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REVIEW ARTICLE

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Crystal-induced inflammation of the kidneys: results from human studies, animal models, and tissue-culture studies

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Abstract
Calcium oxalate (CaOx), calcium phosphate (CaP), and uric acid or urate are the most common crystals seen in the kidneys. Most of the crystals evoke an inflammatory response leading to fibrosis, loss of nephrons, and eventually to chronic renal failure. Of the three, CaOx monohydrate is the most reactive, whereas some forms of CaP do not evoke any discernible response. Reactive oxygen species are produced during the interactions between the crystals and renal cells and are responsible for the various cellular responses. CaOx crystals generally form in the renal tubules. Exposure of renal epithelial cells to CaOx crystals results in the increased synthesis of osteopontin, bikunin, heparan sulfate, monocyte chemoattractant protein 1 (MCP-1), and prostaglandin (PG) E2, which are known to participate in inflammatory processes and in extracellular matrix production. CaOx crystal deposition in rat kidneys also activates the renin–angiotensin system. Both Ox and CaOx crystals selectively activate p38 mitogen-activated protein kinase (MAPK) in exposed tubular cells. CaP crystals can form in the tubular lumen, tubular cells, or tubular basement membrane. Renal epithelial cells exposed to brushite crystals produce MCP-1. Basic CaP and calcium pyrophosphate dihydrate induce mitogenesis in fibroblasts, stimulate production of PGE2, and up-regulate the synthesis of metalloproteinases (MMP) while down-regulating the production of inhibitors of MMPs through activation of p42/44 MAPK. Deposition of urate crystals in the kidneys becomes associated with renal tubular atrophy, interstitial fibrosis, and development of inflammatory infiltrate. Renal epithelial cells exposed to uric acid crystals synthesize MCP-1 as well as PGE2. Monocytes or neutrophils exposed to urate crystals produce tumor necrosis factor α, interleukin-1 (IL-1), IL-6, and IL-8. Expression of IL-8 is mediated through extracellular signal-regulated kinase 1 (ERK-1)/ERK-2 and nuclear transcription factors activated protein 1 and nuclear factor κβ. Urate crystals also stimulate the macrophages to produce MMPs.

Key words Oxalate · Inflammation · Venin-angiotensin · Fibrosis · End stage renal disease

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Introduction

A variety of crystals have been shown to invoke an inflammatory response by diverse cell types. Silica stimulates alveolar macrophages and alveolar type II cells to produce a host of cytokines and chemokines, including monocyte chemoattractant protein 1 (MCP-1).[1,2] The cellular response is mediated in part by oxidant stress and can be attenuated by antioxidant treatment. Both crocidolite and chrysolite asbestos,[3] as well as talc,[4] stimulate mesothelial cells lining the pleura to produce MCP-1. In addition, on interaction with monosodium urate, cells lining the synovium produce the chemokine MCP-1.[5] Intra-articular deposition of hydroxyapatite, octacalcium phosphate, tricalcium phosphate, or calcium phosphate dihydrate leads to inflammation and is an established cause of inflammatory arthritis.[6]

Crystal deposition is a relatively common occurrence in the kidneys and is often associated with inflammation. Calcium phosphate (CaP) and calcium oxalate (CaOx) are the two most common crystals found in the kidneys and both can induce tubulointerstitial (TI) damage and inflammation. Urate crystal deposition in the kidneys during chronic gouty arthritis causes an intense inflammatory reaction. Microtophi containing urate crystals surrounded by macrophages, lymphocytes, and giant cells develop in the renal interstitium. Deposition of cystine crystals in the kidneys of homozygous cystinuric patients provokes an inflammatory response. CaP, CaOx, urate, and cystine crystal deposition in the kidneys can also lead to the development of kidney stones.

