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ORIGINAL PAPER

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Impact of hypoxia and hypercapnia on calcium oxalate toxicity in renal epithelial and interstitial cells

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Abstract Although there is an ongoing controversy about the primary site of calcium oxalate stone (CaOx) formation, there is some evidence for extratubular crystallization. However, the mechanisms leading to such interstitial calcifications are not clear. Anatomical studies have demonstrated a close association between the renal vasculature and renal tubules. It has been hypothesized that disorders of the vasculature may contribute to renal stone formation. The exceptional papillary environment with low oxygen and high carbon dioxide is of interest in this context and its impact on CaOx toxicity to renal cells has to be evaluated. LLC-PK1, Madin-Darby canine kidney (MDCK), human umbilical vein endothelial (HUVEC) and fibroblast cell lines were exposed to hypoxia (3% O_2) alone, hypercapnia combined with hypoxia (3% O_2, 18% CO_2) or standard culture conditions (20% O_2) for 72 h. Cell survival rates were determined microscopically after 4 h of incubation with CaOx at final concentrations of 1, 2 and 4 mM. DAPI staining and western blot were used to evaluate the induction of apoptosis. We confirmed that CaOx leads to concentration-dependent effects on the viability of the cell lines. HUVECs were most vulnerable to CaOx among the four cell lines. Incubation under hypoxia alone had no impact on CaOx toxicity to any of the cell lines in terms of survival. However, under combined hypoxic and hypercapnic conditions, all cell lines displayed a significant reduction of cell survival compared to room air incubation. Again, this effect was most pronounced for HUVECs. The induction of apoptosis could not be demonstrated in any experimental setting. Combined hypoxia and hypercapnia clearly aggravate CaOx toxicity to renal cell lines. As we could not demonstrate the induction of apoptosis, this effect may be a result of toxic necrosis. Especially the CaOx effect on interstitial cell lines might be of interest in the chronic ischemic papillary environment. An increased toxicity may lead to recurrent stone formation, and vice versa, diseases of the vasculature, like arteriosclerosis, may further promote stone formation by induction of local ischemia. This issue has to be clarified by further studies.

Keywords LLC-PK1 · MDCK · HUVEC · Fibroblasts · Urinary calculi

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Introduction

Calcium oxalate (CaOx) is a major constituent of most urinary stones. Nearly 50% of stone formers will have recurrent stone disease within 5 years [1]. Existing theories for CaOx stone formation, such as nucleation and supersaturation, can only partially explain the process, and to date the underlying mechanisms still remain mysterious [2–5]. Although many reports have focused on the crystallization process to clarify lithogenesis [6], several clinical and pathological observations cannot be explained by traditional hypotheses of urinary stone formation. Urine remains within the renal tubules for only 3–5 min and is generally undersaturated with CaOx before reaching the collecting ducts [7]. Observations like exclusive unilateral stone formation or varying levels of salts and nucleation inhibitors in healthy patients suggest a more complicated pathway than supersaturation alone [8]. To explain intratubular crystallization despite a short tubular passage time, the idea of intratubular crystal-cell adhesion has been developed. Retained crystals may initiate stone formation [9, 10].

However, primary crystallization could be demonstrated in the interstitial space close to the bundles of thin loops of Henle and vasa recta [11]. Such data indicate that at least some of the CaOx stones are not formed inside the tubules but in the renal interstitium. This confirms earlier reports by Randall, who described

subepithelial plaques found in renal papillae, indicating that at least some stones are primarily formed extratubular within the renal interstitium [12]. We recently demonstrated that oxalate exerts substantial toxic effects on interstitial cells in vitro [13].

If the process of stone formation takes place in the renal papilla, the environment of this region has to be considered when investigating CaOx stone formation. The close proximity between nephron segments and the renal microvasculature led to the idea of a vascular involvement in stone formation [14]. Beside the high hyperosmolarity, the oxygen tension decreases and the renal papilla has a hypoxic milieu [15]. The local low O_2 and high CO_2 pressure influence the metabolism of interstitial and tubular cells, and may result in cell death promoting further stone formation. The aim of this study was therefore to clarify the impact of ischemia on renal stone formation.

