Urol Res (2006) 34: 26–36
DOI 10.1007/s00240-005-0007-3

ORIGINAL PAPER

Pouran Habibzadegah-Tari · Karen G. Byer
Saeed R. Khan

Reactive oxygen species mediated calcium oxalate crystal-induced expression of MCP-1 in HK-2 cells

Received: 18 October 2005 / Accepted: 5 December 2005 / Published online: 6 January 2006

Abstract Under severe hyperoxaluric conditions calcium oxalate crystals often deposit in the renal interstitium and produce localized inflammation. We have proposed that renal epithelial cells exposed to CaOx crystals produce chemoattractants such as monocyte chemoattractant protein-1 (MCP-1). MCP-1 synthesis is mediated by reactive oxygen species (ROS). HK-2 cells of human renal epithelial line were exposed to CaOx crystals for different lengths of time. The culture media was tested for cell injury marker LDH, and subjected to enzyme-linked immunosorbent assay to determine the secretion of MCP-1 protein. Cell expression of MCP-1 was assessed by Western blot analysis. Gene expression was determined by reverse transcriptase-polymerase chain reaction. The data clearly showed that the HK-2 cells express MCP-1 gene and protein. The MCP-1 mRNA expression was increased following exposure to CaOx crystals, which was reduced upon treatment with free radical scavengers, catalase and superoxide dismutase. Results indicate that CaOx crystals strongly induce MCP-1 synthesis and secretion by the HK-2 cells and production is mediated by intracellular ROS production. Based on these and other data, antioxidant therapy and blockade of rennin–angiotensin system may prove beneficial for the prevention of end stage renal disease caused by hyperoxaluria and CaOx crystal deposition.

Keywords Calcium oxalate · Reactive oxygen species · Oxidative stress · MCP-1 · Kidney stones · Nephrolithiasis

P. Habibzadegah-Tari · K. G. Byer · S. R. Khan (✉)
Department of Pathology and Laboratory Medicine,
University of Florida College of Medicine,
100275, Gainesville, FL 32610-0275, USA
E-mail: Khan@pathology.ufl.edu
Tel.: +1-352-3923574
Fax: +1-352-3928177

Introduction

Calcium oxalate (CaOx) crystals are the major constituents of most human kidney stones worldwide [1]. The mechanism by which a CaOx stone is formed is complex, and many factors are believed to be involved. Crystals form even in urinary system of normal individuals, however, they are excreted as crystalluria [2, 3]. On the other hand, under certain disease states, which increase urinary excretion of oxalate or calcium, the crystals are retained in the kidneys by growing and aggregating with other crystals and/or by adhering to the renal epithelial cells [2, 4–6]. This suggests that growth, aggregation and retention of crystal are important events in the development of kidney stones. The renal tubular epithelial cells can take in the retained crystals. Following endocytosis, the crystals may be destroyed by the cellular lysosomal enzymes and/or transported to the interstitium where they are attacked by the inflammatory cells [4, 6]. Renal epithelial cells are injured upon exposure to oxalate ions and/or CaOx crystals [4, 7, 8]. It has also been shown that oxalate ions (OX) and CaOx crystals induced renal epithelial injury is caused by the production of free radicals and lipid peroxidation of the cell membranes [7, 9].

It is proposed that oxalate and CaOx crystals stimulate the renal epithelial cells to produce chemokines [10], which in turn, attract leukocytes and macrophages inducing localized inflammation [4, 11, 12]. The macrophages and giant multinucleated cells surround the crystals and release their contents. These events are accompanied by the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF-ά) and interleukin-6 (IL-6). Studies have also shown that experimentally induced hyperoxaluria and CaOx crystal deposition in rat kidneys activate rennin–angiotensin system (RAS) [13]. Angiotensin converting enzyme (ACE) inhibitors and angiotensin II (Ang II) type 1 receptor blockers reduced inflammation as well as CaOx crystal deposition in kidney despite similar urinary cal-

cium and oxalate levels [13, 14]. Administration of ACE inhibitor, enalapril, to hyperoxaluric rates also reduced CaOx crystal deposition, M/M infiltration and interstitial fibrosis [15].

Infiltration of leukocytes and macrophages into renal interstitium has been associated with inflammatory diseases and is mediated by chemotactic cytokines and chemokines such as interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1) and regulated on activation, normal T expressed and secreted (RANTES) and macrophage inflammatory protein (MIP)-1α [16–19]. These chemokines are produced by a wide variety of kidney cells such as proximal tubules, renal cortical epithelial cells, glomerular endothelial cells and interstitial fibroblasts after stimulation by proper cytokines and will exert distinct actions on target cells through interacting with their specific receptors.

The first study to show up regulation of MCP-1 by renal epithelial cells in response to CaOx was carried out using a rat renal epithelial cell line, MRK-52E. The NRK-52E cells were exposed to CaOx crystals, OX and catalase, a free radical scavenger [10, 20]. NRK52-E cells expressed MCP-1 mRNA and protein, and the level of expression increased significantly after exposure to CaOx and OX. These responses were significantly reduced following treatment with catalase. The fact that the renal epithelial cells produce MCP-1 and its expression and production is increased when the cells are exposed to high levels of CaOx crystals and oxalate ions, suggests the possibility that this chemokine plays an important role in oxalate-induced inflammatory response of the kidney.

To confirm that cells of human renal epithelium express MCP-1 and respond similar to those of rats we exposed HK-2, a human renal epithelial cell line to CaOx crystals. We postulated that HK-2 cells will express MCP-1 gene and protein and their expression will be altered in the presence of CaOx crystals and that reactive oxygen species will be involved. Our data show that HK-2 cells express MCP-1 and that the expression, production and secretion into the culture medium is increased upon exposure to CaOx crystals in a time and concentration dependent manner. Exposure to CaOx crystals is associated with the production of superoxide. Crystal-induced expression is reduced following treatment with antioxidants and free radical scavengers indicating the involvement of reactive oxygen species (ROS).

Materials and methods

Cell culture

HK-2 cells, a proximal tubular epithelial cell line derived from normal human kidney was purchased from American Type Culture Collection (CRL-2190; Manassas, VA, USA). HK-2 is the only human renal tubular cell line currently available, and has been previously used to investigate the effect of CaOx crystal exposure [21].

Cells were maintained in 75 cm^2 Falcon (Fisher) T-flask in Dulbecco's modified essential medium and F-12 (DEMM/F-12 at 1:1 ratio; Gibco BRL) containing 10% fetal calf serum (FCS), and penicillin and streptomycin at 37°C under 5% CO_2 air atmosphere. The media was changed every 2–3 days. The cells were seeded at 10^6 in 6 and 12-well plates and at 10^5 for 96-well plates, unless otherwise stated. To reduce variability of the results caused by differences in seeding density and/or plating efficiency, the cells were grown to confluence to allow formation of tight junction and well defined cell-to cell contacts as reported earlier [22]. Confluent monolayers (95%) of HK-2 cells were used from day 3 to 4 after plating. Once the cells became confluent, the media was removed and the cells were washed with phosphate saline buffer (PBS). The cells were placed overnight in serum and sodium-pyruvate free media supplemented with insulin/transferin/selenium mix (Gibco BRL), Hydrocortisone (Sigma), triiodo-L-thyronine (Sigma) and Prostaglandin E1 (Sigma) overnight to arrest the growth. The cells were then exposed to the serum and sodium-pyruvate free media supplemented with calcium oxalate monohydrate (COM) crystals (67, 133, and 267 μg/cm^2) for 1, 3, 6, 12, 24 and 48 h at 37°C, unless otherwise stated. Each treatment was done in triplicates, unless otherwise stated. The culture media was collected in 2 ml microfuge tubes and stored at −20°C for further analysis, and total RNA and protein were isolated from the cells using TRIZOL Reagent (Life Technologies) according to the manufacturer procedure and stored at −80°C.

