressed as mean ± standard error of the mean (SEM). A P-value < 0.05 was considered to be statistically significant.

Results

Sodium oxalate led to a time- and concentration-dependent decrease in cell survival that was most prominent in fibroblasts and HUVECs (Table 1). The survival rates of LLCPK1 and MDCK were comparable. In all cell types, 1 mM oxalate exerted relatively moderate toxic effects, while cell survival significantly decreased with higher oxalate levels. Maximum cell toxicity was reached after 4 h; incubation up to 24 h did not further affect cell survival (data not shown).

Cell proliferation of LLCPK1 and fibroblasts showed its highest impairment after oxalate administration (all oxalate concentrations; Fig. 1A–D). Multivariate analysis revealed the highest impairment of proliferation for LLCPK1 at 1 mM and 2 mM, and at 4 mM LLCPK1 and fibroblasts showed comparable behavior. MDCK and HUVEC were more resistant in a comparable degree at all concentrations and incubation times. In contrast to the other cells, MDCK cell proliferation recovered after 24 h.

With the annexin assay, we could not observe induction of apoptosis in any of the oxalate treated cell lines, although application of the positive control cisplatin induced sustained apoptosis in $92.21 \pm 5.83\%$.

In the two-compartment model, both LLCPK1 and MDCK cells displayed a higher vulnerability to oxalate on the basolateral side than on the apical side, as shown in Fig. 2 for cell survival. However, statistical significance was only achieved with high oxalate concentrations (4 mM). Increasing oxalate concentration from 2 to 4 mM, the survival rate decreased to less than 40% of the baseline. MDCK cells showed a tendency to be more sensitive to oxalate than LLCPK1 on the basolateral side, although the difference did not reach significance. Analysis of the proliferation assays gave comparable results (data not shown).

Discussion

The interaction between oxalate and the renal epithelium and interstitium could be the key process in the pathogenesis of CaOx stones. Khan et al. demonstrated that the exposure of LLCPK1 and MDCK cells to oxalate and oxalate crystals led to a decline in cell survival [17]. Other investigators have confirmed these findings [19]. Although several published reports propagate intratubular crystal formation, the site of stone formation is still unclear. Histological and endoscopic examinations have shown intraepithelial and papillary calcifications thought to be precursors of later kidney stones [3, 5, 8]. It is unclear whether these formations are secondary deposits within the interstitium or whether they are a result of primary interstitial stone formation. Obviously, these microliths grow still further within the renal interstitium until they penetrate into the renal collecting system. Information on the biochemical and

Table 1 Cell survival of LLCPK1, MDCK, HUVEC and fibroblasts with 1, 2 and 4 mM sodium oxalate after 2 and 4 h incubation versus controls. All data shown as mean values ± SEM. * indicates $P < 0.05$ compared to control, § $P < 0.01$ compared to control

	2 h incubation			4 h incubation		
	1 mM	2 mM	4 mM	1 mM	2 mM	4 mM
LLCPK1	81.41 ± 3.95	$66.63 \pm 5.19^{*}$	$56.36 \pm 5.57^{*}$	80.68 ± 6.12	$59.55 \pm 9.83^{*}$	$48.72 \pm 6.54^{*}$
MDCK	80.51 ± 4.40	$57.07 \pm 3.67^{*}$	$42.09 \pm 4.35^{\S}$	72.26 ± 6.20	$51.88 \pm 9.23^{*}$	$43.14 \pm 9.78^{\S}$
Fibroblasts	94.65 ± 7.87	$47.82 \pm 7.55^{*}$	$22.79 \pm 8.48^{\S}$	93.89 ± 4.98	$35.05 \pm 6.74^{*}$	$10.08 \pm 3.28^{\S}$
HUVEC	93.68 ± 12.73	67.50 ± 13.18	$15.73 \pm 1.42^{\S}$	75.05 ± 13.38	60.10 ± 5.67	$3.76 \pm 0.99^{\S}$