Materials and methods

Cell culture

Two renal epithelial cell lines were used: LLC-PK1 from porcine kidney, proximal tubular cells, and Madin-Darby canine kidney (MDCK) cells, representing distal tubules and collecting ducts. Both of the two cell lines were supplied by the European Collection of Cell Cultures (ECACC, UK). Renal fibroblast and human umbilical vein endothelial (HUVEC) cell lines were used as interstitial cells, which were purchased from the American Type Culture Collection (ATCC, USA). LLC-PK1 and MDCK cell lines were cultured in DMEM containing 10% FCS and 4 mM glutamine (Gibco, UK). Fibroblasts were cultured in fibroblast culture medium with 10% FCS, 5 mM glutamine, 5 μg/ml insulin and 1 ng/ml basic fibroblast growth factor (PromoCell, Germany). HUVECs were cultured in endothelial cell growth medium with 10% FCS, 10 mM glutamine, ECGS/H 0.4%, 1 ng/ml hydrocortisone and 0.1 ng/ml epidermal growth factor (PromoCell, Germany) at 37°C in an incubator. Cells were subcultured by dissociation with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid (Gibco, UK).

Induction of hypoxia, hypoxia/hypercapnia and CaOx toxicity experiments

LLC-PK1, MDCK, fibroblast and HUVEC cells were seeded into T-25 cm^2 culture flasks. Cells were exposed to either standard culture condition (20% O_2), hypoxia (3% O_2), or hypoxia combined with hypercapnia (3% O_2 and 18% CO_2) for 72 h in an adjustable incubator (Heraeus® BB 6060, Germany). The media were replaced with serum-free media at 48 h. Then, CaOx was added at final concentrations of 1, 2 and 4 mM to the cell media, maintaining the individual air environment. In controls, the media were changed without the addition of CaOx. Every single experiment was repeated ten times.

Cell viability

Cell survival was assessed microscopically by trypan blue staining after incubation for 4 h with calcium oxalate using a Neubauer counting chamber. The results are shown as cell survival rates compared to the control groups cultured at 20% O_2 (controls were fixed at 100%).

Western blot

Total protein was extracted from the cells after various treatments. One hundred micrograms of each lysate was loaded for electrophoresis after protein concentration was determined by BCA reagent (Pierce, Germany). The proteins were transferred onto a nitrocellulose membrane (Bio-Rad, USA). The membrane was incubated overnight at 4°C with caspase-3 antibody (Santa Cruz, USA) following the semi-dry transfer. Results were evaluated after processing the film (Kodak Biomax).

DAPI staining

Cells were cultured in six-well culture plates. The DAPI working solution (Roche, USA) was added to the cells after various treatments as described previously. Cells were washed and fixed with methanol after staining. Cells were observed under the fluorescence microscope (Zeiss, Germany).

Statistical analyses

Statistical analyses were performed with the SAS software package. The following procedures were used: means, GLM (for multivariate analysis), Student's *t* test, Scheffe test. Data were expressed as mean ± standard deviation (SD). A P-value < 0.05 was considered to be statistically significant.

Results

The survival rates of LLC-PK1, MDCK, HUVECs and fibroblasts were compared. All four cell lines displayed CaOx concentration-dependent effects on cell viability. Cell survival rates decreased significantly with the increase of CaOx concentration. From the data, under normal culture conditions, the two tubular cell lines were not impaired to a great extent by CaOx. A significant decrease in cell survival rate was observed in interstitial cells (Fig. 1). HUVECs demonstrated the

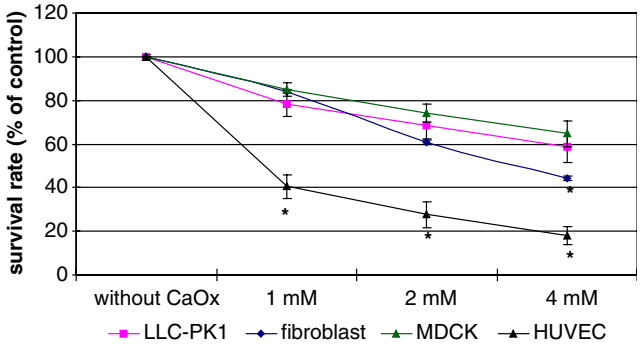

Fig. 1 Comparison of survival rates among the four cell lines incubated with 0, 1, 2, 4 mM CaOx for 4 h at 20% O_2 compared to every other cell line (mean ± SD, *P < 0.001)

lowest cell survival rates among all four cell lines (P < 0.001), fibroblasts also had lower cell survival rates than tubular cells when exposed to high levels of CaOx.