Analysis of MCP-1 by Western immunoblotting analysis

HK-2 cell expression of MCP-1 was assessed by Western blot analysis. The treated cells were exposed to COM, 133 μg/cm^2, for 6 h. Protein was isolated from both the control and treated cells (1×10^8 cells) using TRIzol Reagent (Gibco BRL) according to the manufacturer's procedure. A BioRad protein assay (BioRad) was performed to determine protein concentration according to the manufacture's protocol using bovine serum albumin (BSA) as a standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to show MCP-1 and determine its molecular weight. The protein sample (30 μg) was electrophoresed in 15% criterion Tris–HCl gel (BioRad). Prestained SDS-PAGE Standards (BioRad) were used as described by the manufacturer. After SDS-PAGE, proteins were transferred to 0.2 μm Nitrocellulose membrane (Fisher) using a Bio-Rad Immunoblotting apparatus and blocked for 1 h at room temperature with a 2% solution of milk in Tris-buffered saline containing 0.5% Tween (TBST, Sigma). Non-specific binding sites were further blocked using 5% BSA in the blocking buffer. The membrane was incubated with mouse monoclonal antibody against

human MCP-1 (Sigma) (1:1,000 in TBST/2% milk 1% BSA) overnight at 4°C. Then the membrane was treated with a horseradish peroxidase-linked anti-mouse secondary antibody for one hour at room temperature (1:10,000 in TBST/1% milk 1% BSA) and immunoreactivity was detected using ECL Plus Western Blotting Detection Reagents (Amersham) on a KODAK X-ray. Coomassie and/or silver staining were also done to show the presence of proteins and their relevance molecular weight (data not shown).

Lactate dehydrogenase assay

On the day of experiments, conditioned media was removed from the experimental wells and assayed for lactate dehydrogenase (LDH) activity or stored at −20°C for later use. LDH, is a stable cytosolic enzyme that is released when the cell is lysed or the cell membrane is injured. A colorimetric assay kit (Proteins International) has been used to measure the LDH. The samples were centrifuged to remove crystals and cellular debris. Aliquots of the conditioned media (50 µl) in duplicate were transferred to 96-well flat plates. A positive control (cells lysed with lysis buffer supplied with kit) and blank (media) were also aliquoted to the designated wells. The substrate, 50 µl, (provided with the kit) was added to all the samples and incubated at room temperature in dark for half an hour. Following the incubation, stop solution was added and plate read at 490 nm on a microplate reader (BioRad 3550 microplate reader) (BioRad). The comparisons were made against appropriate blanks.

Effect of free radical scavengers

In order to evaluate the protective effect of free radical scavengers, cells were exposed to COM crystals following pretreatment with catalase (2,000 u/ml, Sigma) or SOD (400 u/ml, Sigma) for 8–12 h. The media was removed and replaced with fresh serum free media containing COM (133 µg/cm^2) alone and/or in combination with catalase or SOD for 3 and 6 h, unless otherwise stated. The conditions, such as concentration of SOD and catalase and time of exposure were chosen from previously published values [10, 23]. The exposure time (8–12 h) was shorter than that reported earlier (24 h) and had no effect on cell viability.

RNA isolation and PCR

In order to determine MCP-1 mRNA expression in HK-2 cells, the total cellular RNA was isolated from treated and untreated cells (1×10^8 cells) using TRIzol Reagent (Gibco BRL) according to the manufacturer's procedure and was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR). A 20-µl reaction volume was used for 2 µg of total RNA. The following components, 1 µl of 10-mmol/l dNTP mix (Eppendorf), 1 µl of Oligo d (T) $_{12–18}$ (Invitrogen), and mRNA were added to a nuclease free microfuge tube. Enough DNase /RNase-free H_20 was added to bring the final volume to 10 µl. The sample was mixed by pipetting up and down, placing it at 65°C for 5 min and chilling it on ice. The tubes were briefly centrifuged to collect contents to which the following were added: 4 µl of 5× first-strand buffer (Invitrogen), 2 µl 100 mmole/l DTT (Invitrogen), 1 µl RNasin (Promega), 2 µl of acetylated BSA (Promega) and 1.0 µl of reverse transcriptase (Invitrogen). The sample was mixed gently and incubated at 42°C for 5 min, then at 70°C for 15 min in the thermocycler. PCR was carried out in a reaction mixture consisting of 1 µl of RT product, 5 µl of 10× PCR reaction buffer (Qiagen), 2.5 µl of 10 mmol/l dNTP (Eppendorf) mix, 2.5 µl of 25 mmol/l MgCl2 (Qiagen), 0.5 U of recombinant Taq polymerase (Qiagen) and 2 µl of each of the 5′ and 3′primers (50 picomoles/µl) of human MCP-1 and β-actin. The primers from the published cDNA sequence are hMCP-1: 5′-AGTCTCTGCCGCCCTTCTGTG-3′ and 5′-TTGGGTTGTGGAGTGAGTGTT-3′, amplifying a 315-bp product; and β-actin: 5′-GTGGGGCGC CCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACG-CACGATTTC-3′, amplifying a 590 pb product. MCP-1 and β-actin were amplified for 36 cycles at 94°C for 1 min, annealing at 70°C for 45 s, followed by extension at 72°C for 1 min. The product (10 µl) was resolved on a 2% agarose gel + ethidium bromide using 100 base pair DNA ladder (Promega) as the molecular weight marker. The PCR band intensity was analyzed using quantitative densitometry.

Nitro blue tetrazolium assay

A modified version of a previously described assay for conversion of nitro blue tetrazolium (NBT) to formazan by superoxide anion was used to measure the generation of ROS [24]. For this set of experiments, the HK-2 cells were grown in 96-well plates. Once they reached confluence (95%), the media was removed and cells were washed and placed in serum-free media as described before. The cells were then exposed to the serum-free media (0.2 ml) supplemented with 1 mg/ml NBT. COM (0, 67, 133, and 267 µg/cm^2) was also added, and incubated at 37°C for 0, 2, 4, 6, 12, 24 and 48 h. At specified times, the supernatant NBT solution was aspirated from the wells, and the wells were thoroughly washed with 75% methanol to halt the reaction. The wells were then washed four times with 100% methanol to remove unreduced NBT dye and allowed to air-dry. The reduced formazan precipitate remained visible as purple granules on the bottom of the wells. After air-drying, 70 µl of 2 M potassium hydroxide was added to each well to lyse the cells. The formazan was then solubilized by the addition of 82 µl of dimethyl sulfoxide to the KOH (at a ratio of 1:1.17 volume per volume). The content of the

wells was then mixed by pipeting to complete solubilization. The O.D.^{655} of the solution was read on the ELISA reader (BioRad 3550 microplate reader) (Bio-Rad). The blanks consisted of wells with no cells which were incubated with NBT solution and subjected to the same processing.

Enzyme linked immunoosorbent assay

The content of MCP-1 in the liquid culture conditioned media was determined using human specific enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Biosource, Camarillo, CA, USA). Total protein content of the culture media from treatments and control were determined using BCA protein Assay Kit (Pierce, Rockford, IL, USA).