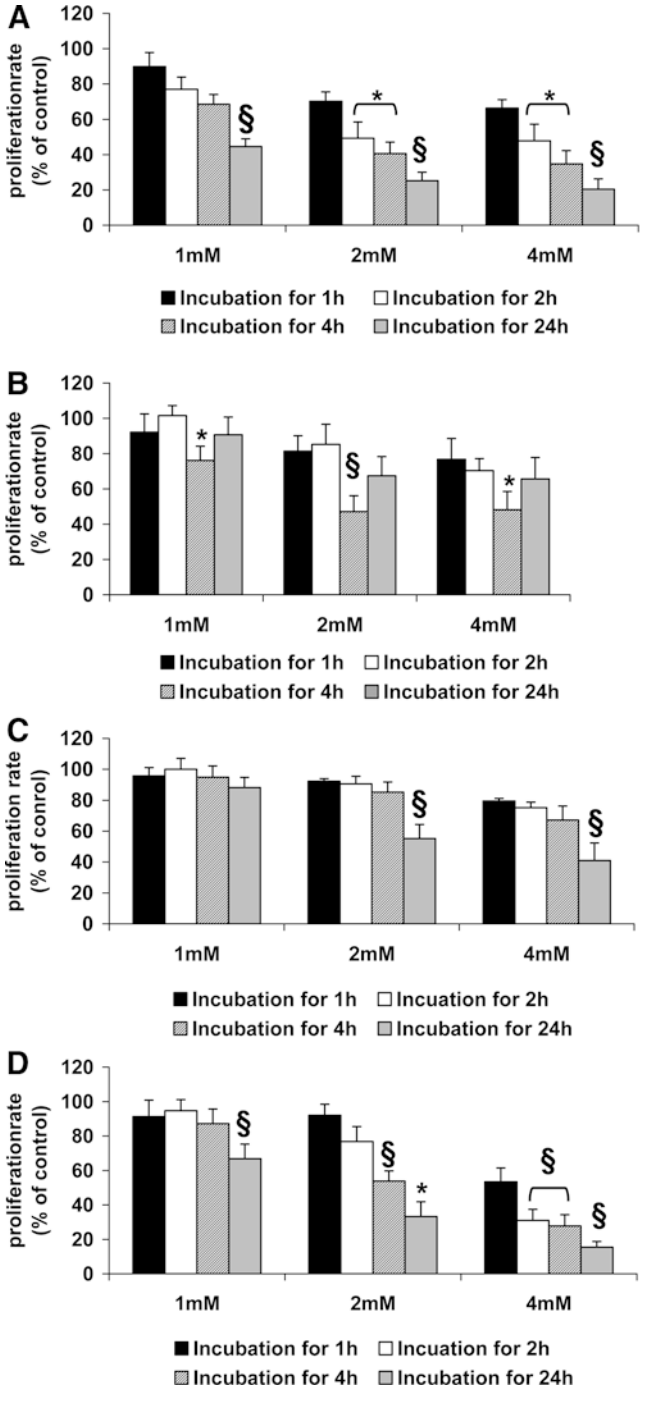

Fig. 1 Cell proliferation of: **A** LLCPK, **B** MDCK, **C** HUVEC and **D** fibroblasts Cells were incubated for 1, 2, 4 and 24 h. All data are shown as mean values ± SEM. *Asterisks* indicate $P < 0.05$, *paragraph symbols* $P < 0.01$ compared to the control

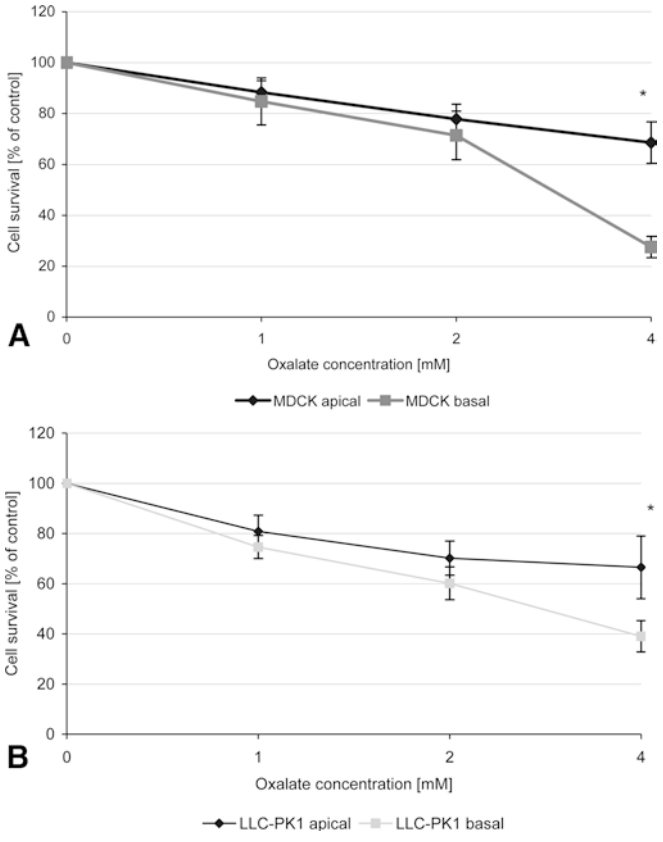

Fig. 2 Cell survival of **A** MDCK and **B** LLCPK1 cells with 1, 2, and 4 mM sodium oxalate concentration in a two-compartment model (2 h incubation) versus controls. All data are shown as mean values ± SEM. *Asterisks* indicate $P < 0.05$ compared to lower concentration