Following exposure to hypoxic condition (3% O_2) for 72 h, the four cells lines were incubated with CaOx for 4 h more. The survival rates of the four cell lines did not show any difference when exposed at hypoxia without CaOx. Following the increase of CaOx levels, survival cell numbers decreased significantly in HUVEC cells compared to the two tubular cell lines. Furthermore, fibroblasts showed lower cell viability than the two tubular cells at 4 mM CaOx (P < 0.0001) (Fig. 2). All cell lines demonstrated a mild decline of cell survival when cells were cultured at hypoxia instead of standard culture conditions. LLC-PK1, MDCK and HUVEC cells had lower cell survival rates at hypoxia than under standard culture conditions whereas no significant decrease was found in fibroblast cells. Hypoxia did not enhance the toxicity of CaOx on renal cells. However, after being cultured at hypoxia and hypercapnia for 72 h both tubular cells and interstitial cells showed significant lower cell survival rates than the cells in standard culture air or at hypoxia. Furthermore, all of the four cell lines except for HUVEC displayed significantly lower survival rates than cultured at 20% O_2 in air with the addition of CaOx (Figs. 2, 3) (P < 0.001). Different cell lines showed different levels of impairment to hypercapnia and hypoxia. HUVEC showed the lowest cell survival rates in all cell lines (Fig. 3) (P < 0.0001). Under low O_2 and high CO_2 air conditions, only about 63% cell survival rate was found in HUVEC, which suggested highest impairment among the four cell lines. Although cell death was confirmed by the evaluation of cell viability, no evidence of apoptosis in any cell line was observed from the results of DAPI staining and western blot (Figs. 4, 5) after CaOx incubation under hypoxia alone or hypoxia combined with hypercapnia.

Discussion

In 1937, Randall [12] described calcium-containing calcifications within the renal papilla. He proposed the basement membrane of collecting ducts being calcified in a circumferential fashion, eventually eroding to the papillary surface [16]. During the last 15 years, most studies have focused on intratubular crystal formation [17]. However, some investigators favoured the idea of interstitial crystal formation [6, 11, 13]. In an animal study, papillary ^{14}C-oxalate was detected tenfold higher in the interstitial tissue than in the intraluminal spaces [18]. Therefore, it was supposed that the interstitial tissue in the papilla might be considered to be the primary site of stone formation. Stoller et al. [14] hypothesized that the primary event in stone formation may be associated with the renal vasculature. This idea is supported by the unique arrangement of the medullary perfusion [19, 20], which is prone to injury at the papillary tip in a hypoxic and hyperosmolar environment.

The interaction between CaOx and the renal epithelium and interstitium could be the key process in the pathogenesis of CaOx stones. Many investigations have

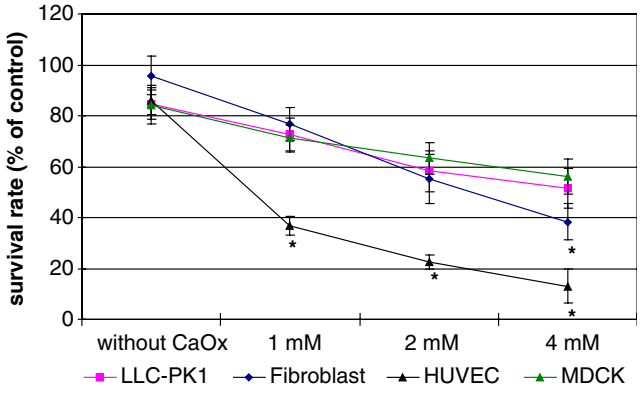

Fig. 2 Comparison of survival rates among the four cell lines incubated with 0, 1, 2, 4 mM CaOx for 4 h at 3% O_2 compared to every other cell line (mean ± SD, *P < 0.0001). Baseline survivals normalized to normoxic conditions

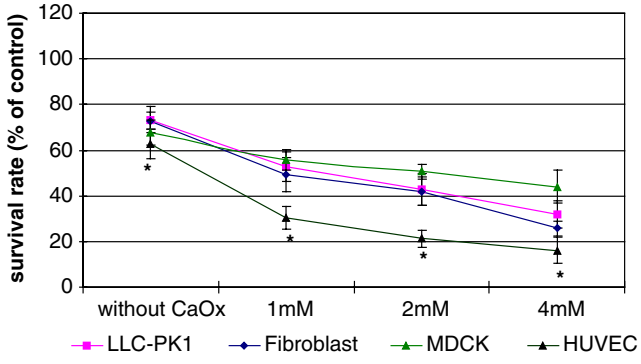

Fig. 3 Comparison of four cell lines survival rates incubated with 0, 1, 2, 4 mM CaOx for 4 h at 3% O_2 and 18% CO_2 compared to every other cell line (mean ± SD, *P < 0.0001). Baseline survivals normalized to normoxic conditions