Statistical analyses

The factors considered during statistical analyses included cell line, HK-2 cells, treatments (control, CaOx alone or in combination with catalase and SOD and treatment time (0, 3, 6, and 12 h). Each experiment was done at least three times. The analysis was done using one-way analysis of variance (ANOVA) with Boneferri/Dunn to look at the variability within an experiment (intra) and among the experiments (inter). A P value of < 0.05 was considered significant.

Results

LDH release and lipid peroxidation

Figure 1 shows LDH release by cells under confluent conditions. In general, there was a time and concentration dependent increase in LDH release by HK-2 cells. There was little to no increase in LDH release when exposed to 67 μg/cm^2 COM as compared to control for up to 6 h. However, LDH release increased in 12 h and declined to the base line in 24 h. There was a significant increase in LDH release at 3 h ($P < 0.05$) following exposure to 133 μg/cm^2 COM, which declined within 24 h. At 267 μg/cm^2 level, LDH release increased with time and reached a peak in 12 h ($P < 0.05$). The increase in LDH release into the medium of cells exposed to high crystal load and for longer duration is most likely due to cell death which releases cell contents into the medium.

Production of superoxide

The production of superoxide anion by confluent HK-2 cells was measured following exposure to COM using nitro blue tetrazolium assay (Fig. 2). When cells were exposed to COM crystals, there was a highly significant increase in superoxide production initially, in a time and concentration dependent manner. However, after 6 h exposure, all concentration levels caused similar increases and superoxide production reached a plateau after 12 h.

Expression and production of MCP-1 by RT-PCR

Reverse transcriptase-polymerase chain reaction was performed to determine MCP-1 mRNA expression in HK-2 cells. Exposure to COM crystals stimulated the expression of MCP-1 mRNA. Peak expression occurred at 3 h and stayed the same for up to 6 h, and then declined within the next 12 h. Figure 3 shows the level of expression of MCP-1 mRNA by RT-PCR in HK-2 cells exposed to 133 or 267 μg/cm^2 concentrations of COM for 0–24 h, demonstrating the predicted 315 bp fragment of MCP-1 (lower bands) and 590 bp fragment of β-actin (upper bands).

Western blot analysis

The cell extract of control and treated cells (133 μg/cm^2 COM) was subjected to Western blotting with mouse monoclonal antibody against human MCP-1 (Fig. 4). The molecular weight of hMCP-1 is about 8.7 kDa.

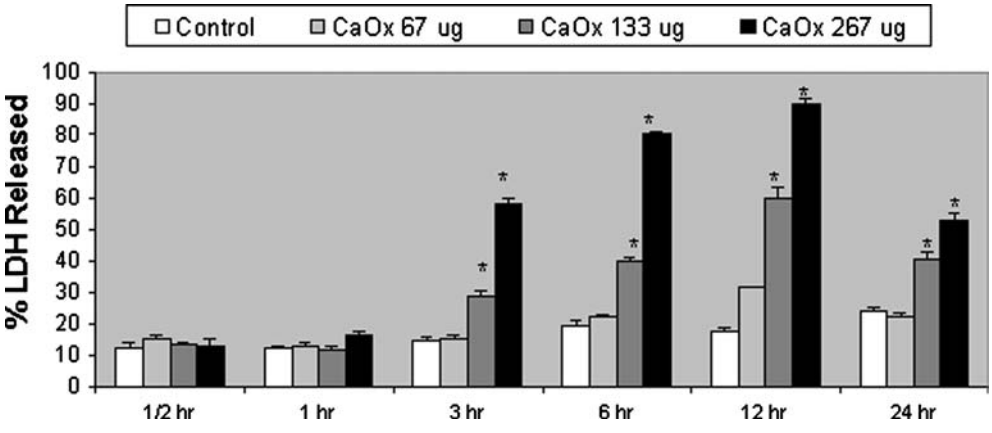

Fig. 1 Percent increase in lactate dehydrogenase (*LDH*) release against control by HK-2 cells exposed to 67, 133 and 267 μg/cm^2 of CaOx using confluent cells for 0.5, 1, 3, 6, 12 and 24 h. A positive control (cells lysed with lysis buffer supplied with kit) and blank (media) were also aliquoted to the designated wells. Data shown are mean ± SD of three separate experiments with three independent replicates. *$P < 0.05$ versus 133 and 267 μg/cm^2 of CaOx, 3, 6, 12 and 24 h

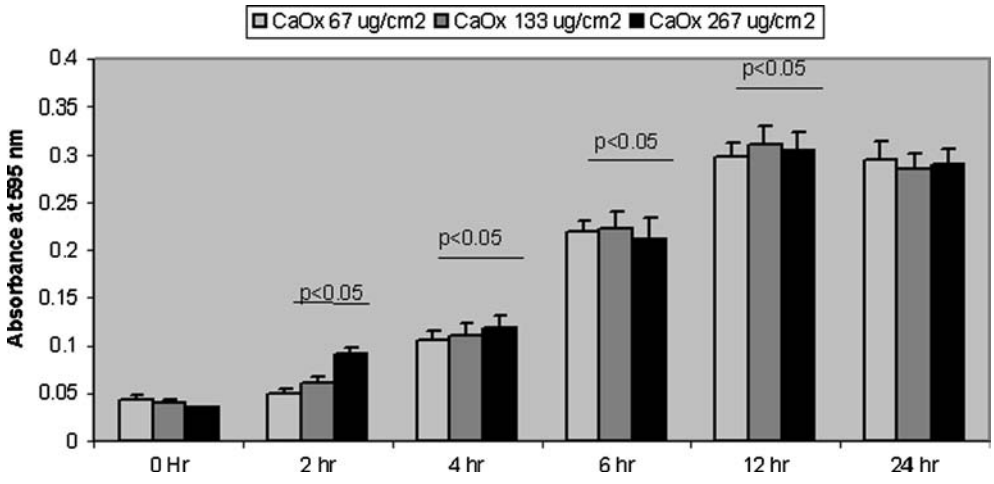

Fig. 2 Time dependent effect of COM on production of superoxide anion following exposure to 67, 133 and 267 μg/cm^2 of CaOx using confluent cells for 0, 2, 4, 6, 12, 24 h. Data shown are mean ± SD of three separate experiments with 12 independent replicates. $P < 0.05$ versus 67, 133 and 267 μg/cm^2 of CaOx, 2, 4, 6 and 12 h. After 12 h, they all reached a plateau

When the cell extract was electrophoresesd by SDS-PAGE, the antibody cross-reacted with a band corresponding to approximately 8–10 kDa. There was one more band corresponding to approximately 20 kDa. This band could be MCP-1 dimer or the result of a nonspecific reaction with albumin. No band was detected with the control, untreated cells.

Enzyme linked-immunoosorbent assay

Exposure of HK-2 cells to COM crystals was associated with concentration and time dependent changes in the production and release of MCP-1. Exposure to 67 μg/cm^2 crystals resulted in a highly significant increase in MCP-1 in the medium, which was even higher when cells were exposed to more crystals at a concentration of 133 μg/cm^2. However, a further increase in crystal amount did not produce any more MCP-1, it actually went down (Fig. 5).

Increase in cellular as well as media levels of MCP-1 was also time dependent (Fig. 6a, b). There was continuous and manifold increase in cellular MCP-1 within the first 12 h. After a 24 h exposure, cellular MCP-1 stayed significantly higher than control but not significantly different from that after12 h. After 48 h it was still higher than control, but significantly lower than after 24 h exposure. Increases in MCP-1 levels in the culture medium followed a similar time dependent although slightly different pattern. There was a continuous and significant increase in MCP-1 levels for up to 24 h followed by significant decrease after 48 h of the exposure.