Necropsy studies of human kidneys have revealed calcification (CaP) in renal interstitium, mostly around the renal

tubules. Anderson and McDonald reported interstitial CaP crystal deposits in almost all the autopsied kidneys they examined.[7] In addition they found phagocytic cells in the process of ingesting calcareous material. Light and electron microscopic studies of a renal papilla and associated CaOx kidney stones showed CaP deposition in the interstitium and CaOx in the ducts of Bellini.[8] Distinct signs of injury and inflammation included necrosis of tubular epithelium and capillary endothelium, the presence of myofibroblasts, and a large amount of collagen and large numbers of leukocytes in the interstitium. Recently,[9] renal cortical and medullary tissue biopsies obtained from 19 idiopathic CaOx stone patients and four CaOx stone formers with intestinal bypass surgery were analyzed by light and electron microscopy. Idiopathic stone formers showed distinct subepithelial Randall's plaques.[10] Deposits of poorly crystalline apatite mixed with some calcium carbonate were seen in the interstitium close to the collagen fibers, surrounding the Loops of Henle, the vasa recta, and interstitial cells. Crystal deposition near the plaques was primarily limited to the basement membrane of the Loops of Henle and of some vasa recta. Tubular epithelial cells appeared, for the most part, unharmed and fibrosis was not detected in the surrounding interstitium. Kidneys of intestinal bypass surgery patients did not show any plaques or interstitial crystal deposits, only apatitic crystal deposits in some renal tubules. Tubules with crystals showed extensive damage and the surrounding interstitium appeared fibrotic.

Renal epithelial cells occupy approximately 80% of the total kidney volume and are particularly vulnerable to the changing environment in the glomerular filtrate and tubular fluid.[11] The interstitium contains a variety of resident and infiltrating cells, is associated with the tubular epithelium as well as the vascular network of the kidney, and is greatly influenced by pathological events in neighboring locations. Injury to the tubulointerstitium leads to inflammation, renal fibrosis, and progressive renal failure.[12–14] Pathogenesis involves production of chemoattractants causing interstitial infiltration by cells of monocyte/macrophage (M/M) lineage. Expression of transforming growth factor β_1 (TGF-β_1) is increased. There is increased synthesis of extracellular matrix (ECM) proteins such as collagen I, III, and IV; fibronectin; and laminin. Expression of metalloproteinases is decreased while that of protease inhibitors, including tissue inhibitors of metalloproteinases (TIMP), is increased, causing a reduction in degradation of ECM components. The renin–angiotensin system (RAS) plays an important role because angiotensin II (Ang II) participates in key events of the inflammatory response such as vascular permeability, infiltration of inflammatory cells, and tissue repair and remodeling. In addition, oxidative stress is considered a key element in the development of chronic disease. Various actions of Ang II and TGF-β are mediated by oxidative stress. Regulation of nuclear factor κβ (NF-κβ), which is intimately involved in inflammation, is also redox dependent.

A number of animal model and tissue culture studies have investigated the interactions between crystals and renal cells to better understand tubulointerstitial damage caused by crystal deposition in the kidneys. This report discusses observations from human kidneys and the consequences of experimentally induced renal epithelial exposure to CaOx, CaP, and urate crystals both in vivo in animal models and in vitro in tissue cultures.

Calcium oxalate crystals

Human studies

Calcium oxalate crystals have been observed in the kidneys of patients with a variety of disorders in which production and excretion of oxalate (Ox) is increased. In a biopsy from a patient with primary hyperoxaluria, crystals were seen within tubular epithelial cells as well as in the interstitium of the transplanted kidney.[15] Crystal endocytosis was associated with cell proliferation and the formation of multinucleated giant cells as well as with vascular and interstitial inflammation. Similar observations have been made in other cases of increased urinary excretion of Ox secondary to enteric hyperoxaluria, Crohn's disease, and after intestinal bypass.[16–18]

Higher than normal levels of renal enzymes gamma-glutamyl transpeptidase (GGTP), angiotensin 1 converting enzyme (ACE), β-galactosidase (GAL), and *N*-acetyl-β-glucoseaminidase (NAG) were found in the urine of idiopathic CaOx stone formers.[19] Because elevation of these enzymes in the urine is considered an indication of renal proximal tubular injury, it was concluded that stone patients had damaged renal tubules. Results of a recent study also showed that CaOx kidney stone patients excrete significantly higher levels of GAL and NAG in the urine.[20] In addition, urine from stone patients had significantly higher levels of α-glutathione *S*-transferase (α-GST), malondialdehyde (MDA), and thiobarbituric acid-reactive substances (TBARS) indicating that CaOx kidney stone-associated renal injury is most likely caused by the production of reactive oxygen species.