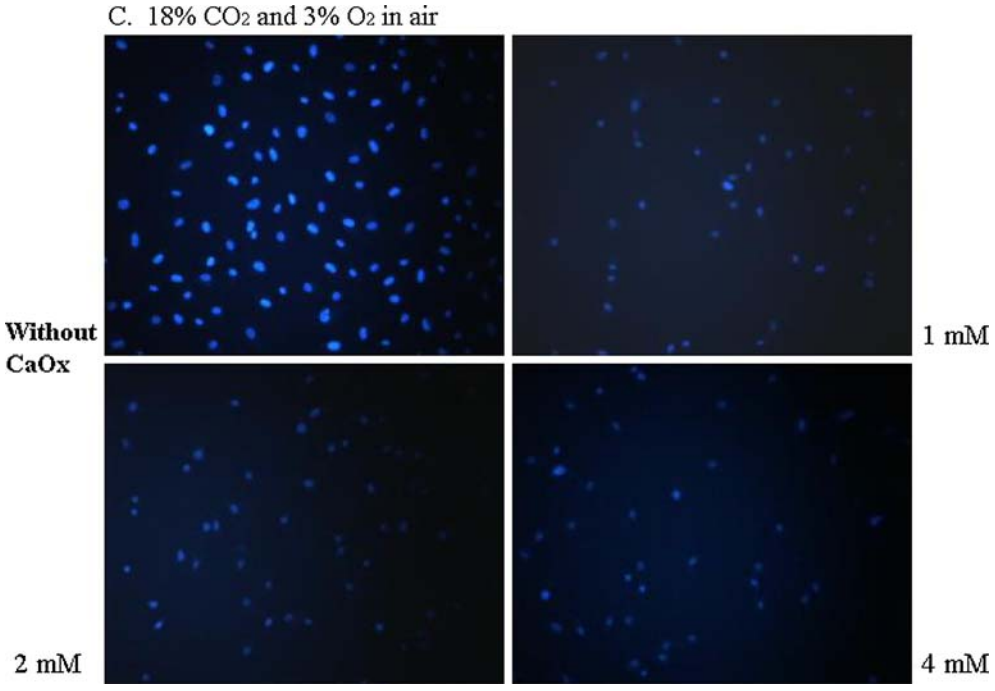

Fig. 4 Apoptotic morphological changes could not be detected by DAPI staining. HUVEC cells incubated with various concentrations of CaOx at 18% CO_2 and 3% O_2 are shown as an example (magnification 20×)

demonstrated that the exposure of LLC-PK1 and MDCK cells to CaOx crystals led to a decline in cell survival [21]. Our previous study demonstrated that oxalate not only exerted pronounced toxic effects on epithelial cells, but also on interstitial and endothelial cells [13].

From our investigations, both epithelial and interstitial cell lines showed a marked decline in cell survival when exposed to CaOx in room air. Tubular cells and interstitial cells showed different vulnerability to either varying levels of CaOx or oxygen tension. We could not observe a significant difference in viability between LLC-PK1 and MDCK cells, although other studies demonstrated that LLC-PK1, representing proximal tubular cells, are more sensitive to oxalate than MDCK, representing distal tubular cells [22, 23]. This might be due to the relatively high CaOx crystal concentrations of 1–4 mM (equivalent to the range of 0.28 and 1.12 mg/ml). Other studies utilized CaOx crystal load varying from 10 μg/ml to 2 mg/ml [24]. Higher oxalate concentrations in the renal papilla than in the renal cortex and medulla may indicate a higher CaOx concentration in the interstitium than expected in the tubular system. Furthermore, to our knowledge, no data is available on the exact CaOx or oxalate concentrations in the nephron and the renal tissue. Beside oxalate toxicity, increased calcium influx into the cells may lead to cell death by Ca^{2+}-induced mitochondrial permeability transition and mitochondrial Ca^{2+} accumulation. In an earlier publication, we demonstrated low amounts of free oxalate and Ca^{2+} by EQUIL2 calculation [13, 25]. Direct effects of free calcium or oxalate were therefore not considered.