Effect of free radical scavengers on LDH release

Catalase or SOD was used to evaluate the effect of these free radical scavengers (Fig. 7a, b). Briefly, cells were pretreated with catalase or SOD for 8–12 h. After treatment, the media was removed and replaced with fresh serum free media containing COM alone and/or in combination with catalase or SOD for 3 or 6 h. There was a significant increase in LDH release (72%) at 3 h following exposure to 133 μg/cm^2 COM in the absence of catalase (Fig. 7a) over that of the control. However,

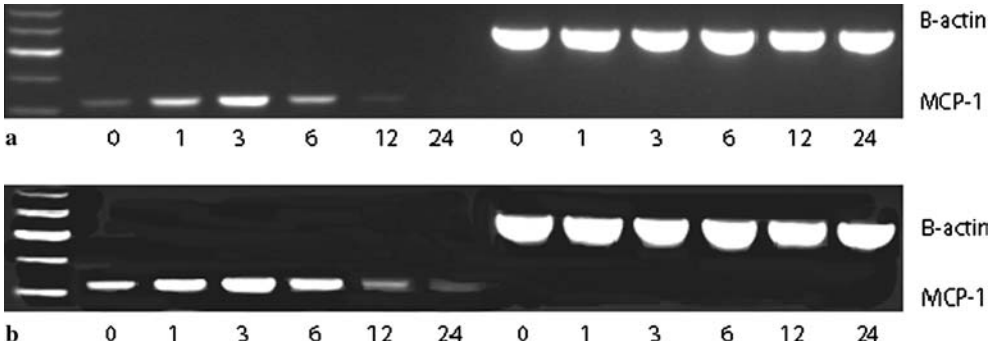

Fig. 3 **a** Expression of MCP-1 mRNA relative to β-actin by RT-PCR in HK-2 exposed to COM (133 μg/cm^2) for 0–24 h. The lower band is the predicted 315 bp fragment of MCP-1 and the upper band is predicted 590 bp fragment of β-actin. Two independent experiments showed similar results. **b** Expression of MCP-1 mRNA relative to β-actin by RT-PCR in HK-2 exposed to COM (267 μg/cm^2) for 0–24 h. The lower band is the predicted 315 bp fragment of MCP-1 and the upper band is predicted 590 bp fragment of β-actin. Two independent experiments showed similar results

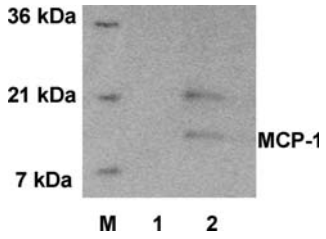

Fig. 4 Detection of MCP-1 protein by Western blot analysis in HK-2 cells. The treated cells were exposed to CaOx (133 μg/cm2) for 6 h. Protein was isolated from control and treated cells using TRIzol Reagent and subjected to electrophoresis and analyzed using anti MCP-1 monoclonal antibody. *M* prestained molecular weights marker; *lane 1* is the control and *lane 2*, treated cells. The position of monomer MCP-1 is indicated (10 Kd). Two independent experiments showed similar results

when cells were exposed to COM in the presence of catalase, the level of LDH released decreased, but did not reach that of the control (received no catalase at any time) (Fig. 7a). It is also interesting to note that the level of LDH release in cells exposed to catalase alone is lower than that of control (received no catalase at any time).

Similar results were obtained when cells were exposed to COM (133 μg/cm^2) in the absence of SOD (Fig. 7b). LDH increase was approximately 60%, which dropped in the presence of SOD. However, the level of inhibition with SOD did not drop to that of the control level. When cells were incubated for 6 h, the results were similar to that of the incubation at 3 h but slightly higher LDH release (data not shown).

Effect of free radical scavengers on MCP-1 mRNA expression

Reverse transcriptase-polymerase chain reaction was used to determine the effect of free radical scavengers on MCP-1 mRNA expression in HK-2 cells (Fig. 8a, b). The MCP-1 mRNA level was reduced when cells were exposed to COM in the presence of catalase compared to the state when cells were exposed to COM alone. Endogenous (constitutive) MCP-1 mRNA was also reduced when cells were incubated with catalase as compared to the control cells that received no catalase (Fig. 8a). The pattern of inhibition of MCP-1 mRNA level was similar when SOD was used (Fig. 8b). The level of MCP-1 mRNA was reduced when cells were exposed to COM in the presence of SOD.

Discussion

Monocyte chemoattractant protein-1, a member of CC chemokines, is expressed and produced by different cell types including endothelial cells, vascular smooth muscle cells and renal cells. It is barely detectable in normal kidneys. However, MCP-1 gene expression and protein production is greatly increased in the disease states. MCP-1 mRNA was shown to increase significantly in chronic relapsing (CR)-experimental autoimmune encephalomyelitis (EAE) lesions rather than in acute EAE lesions. More importantly, the mRNA induction during the remission of CR–EAE was higher than during the recovery phase of acute EAE, suggesting that the high level of MCP-1 in CR–EAE is associated with relapse of the disease [25]. When urinary MCP-1 levels were measured in patients with glomerulopathies, the data demonstrated that MCP-1 was present in the urine of patients with glomerular disease while those with inflammatory glomerulopathies had higher urinary MCP-1 [26]. The urinary MCP-1 was biologically active in chemotaxis assay, which was attenuated by anti MCP-1 antibody. MCP-1 expression has also been shown in human crescentic glomerulonephritis [27], acute lung injury in rats [28], lupus nephritis in mice [29], etc. The activation of MCP-1 gene is mediated by redox sensitive mechanisms. For example, superoxide and H_2O_2 are required for TNF-α-induced MCP-1 gene

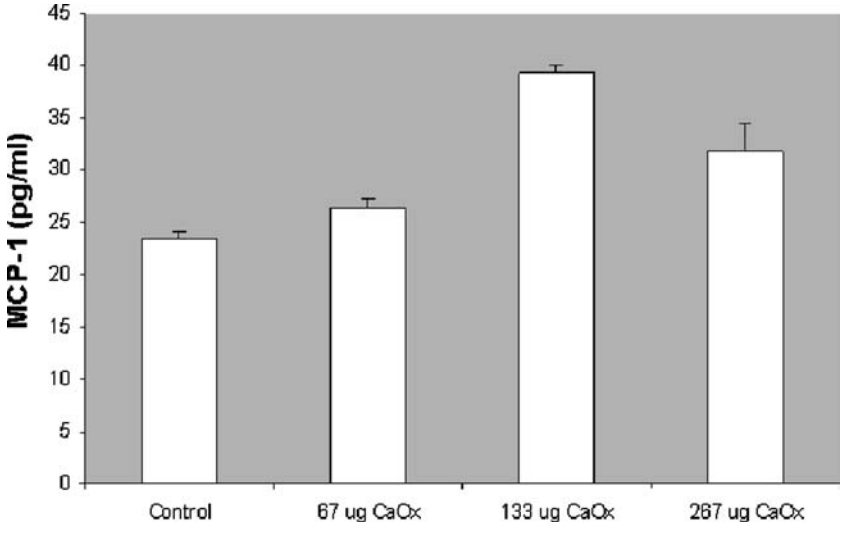

Fig. 5 Dose dependent effect of COM crystals on MCP-1 production released into the culture media of HK-2 cells after 6 h of exposure. The data are presented as mean ± SD. Exposure to all concentrations of crystals was associated with significant ($P < 0.05$) increase in MCP-1 production compared to control. Differences between responses to various crystal concentrations were also significant ($P < 0.05$)