immunological processes induced by interstitial calcifications is scanty. De Water et al. demonstrated that, in humans, crystalline substances within the renal interstitium are surrounded by inflammatory cells such as monocytes, macrophages and lymphocytes [12, 13]. Necrotic cells have been shown inside urinary stones and are thought to be an initial point of crystallization. The influence of oxalate on cells such as fibroblasts or endothelial cells has not yet been investigated. We examined the influence of oxalate on renal epithelial and interstitial cells with respect to cell survival, proliferation and the induction of apoptosis. We clearly demonstrated that oxalate not only exerts pronounced toxic effects on epithelial cells, but also on interstitial and endothelial cells. Renal fibroblasts and HUVECs reacted with a marked decline in cell survival and metabolism when oxalate was delivered to the culture medium. A previous publication [18] demonstrated that LLCPK1 cells are more sensitive to oxalate than MDCK cells. In our study, we could confirm these findings partly in terms of cell survival and cell proliferation. Although LLCPK1 showed a tendency to be more vulnerable, significance was only achieved for 4 mM oxalate and 2 h incubation (cell survival) and incubation times over 2 h for cell proliferation. An explanation for this could be the lower physiological oxalate concentration found in the proximal nephron segments, indicating that MDCK cells have possibly adapted to higher oxalate concentrations and have developed yet unidentified protective mechanisms.

In our investigations, oxalate toxicity was induced at a threshold value of approximately 1 mM. Although it is likely that this value represents a (patho-) physiological

level, to our knowledge there are no data on the exact total oxalate concentration in the nephron and the renal tissue. In our opinion, the amount of oxalate necessary to induce stone formation is only found within the distal collecting ducts and the renal papilla. Recently, radioluminography depicted a tenfold higher oxalate concentration in the renal papilla than in the renal cortex and medulla [20]. The oxalate concentrations in this area possibly exceed those expected in the tubular system. Belliveau et al. demonstrated that the addition of soluble oxalate at concentrations of 10^{-4} M causes $>50\%$ precipitation of the oxalate resulting in equilibrium oxalate concentrations of less than 6×10^{-5} M [21]. Therefore, the soluble oxalate concentration would be less than that calculated by the amount of sodium oxalate added to the medium. Furthermore, the precipitation of oxalate reduces the extracellular calcium content (due to the formation of CaOx). In the calculation of supersaturation with the EQUIL2 program, these processes are not considered.

The induction of apoptosis by oxalate in renal epithelial cells has already been described [7]. With the annexin assay used by us, which is highly specific for apoptosis, no induction of apoptotic processes was observed, indicating a necrotic death mechanism. This hypothesis was confirmed by Miller et al. [2] who demonstrated that oxalate-induced cell death exhibits certain characteristics similar to necrotic cell death, such as cellular and nuclear swelling in the majority of cells with only little evidence of DNA cleavage. However, certain characteristics of apoptotic cell death were also detected, suggesting that toxic concentrations of oxalate trigger both forms of cell death in renal epithelial cells.

The deposition of CaOx within the renal interstitium could exert toxic effects on both interstitial cells and renal tubular cells. So far, investigations on oxalate toxicity have focused on the tubular (apical) side of the cells; it is conceivable, that the basolateral side is more sensitive. Oxalate could maintain inflammatory processes and cell necrosis in the interstitium, a mechanism that can further promote stone growth. Kageyama et al. demonstrated that MDCK cells produce microliths consisting of calcium phosphate on the basolateral side but not on the lumen side when cultured in DMEM with 10% FBS medium [22]. Although the mechanism of crystal deposition is still unclear, this observation might be of relevance in understanding CaOx lithogenesis, as CaPh microcrystals are thought to be precursors of CaOx stones. The hypothesis of extraluminal stone formation was further supported by the findings of Evan et al., who demonstrated that in CaOx stone patients, precursors of later urinary stones are found near the thin loops of Henle within the interstitium [14]. However, further investigations are needed to clarify the mechanism of oxalate transport at the distal tubule. Although crystal formation usually occurs beyond the renal proximal tubule, most studies investigating oxalate transport mechanisms have focused on proximal nephron parts [23, 24].

In this study, we have demonstrated, for the first time, that tubular cells definitely showed diverging sensitivity to oxalate with respect to the side of application. Cells of the renal epithelium were found to be less sensitive to oxalate on their apical (tubular) side. Although the results did not reach statistical significance, MDCK showed a clear tendency to be more vulnerable to oxalate than LLCPK1. Differences in cell sensitivity compared to standard flasks are most probably a result of the different growth conditions. The reason for the discrepancy in oxalate toxicity would be the existence of protective cell surface molecules on the apical side, which has not been described so far. Further investigations are needed to clarify this issue.

In conclusion, we have demonstrated the impact of oxalate on renal interstitial cell lines as well as on the basolateral side of epithelial cells. Our findings imply an involvement of the renal interstitium in stone formation.

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