Animal model studies

The interactions between the renal epithelium and high levels of Ox, CaOx crystals, or both have been examined by inducing CaOx crystal deposition in animal models.[21–25] Experimental CaOx crystal deposition in the kidneys or nephrolithiasis can be induced by the administration of hyperoxaluria-causing agents such as sodium oxalate, ammonium oxalate (AOx), ethylene glycol (EG), or hydroxy-L-proline (HLP). With various coworkers, I have studied many aspects of urolithiasis in male and female Sprague–Dawley rats.[26–32] When hyperoxaluria is induced by administration of 0.75%–1% EG, there is an increase in urinary excretion of Ox within 2 days, hyperoxaluria is established within 3 days, CaOx crystalluria develops within 2 weeks, and CaOx nephrolithiasis develops within 4–6 weeks.[23] Urinary pH and excretion of citrate are significantly decreased. Other urinary factors and renal creatinine clearance remain

within the normal range. Crystal deposition starts in the collecting ducts of the renal papilla and large stone-like deposits are formed associated with the renal papillae, fornices, or both.

Kidneys of nephrolithic rats showed deposition of CaOx crystals in renal calyces and at the papillary tips. Many were located subepithelially, often anchored to the basement membrane.[33,34] Ultrastructural examination of the kidneys revealed damage to the epithelial cells lining the renal tubules, which contained crystals. In addition, intracellular edema, widened intercellular spaces, and many cells containing dividing nuclei were present. The clubbing of microvilli and focal loss of the brush border distorted the luminal cell surfaces. Cells often appeared to burst open and release their contents into the tubular lumen. Evidence has also been provided for apoptotic cell death of the tubular epithelial cells in the presence of high levels of Ox and CaOx crystals.[35]

The earliest noticeable changes were detected in cells of the proximal tubules, which appeared more sensitive. This injury resulted in death and detachment of many epithelial cells, thus resulting in exposure of the basal lamina. Most crystals were intraluminal and invariably associated with cellular degradation products, indicating the possibility of membrane-induced nucleation of CaOx crystals. Crystal deposition was associated with inflammation. Inflammatory cells such as monocytes, macrophages, and polymorphonuclear leukocytes migrated to the adjacent interstitium, and interstitial crystals were often seen surrounded by macrophages and giant cells.[33]

Our study in which kidneys were examined at different times after sodium oxalate injection-induced acute hyperoxaluria showed that crystals appeared first in the tubular lumen, then moved to inter- and intracellular locations, and eventually moved into the interstitium. After a few weeks, interstitial crystals disappeared, indicating the existence of a mechanism to remove the CaOx crystals.[28] Studies of de Bruijn et al.[24] also showed that in experimental CaOx nephrolithiasis, crystals form in the tubular lumen and eventually move into the interstitium causing inflammation and attracting many inflammatory cells including leukocytes, monocytes, and macrophages.[36]

Multinucleated giant cells were also identified in the interstitium. The interstitial infiltrate around crystals consisted mainly of lymphocytes and macrophages.[36–38] These cells may play an important role in renal tissue damage through the production of proteolytic enzymes, cytokines, and chemokines.[39,40] The mechanism by which inflammatory cells enter the renal interstitium is not known, but it is likely that chemotactic factors and adhesion molecules are involved. Products of interaction between renal tubular cells and Ox or CaOx crystals under hyperoxaluric conditions might play an important role in the attraction and accumulation of infiltrating inflammatory cells.

Mild renal injury is detectable in hyperoxaluric rats without CaOx crystal deposition in the kidneys, indicating the nephrotoxic nature of the Ox ions, whereas morphologically discernible damage is associated with crystal deposition. This attests to the detrimental effect of epithelial exposure to the dual toxins, Ox and CaOx crystals. Results from one of our studies[26] showed a gradual increase in urinary levels of alkaline phosphatase (AP), gamma-glutamyl transpeptidase (GGTP), and *N*-acetyl-β-glucoseaminidase (NAG), enzymes often reflective of proximal tubular injury. Urinary excretion of NAG was most significantly increased and correlated highly with urinary excretion of Ox.

Free radicals and metabolites of arachidonic acid such as prostaglandins are known chemical mediators of inflammation. We have demonstrated that hyperoxaluric rats had increased urinary excretion of lactate dehydrogenase (LDH) and lipid peroxides indicating the involvement of free radicals in Ox- and CaOx-associated renal toxicity.[29] In addition, administration of antioxidants such as vitamin E resulted in reduced levels of lipid peroxides in the kidneys and decreased urinary excretion of LDH and lipid peroxides. After 8 weeks on a hyperoxaluric diet, male Sprague–Dawley rats excreted higher amounts of arachidonic acid and prostaglandin E2 than the normal controls (Fig. 1). We investigated MCP-1 production by renal epithelial cells in response to an exposure to Ox and CaOx crystals. Immunohistochemical investigation of the paraffin-embedded sections of rat kidneys using a rabbit anti-rat MCP-1 antibody showed cells of crystal-containing tubules to stain positive for MCP-1 (unpublished results).