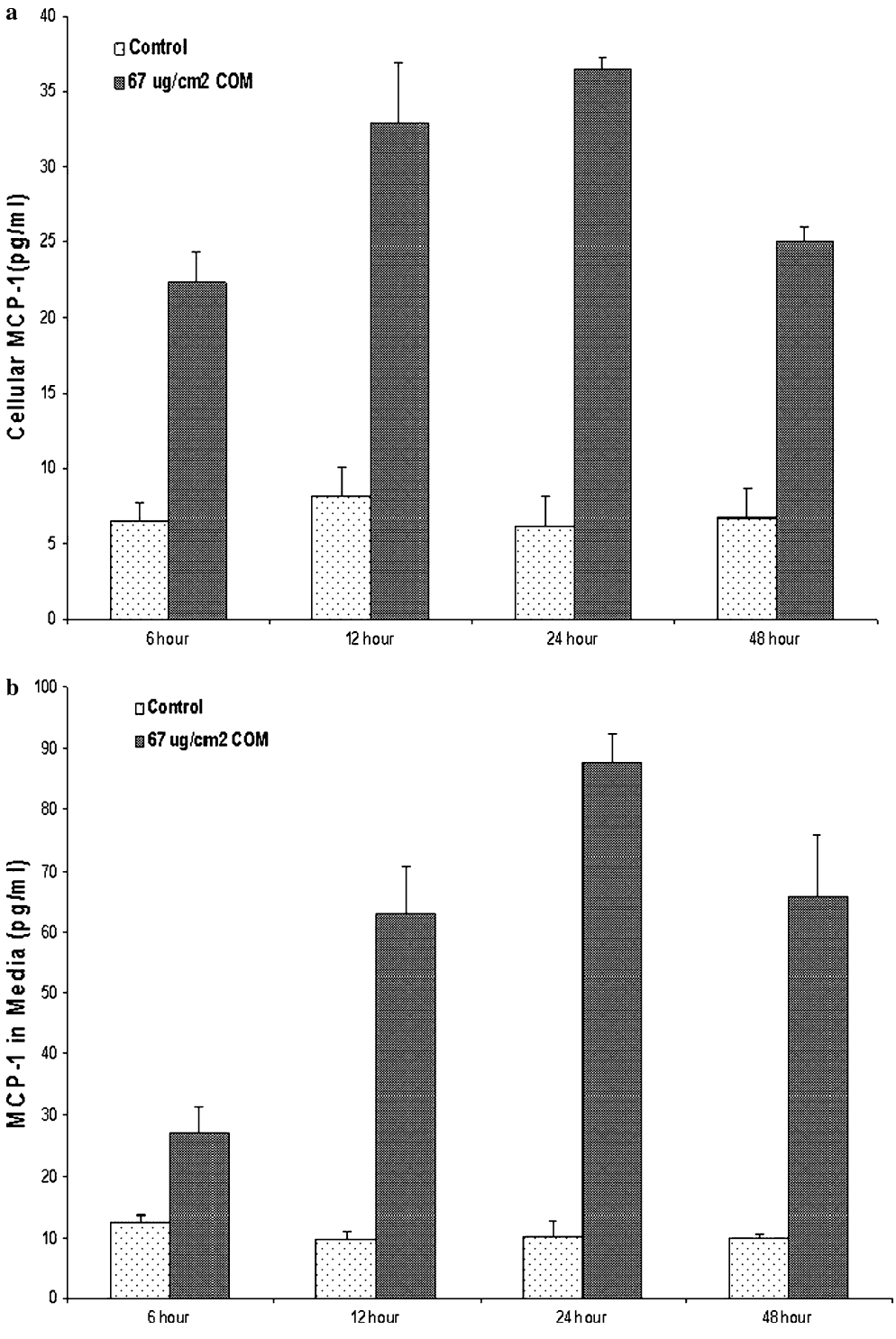

Fig. 6 Time dependent changes in MCP-1 production (**a**) and release (**b**) into the medium. MCP-1 production and release are significantly increased for the first 24 h of exposure and then decreased after 48 h

expression in the endothelial cells [30]. Enhanced MCP-1 expression during ischemia/reperfusion injury is also mediated by oxidative stress [31].

Other studies have shown that MCP-1 is expressed when the cells are exposed to crystals and increased expression is associated with crystal induced oxidative stress [32]. Using a murine alveolar type II cell line, murine lung epithelial cell (MLE)-15, the changes in several chemokines including MCP-1 were measured following exposure to silica in combination with antioxidant [33]. It was shown that MCP-1 was expressed after exposure to silica and expression was attenuated by antioxidant treatment. In another study, rat pleural mesothelial cells were exposed to chrysotile and crocidolite asbestos fibers [34]. Both upregulated MCP-1 mRNA expression and protein synthesis.

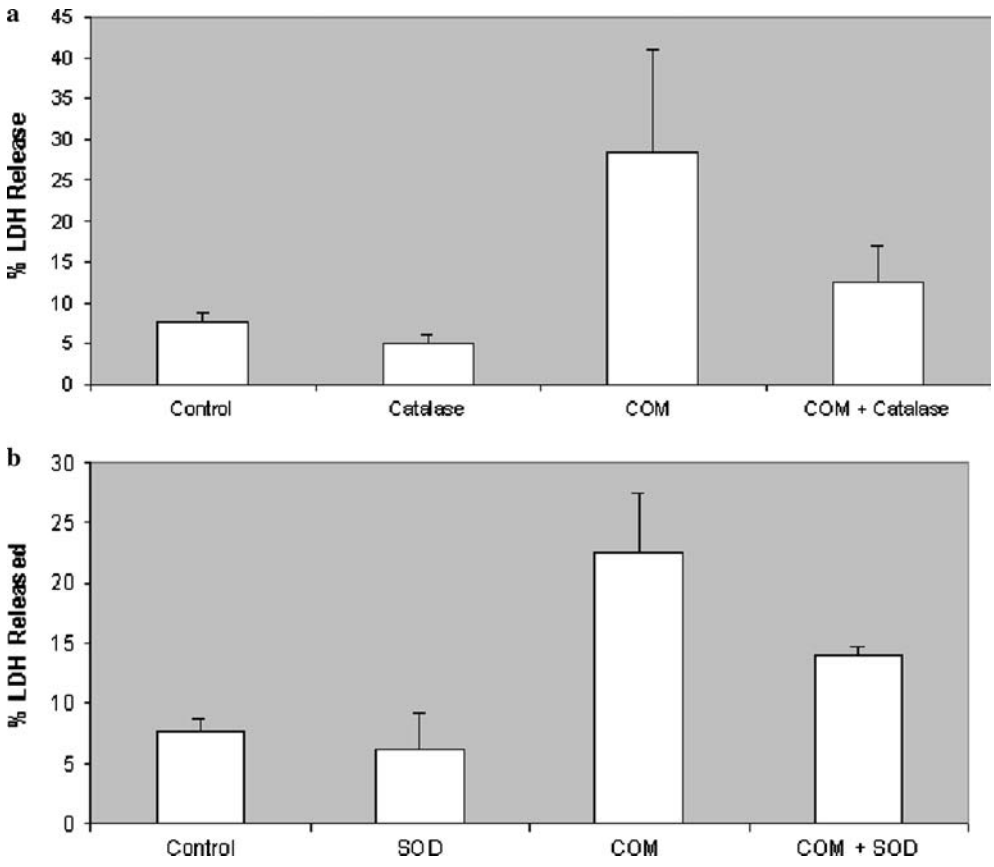

Fig. 7 Percent increase in lactate dehydrogenase (*LDH*) release against control by HK-2 cells exposed to CaOx monohydrate (*COM*) with or without **a** catalase or **b** superoxide dismutase (*SOD*) for 3 h. The bar graphs show the mean ± SED of two separate experiments performed in triplicates. Crystal exposure caused significant increase ($P < 0.05$) in LDH release. LDH release was significantly reduced ($P < 0.05$) in the presence of both catalase and SOD

Results of this study provided evidence that HK-2 cells constitutively express MCP-1 mRNA, and its expression is significantly increased following exposure to COM crystals. The increase in MCP-1 gene expression was time and concentration dependent. Earlier studies with NRK-52E cells have also shown a time and concentration dependent increase in crystal-induced MCP-1 expression. The gene expression started to increase as early as 1 h after the exposure. Production and secretion as indicated by the cellular and media levels of MCP-1 also went up quickly.