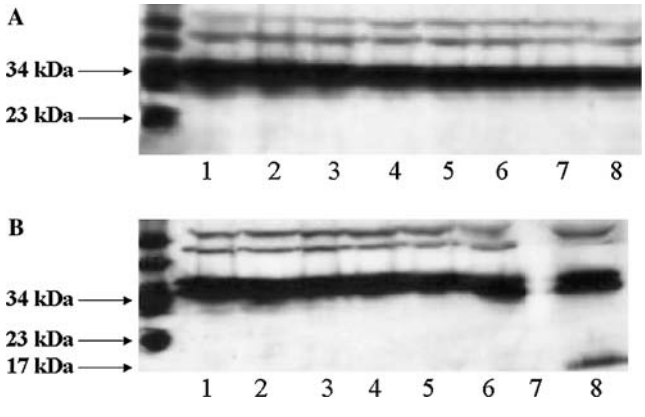

Fig. 5 Active caspase-3 was not observed in any of the cell lines with or without CaOx treatment and after exposure to low oxygen and high carbon dioxide for 72 h. HUVEC western blot results are shown as an example (**a** *Lanes 1–4*: 0, 1, 2, 4 mM CaOx for 4 h at 3% O_2. *Lanes 5–7*: 0, 1, 2 mM CaOx for 4 h at 20% O_2. *Lane 8*: negative control. **b** *Lane 1*: 4 mM CaOx for 4 h at 20% O_2. *Lanes 2–5*: 0, 1, 2, 4 mM CaOx for 4 h at 18% CO_2 and 3% O_2. *Lane 6*: negative control. *Lane 7*: blank. *Lane 8*: positive control)

Our results confirm that both proximal and distal tubules could be injured by a high concentration of CaOx. HUVECs showed the highest vulnerability to CaOx crystals among all cell lines, which may confirm the higher susceptibility of the renal interstitium.

Hypoxia occurs at the early stage of ischemia and hypercapnia is induced when the ischemic condition lasts for a longer time. We demonstrated that combined hypoxia and hypercapnia significantly inhibited the viability of epithelial and interstitial cell lines whereas only a moderate influence was found under hypoxia alone. Again, HUVECs showed the highest impairment among all cells when exposed to hypoxic and hypercapnic conditions. This may indicate that disorders of

microvasculature potentially play an important role in the development of urolithiasis and the injury renal cells induced by ischemia are more sensitive to the CaOx crystal challenges, which may contribute to the stone formation.

CaOx could maintain inflammatory processes and cell necrosis in the interstitium, a mechanism that can further promote stone growth. The induction of apoptosis by oxalate in renal epithelial cells has already been reported [10]. However, characteristics of necrotic cell death, such as cellular and nuclear swelling, are as well described after incubation with oxalate or CaOx [26]. Hypoxia-induced cell death may result from both apoptosis as well as necrosis, as demonstrated in experimental models of renal injury in vivo and in vitro [27]. In general, the mechanism of cell death either by necrosis or apoptosis is determined by the severity of the injury. Schumer [28] reported the occurrence of apoptosis in acute renal ischemia/reperfusion in rat kidney. However, the literature on this topic is conflicting, reporting necrotic changes rather than apoptotic features and vice versa [29–31]. Both apoptosis and necrosis seem to occur at early stages of hypoxia but a shift to necrosis is seen at later stages [30, 32]. We could not observe apoptotic changes in any cell line, either in room air or at hypoxia or hypoxia combined with hypercapnia. This confirms data reporting both apoptosis and necrosis to occur at early stages of hypoxia but a shift to necrosis is seen at later stages [30, 32]. Our observation might suggest that under chronic ischemia conditions apoptotic processes do not play an important role in CaOx-induced toxicity of renal cell lines. In addition to the mechanism of cell death, the hypoxia-induced molecular mechanisms are only partially understood. It has been demonstrated that hypoxia-inducible factor (*HIF*)-alpha protein levels are increased by hypoxia, leading to alterations of NADPH oxidases (NOXs), endothelial PDGF and VEGF [33–36]. Further studies will have to characterize the changes in signalling pathways and to determine the molecular impact of hypoxia. Such studies should as well aim to determine the oxygen and carbon dioxide concentrations at the cell level. Pettersen et al. [37] demonstrated that even normal culture conditions induce moderate hypoxia as a result of the low diffusion rate of oxygen through cell culture media. It is therefore conceivable that the O_2 and CO_2 levels at the monolayer are different to those measured in the gas. However, our study demonstrated a clear impact of hypoxia and hypercapnia on the vulnerability of renal cells to calcium oxalate.

In conclusion, our findings demonstrate that the renal interstitium might play a role in CaOx stone formation. The unique papillary environment with local ischemia under physiological conditions has to be taken into account when investigating the mechanisms of CaOx stone formation. Furthermore, an involvement of the renal vasculature is probable when considering the close anatomical association of renal vasculature and epithelium. Our results may support this idea.

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