CaOx crystal deposition in the kidneys increases the expression of Tamm–Horsfall protein (THP),[41,42] osteopontin (OPN),[30] inter-alpha-inhibitor (ITI),[31,32] prothrombin

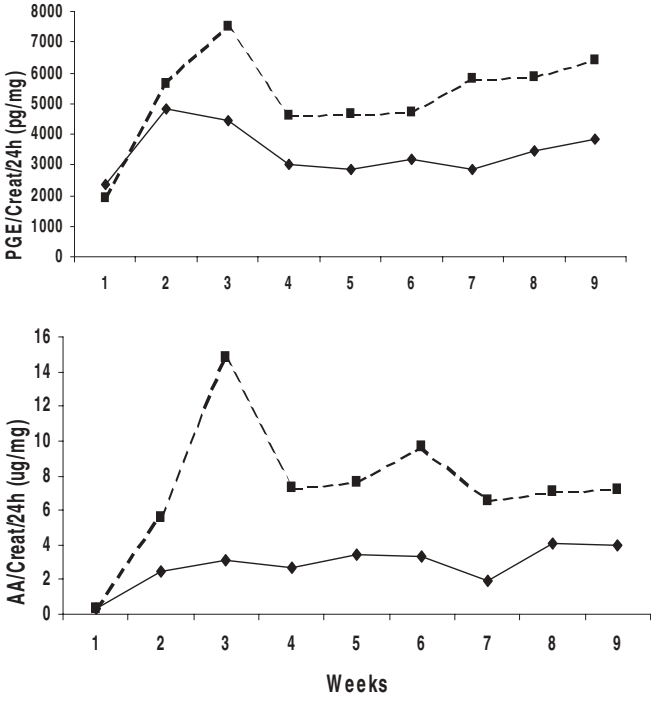

Fig. 1. Calcium oxalate (CaOx) nephrolithiasis was induced in four male Sprague–Dawley rats by the administration of 0.75% ethylene glycol (EG). Urine was collected once a week. After 1 week of treatment, the nephrolithic rats on diets with EG excreted significantly more ($P < 0.05$) arachidonic acid (*AA*) and prostaglandin E2 (*PGE*) in their urine than three control male rats on a normal diet. *Diamonds*, control rats; *squares*, rats treated with EG; *creat*, creatinine

(PT),[43] and heparan sulfate (HS)[44] as determined by immunocytochemical localization using specific antibodies. Some studies indicate that there is no increase in either the production or excretion of THP,[41,42] only increased retention in the kidneys where THP surrounded the retained crystals. Other studies have, however, shown either a decrease[45] or an increase[46] in THP expression and production. Production and urinary excretion of OPN,[30,42] PT,[43] various ITI-related proteins,[31,32] and HS[44] is substantially increased as determined by detection of their specific mRNAs using in situ hybridization, reverse transcription polymerase chain reaction, or both.

Other studies have provided evidence for the activation of the renin–angiotensin system during the development of tubulointerstitial lesions of CaOx crystals.[37,38] Reduction of angiotensin production by inhibiting the angiotensin-converting enzyme as well as blocking the angiotensin receptor reduced crystal deposition and ameliorated the associated inflammatory response. Recently, we have shown that CaOx crystal deposition in rat kidneys activates the renin–angiotensin system and increases renin expression in the kidneys and serum.[47]

Recent studies have shown OPN to be a monocyte chemoattractant specifically for the renal interstitium and that up-regulation of OPN precedes the interstitial monocyte infiltration.[48,49] OPN knockout studies demonstrated a reduced influx of macrophages into obstructed kidneys of knockout mice compared to wild-type mice at days 4 and 7 but not at day 14. It was concluded that OPN mediates early interstitial macrophage influx.[49] Ethylene glycol administration to OPN knockout mice resulted in intratubular deposition of CaOx, whereas there was no deposition in the wild-type mice given the same treatment.[50]

Other up-regulated macromolecules are also involved in the inflammatory process.[51] HS regulates extracellular matrix production. Bikunin, a constituent of ITI, is a proteinase inhibitor. Acute inflammatory conditions are known to up- or down-regulate transcription of ITI genes. Bikunin is associated with inflammation and stabilizes the extracellular matrix.[52,53] THP is seen in the renal interstitium in several forms of tubulointerstitial diseases. The administration of THP is shown to produce interstitial inflammation and scarring and can activate alternate pathways, interact with neutrofils, and bind to certain cytokines. Prothrombin is the precursor of thrombin and fragments 1 and 2. Thrombin is involved in platelet aggregation and blood coagulation and plays a major role in the recruitment and activation of infiltrating immune cells.