The reason for continuous expression of this chemokine and the factors involved are not known, although ROS seem to play a role. When the cells were treated with catalase or SOD in the absence of any stimuli, the constitutive expression of MCP-1 was decreased. Furthermore, investigating the effect of free radical scavenger on MCP-1 mRNA expression in HK-2 cells exposed to COM showed that the expression of MCP-1 gene was reduced upon treatment with catalase and/or SOD. The fact that COM induces MCP-1 gene expression that can be suppressed by SOD provides evidence that superoxide anion is involved in regulation of MCP-1 gene expression. Treatment with catalase also reduced MCP-1 expression indicating the involvement of H_2O_2. SOD dismutates superoxide to H_2O_2, which is further degraded by either catalase or glutathione peroxidase. However, COM crystal induced expression of MCP-1, in the presence of free radical scavengers SOD and catalase, was higher than that of the control suggesting other ROS may also be produced and/or a different mechanism may also be involved [30, 35, 36]. For example, O_2^- can react with NO, yielding $ONOO^-$ which is highly reactive [30], and is also capable of generating reactive OH. ROS including O_2^-, HO, $ONOO^-$, as well as H_2O_2 are currently considered as signaling molecules, which are mobilized in response to pro-inflammatory stimuli. Although several studies have clearly shown a major role for ROS in the development and progression of diseases, the relative roles of each ROS and their connection to inflammatory signaling is not yet fully understood.

Calcium oxalate monohydrate-induced production of superoxide was also injurious to cells as indicated by the release of LDH. LDH release was significantly reduced in the presence of extracellular catalase and SOD, which provide protection against H_2O_2 and superoxide anion. The catalase treatment had a more salutary effect on COM induced LDH release than SOD treatment. Although SOD is not really a detoxifying enzyme, its product H_2O_2 is a toxic agent. Dismutation of superoxide anion, O_2^-, into H_2O_2 is the first step of the enzymatic process. Further conversion of H_2O_2 into water results in the inactivation of the ROS. H_2O_2 is degraded by the enzyme catalase and glutathione peroxidase. In addition, cells also contain compounds such as glutathione (GSH), ascorbic acid, NADPH and α-tocopherol, which donate hydrogen to neutralize ROS. Earlier

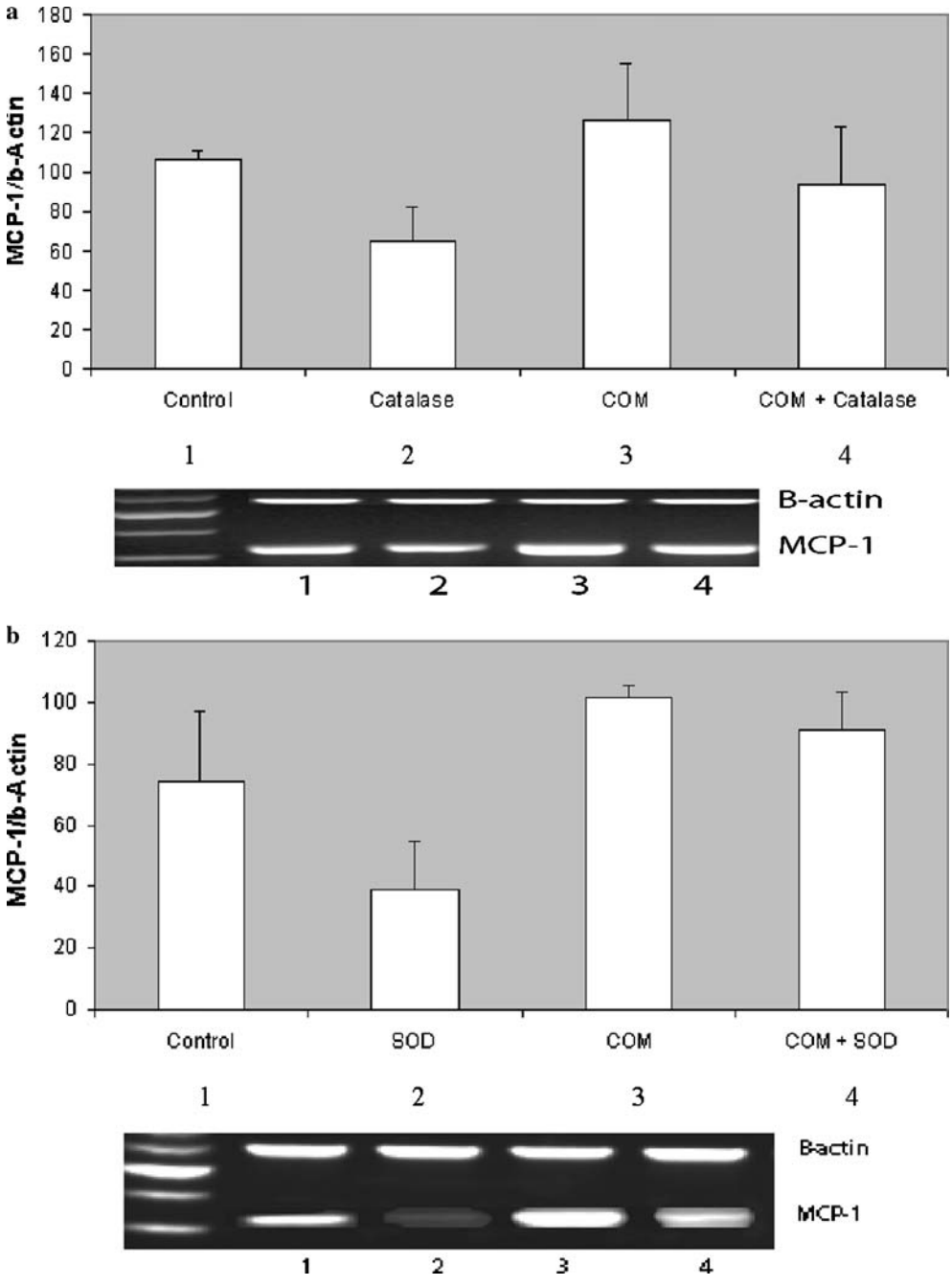

Fig. 8 Expression of MCP-1 mRNA by RT-PCR in HK-2 exposed to with or without catalase (**a**) and superoxide dismutase (*SOD*) (**b**) for 3 h. Samples are as follows **a**: *1* control, *2* catalase alone, *3* COM, *4* COM + Cat **(b)**: *1* Control, *2* SOD alone, *3* COM, *4* COM + SOD. The lower band is the predicted 315 bp fragment of MCP-1 and the upper band is predicted 590 bp fragment of β-actin. The *bar graphs* show the MCP-1/β-actin ratio. The results are the representative of two separate experiments performed. Endogenous or coonstituitive MCP-1 was significantly reduced in the presence of both SOD and catalase while reduction of COM induced expression of MCP-1 mRNA did not reach significant levels

studies have shown that cellular glutathione levels are reduced when renal epithelial cells are exposed to COM [37, 38].

These results are in accordance with previous studies providing evidence that COM as well as other crystals can be injurious to LLC-PK1, a line of porcine renal epithelial cells, NRK52E cells, a line of murine renal epithelial cells, and MDCK, a line of canine renal epithelial cells [9, 10, 22]. It has previously been shown that exposure of HK-2 cells to oxalate produce time and concentration dependent changes and show marked sensitivity to high oxalate concentrations [21, 39]. Oxalate exposure is associated with significant increase in superoxide production. Membrane permeability as determined by trypan blue staining, is significantly increased and DNA synthesis is reinitiated. Prolonged exposure to high oxalate concentrations results in cell death.

To date, the general agreement is that the main role of MCP-1 is the chemotactic activity, attracting monocytes to the site of injury [40–43]. Role of chemokines such as MCP-1 and IL-10 and RANTES in the trafficking of leukocytes has been demonstrated in disorders such as rheumatoid arthritis [40], lung allergic inflammation [41], pulmonary *Cryptococcus neoformans* infection [42], and Human cytomegalovirus (HMCV)

infection [43]. These infiltrated cells may in turn initiate an inflammatory response by producing cytokines, chemokines and toxic substances such as ROS leading to further injury. In a study involving thrombin, it was demonstrated that thrombin-activated tissues might induce a cascade of events characterized by IL-8 secretion, neutrophil local infiltration, and the release of IL-6Rα from neutrophil membranes. sIL-6Rα may then complex with IL-6 and increase the amount of MCP-1 secretion, favoring mococyte infiltration and a shift from acute into chronic inflammation [44]. It has also been demonstrated that MCP-1, a major contributor to monocyte infiltration into breast tumors, and monocytic secreted TNFα upregulate each other resulting in vivo in a positive feedback loop that supports disease progression [45].

To date no study has so far addressed the definite role of the MCP-1 in oxalate and CaOx-induced injury, inflammation and stone formation. Support for the concept of inflammation playing a significant role in CaOx nephrolithiasis comes from studies investigating the involvement of RAS in CaOx crystal deposition in kidneys of experimental rats. As mentioned earlier, recent studies show that experimentally induced hyperoxaluria and CaOx crystal deposition in rat kidneys activate RAS. ACE inhibitors and Ang II type 1 receptor blockers reduced inflammation as well as CaOx crystal deposition in the kidney [13–15], and reductions were associated with diminished oxidative stress [13, 14]. Reduction in inflammation and crystal deposition is associated with reduced oxidative stress. Ang II type 1 receptor blockage by the administration of candesartan to hyperoxaluric rats increased the expression of mRNA for osteopontin, renin and ACE and reduced CaOx crystal deposition and infiltration of monocyte/macrophages (M/M) into renal interstitium, despite similar urinary calcium and oxalate levels. In addition, candesartan administration resulted in improved albumin excretion and serum creatinine level. Ang II type 1 receptor blockade by 1osartan administration also resulted in reduced CaOx crystal deposition, M/M infiltration, and interstitial fibrosis. Ang II type 1 receptor blockage also reduced the oxidative stress. Similarly, the administration of ACE inhibitor, enalapril, to hyperoxaluric rates also reduced CaOx crystal deposition, M/M infiltration and interstitial fibrosis [15]. Ang II-induced MCP-1 gene and protein expression in mouse proximal tubular cells have also been shown to be mediated by ROS [46].

Injuries associated with severe hyperoxaluria and CaOx crystal deposition in the kidneys may be caused by stimulation of RAS and overproduction of chemokines. Our present as well as earlier studies demonstrate that CaOx crystals strongly induce MCP-1 synthesis and secretion by the renal epithelial cells. Intracellular ROS production appears to be critical for crystal induced inflammatory response. Progression of tubulointerstitial damage may determine the final outcome. Therefore, antioxidant therapy and blockade of RAS may prove beneficial for the prevention of end stage renal disease and failure.

Acknowledgements Supported by NIH grants # RO1 DK 053962, 059765, and 065658.

References

1. Parmar MS (2004) Kidney stones. BMJ 328:1420–1424
2. Kok DJ, Khan SR (1994) Calcium oxalate nephrolithiasis, a free or fixed particle disease. Kidney Int 46:847–854
3. Finlayson B, Reid F (1978) The expectation of free and fixed particles in urinary stone disease. Invest Urol 15:442–448
4. Khan SR (1995) CaOx crystal interaction with renal epithelium, mechanism of crystal adhesion and its impact on stone development. Urol Res 23:71–79
5. Finlayson B (1977) Calcium stones: some physical and clinical aspects. In: David DS (ed) Calcium metabolism in renal failure and nephrolithiasis. Wiley, New York, pp 337–382
6. Lieska JC, Toback FG (1997) Renal cell–urinary crystal interactions. Curr Opin Nephrol Hypertens 9:349–355
7. Thamilselvan S, Hackett RL, Khan SR (1997) Lipid peroxidation in ethylene glycol induced hyperoxaluria and calcium oxalate nephrolithiasis. J Urol 157:1059–1063
8. Wiessner JH, Hasegawa AT, Hung LY, Mandel NS (1999) Oxalate-induced exposure of phosphatidylserine on the surface of renal epithelial cells in culture. J Am Soc Nephrol 14(10 Suppl):S441–S445
9. Scheid CR, Koul H, Hill WA, Luber-Narod J, Kennington L, Honeyman T, Jonassen J, Menon M (1996) Ox toxicity in LLC-PK1 cells: role of free radicals. Kidney Int 49:413–419
10. Umekawa T, Chegini N, Khan SR (2002) Oxalate ions and calcium oxalate crystals stimulate MCP-1 expression by renal epithelial cells. Kidney Int 61:105–112
11. DeWater R, Noordermeer C, van der Kwast TH, Nizze H, Boeve ER, Kok DJ, Schroeder FH (1999) CaOx nephrolithiasis in rats: the effect of renal crystal deposition on the cellular composition of the renal interstitium. Am J Kidney Dis 33:761–771
12. De Water R, Leenen PJ, Noordermeer C, Nigg AL, Houtsmuller AB, Kok DJ, Schroder FH (2001) Cytokine production induced by binding and processing of calcium oxalate crystals in cultured macrophages. Am J Kidney Dis 38:331–338
13. Umekawa T, Hatanaka Y, Kurita T, Khan SR (2004) Effect of angiotensin II receptor blockage on osteopontin expression and calcium oxalate crystal deposition in rat kidneys. J Am Soc Nephrol 15:635–644
14. Toblli JE, Ferder L, Stella I, Cavanagh EMV, Angerosa M, Iserra F (2002) Effects of angiotensin II subtype 1 receptor blockade by 1osatan on tubulointerstitial lesions caused by heperoxaluria. J Urol 168:1550–1555
15. Toblli JE, Ferder L, stella I, Angerosa M, Inserra F (2002) Protective role of enalapril for hyperoxaluric tubulointerstitial lesions of hyperoxaluria. J Urol 166:275–280
16. Kaneto H, Fukuzaki A, Ishidoya S et al (2000) mRNA expression of chemokines in rat kidneys with ureteral obstruction. Nippon Hin Gak Zas 91:69–74
17. Schmouder RL, Strieter RM, Kunkel SL (1993) Interferon-gamma regulation of human renal cortical epithelial cell-derived monocyte chemotactic peptide-1. Kidney Int 44:43–49
18. Kakizakai Y, Waga S, Sugimoto K et al (1995) Production of monocyte chemoattractant protein-1 by bovine glomerular endothelial cells. Kidney Int 48:1866–1874
19. Stephan S, Nelson PJ, Schlondorff D (2000) Chemokines, chemokine receptors and renal diseases: from basic science to pathophysiologic and therapeutic studies. J Am Soc Nephrol 11:152–176
20. Umekawa T, Chegini N, Khan SR (2003) Increased expression of monocyte chemoattractant protein-1 (MCP-1) by renal epithelial cells in culture on exposure to calcium oxalate, phos-

phate and uric acid crystals. Nephrol Dial Transplant 18:664–666
21. Bhandari A, Koul S, Sekhon A, Pramanik SK, Chaturvedi LS, Huang M, Menon M, Koul HK (2002) Effects of oxalate on HK-2 cells, a line of proximal tubular epithelial cells from normal human kidney. J Urol 168:253–259
22. Hackett RL, Shevock PN, Khan SR (1995) Alterations in MDCK and LLC-PK1 cells exposed to oxalate and calcium oxalate monohydrate crystals. Scanning Microsc 9(2):587–596
23. Thamilselvan S, Byer KJ, Hackett RL, Khan SR (2000) Free radical scavengers, catalase and superoxide dismutase provides protection from oxalate-associated injury to LLC-PK1 and MDCK cells. J Urol 164:224–229
24. Rook GAW, Steele J, Umar S, Dockrell HM (1985) A simple method for the solubilisation of reduced NBT, and its use as a colorimetric assay for activation of human macrophages by γ-interferon. J Immunol Methods 82:161–167
25. Jee Y, Yoon WK, Okura Y, Tanuma N, Matsumoto Y (2002) Upregulation of monocyte chemotactic protein-1 and CC chemokine receptor 2 in the central nervous system is closely associated with relapse of autoimmune encephalomyelitis in Lewis rats. J Neuroimmunol 128:49–57
26. Rovin BH, Doe N, Tan LC (1996) Monocyte chemoattractant protein-1 levels in patients with glomerular disease. Am J Kidney Dis 27:640–646
27. Segerer S, Nelson PJ, Schlondorff D (1999) Expression of the C–C chemokine receptor 5 in human kidney diseases. Kidney Int 56:52–64
28. Bless NM, Huber-Lang M, Guo RF, Warner RL, Schmal H, Czermak BJ, Shanley TP, Crouch LD, Lentsch AB, Sarma V, Mulligan MS, Friedl Hp, Ward PA (2000) Role of CC chemokines (macrophage inflammatory protein-1 beta, monocyte chemoattractant protein-1, RANTES) in acute lung injury in rats. J Immunol 164:2650–2659
29. Perez de Lema G, Maier H, Nieto E, Vielhauer V, Luckow B, Mampaso F, Schlondorff D (2001) Chemokine expression precedes inflammatory cell infiltration and chemokine receptor and cytokine expression during the initiation of murine lupus nephritis. J Am Soc Nephrol 12:1369–1382
30. Misko TP, Highkin MK, Veenhuizen AW, Manning PT, Stern MK, Currie Mg, Salvemini D (1998) Characterization of the cytoprotective action of peroxynitrite decomposition catalysts. J Biol Chem 273:15646–15653
31. Snug FL, Zhu TY, Au-Yeung KKW, Siow YL, Karmin O (2002) Enhanced MCP-1 expression during ischemia/reperfusion injury is mediated by oxidative stress and NF-kB. Kidney Int 62:1160–1170
32. Aihara K, Byer K J, Khan SR (2003) Calcium phosphate-induced renal epithelial injury and stone formation: involvement of reactive oxygen species. Kidney Int 64:1283–1291
33. Barrett EG, Johnston C, Oberdorster G, Finkelstein JN (1999) Silica-induced chemokine expression in alveolar type II cells is mediated by TNF-alpha-induced oxidant stress. Am J Physiol 276:L979–L988
34. Nasreen N, Hartman DL, Mohammed KA, Antony VB (1998) Talc-induced expression of C–C and C–X–C chemokines and intercellular adhesion molecule-1 in mesothelial cells. Am J Respir Crit Care Med 158:971–978
35. Chen, Xi-Lin, Zhang Q, Zhao R, Medford RM (2004) Superoxide, H_2O_2 and iron are required for TNF-α-induced MCP-1 gene expression in endothelial cells: role of Rac1 and NADPH oxidase. Am J Physiol Heart Circ Physiol 286:H1001–H1007
36. Matata BM, Galinanes M (2002) Peroxynitrite is an essential component of cytokines production mechanism in human monocyte through modulation of nuclear factor-kappa B DNA binding activity. J Biol Chem 277:2330–2335
37. Khand FD, Gordge MP, Robertson WG, Notonha-Dutra AA, Hothersall JS (2002) Mitochondrial superoxide production during oxalate mediated stress in renal epithelial cells. Free Radic Biol Med 32:1339–1350
38. Umekawa T, Byer K, Kurita T, Khan SR (2005) Diphenyleneiodium (DPI) reduces oxalate ions and calcium oxalate monohydrate and brushite crystal-induced up-regulation of MCP-1 in NRK-52E cells. Nephrol Dial Transplant 20(5):870–878
39. Koul S, Fu S, Koul H (2003) Oxalate exposure promotes re-initiation of the DNA synthesis and apoptosis of KH-2 cell, a line of human renal epithelial cells. Ann N Y Acad Sci 1010:292–295
40. Hosaka S, Akahoshi T, Wada C, Kondo H (1994) Expression of the chemokine superfamily in rheumatoid arthritis. Clin Exp Immunol 97:451–457
41. Gonzalo JA, Lloyd CM, Wen D, Albar JP, Wells TN, Proudfoot A, Martinez-A C, Dorf M, Bjerke T, Coyle AJ, Gutierrez-Ramos JC (1998) The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyper responsiveness. J Exp Med 188:157–167
42. Huffnagle GB, Strieter RM, Standiford TJ, McDonald RA, Burdick MD, Kunkel SL, Toews GB (1995) The role of monocyte chemotactic protein-1 (MCP-1) in the recruitment of monocytes and CD4+ T cells during a pulmonary *Cryptococcus neoformans* infection. J Immunol 155:4790–4797
43. Momma Y, Nagineni CN, Chin MS, Srinivasan K, Detrick B, Hooks JJ (2003) Differential expression of chemokines by human retinal pigment epithelial cells infected with cytomegalovirus. Invest Ophthalmol Vis Sci 44:2026–2033
44. Marin V, Montero-Julian FA, Gres S, Boulay V, Bongrand P, Farnarier C, Kaplanski G (2001) The IL-6-soluble IL-6 R alpha autocrine loop of endothelial activation as an intermediate between acute and chronic inflammation: an experimental model involving thrombin. J Immunol 167:3435–3442
45. Neumark E, Sagi-Assif O, Shalmon B, Ben-Baruch A, Witz IP (2003) Progression of mouse mammary tumors: MCP-1-TNF-alpha cross-regulatory pathway and clonal expression of promalignancy and antimalignancy factors. Int J Cancer 106:879–886
46. Tanifuji C, Suzuki Y, Geot WM, Horikoshi S, Sugaya T, Ruiz-Ortega M, Egido J, Tomino Y (2005) Reactive oxygen species-mediated signaling pathway in angiotensin II-induced MCP-1 expression of proximal tubular cells. Antioxid Redox Signal 7:1261–1268