Tissue-culture studies

Tissue-culture studies in which renal epithelial cells were exposed to Ox, CaOx crystals, or both have generally confirmed the results obtained from animal model studies and have provided new insights into renal responses to exposure to Ox and CaOx crystals. The response is time and concentration dependent and cell specific. Both Ox and CaOx crystals are injurious to renal epithelial cells in culture.[54–59] porcine kidney (LLC-PK1) cells, which represent the proximal tubular cells, are more susceptible to injury than Madin-Darby canine kidney (MDCK) cells, which represent epithelial cells of the more distal sections of the renal tubules. Lower Ox levels induce expression of immediate early genes, stimulate DNA synthesis, and promote cellular proliferation, whereas higher Ox levels induce cell damage and death. Epithelial injury promotes attachment of CaOx crystals.[60–61] The attachment is mediated by Ox-induced exposure of phosphatidylserine (PS) on cell surfaces.[62,63] Enrichment of cell membranes with PS also resulted in increased attachment of CaOx crystals.[64] Several studies have, however, indicated that injury and exposure to PS may not be essential for the attachment of CaOx crystals to epithelial cells.[65–68] CaOx crystals bind rapidly to the surfaces of non-transformed monkey renal epithelial (BSC1) cells and are internalized. A variety of anionic cell surface molecules, which mediate crystal attachment, are exposed during cell proliferation or on exposure to Ox. Specific urinary substances such as citrate, glycosaminoglycans, OPN, and bikunin[69] regulate crystal attachment. In addition, crystals adhere to proliferating and subconfluent but not to confluent cultures of MDCK cells.

Results of studies of renal epithelial cell exposure to Ox, CaOx, or both also confirmed the involvement of free radicals in Ox toxicity.[58,70,71] The possibility of free radicals being involved in Ox toxicity was suggested by Scheid et al.,[58] who exposed LLC-PK1 cells to Ox. The free radical production was determined by monitoring the reduction of nitroblue tetrazolium to a blue reaction product and by following the conversion of dihydrorhodamine 123 to its fluorescent derivative rhodamine 123. Studies demonstrated that Ox induced a concentration-dependent increase in dye conversion that was blocked by the free radical scavengers catalase and superoxide dismutase. Free radical scavengers also prevented Ox-induced loss of membrane integrity. Human renal proximal tubular epithelial cells exposed to Ox also showed membrane damage, cell loss, and expression of egr-1 mRNA.[70] These responses were attenuated by a superoxide dismutase mimetic, Mn(III) tetrakis porphyrin, indicating the involvement of free radicals in Ox nephrotoxicity.

The response of renal epithelial cells to calcium oxalate monohydrate (COM) crystals is characterized by increased expression of specific genes that encode the following: (1) transcriptional activators such as early growth response-1, c-*myc*, Nur-77, c-*jun*; (2) a regulator of the extracellular matrix composition, the fast-acting plasminogen activator inhibitor-1; and (3) growth factors that can stimulate fibroblast proliferation in a paracrine manner such as the platelet-derived growth factor-A chain, a connective tissue growth factor.[72] Exposure to CaOx crystals increased OPN mRNA and stimulated MDCK and BSC-1 cells to produce OPN.[73] The bikunin gene was also expressed and bikunin was produced when MDCK cells were exposed to Ox.[74]

Because crystal deposition in kidneys is associated with migration of monocytes/macrophages (M/M) into the interstitium, we hypothesized that in response to crystal exposure, renal epithelial cells produce chemokines, which

attract the M/M to the sites of crystal deposition.[75,76] We investigated the expression of MCP-1 mRNA and protein by normal rat kidney (NRK52E) rat renal tubular epithelial cells exposed to CaOx crystals. Confluent cultures of NRK52E cells were exposed to CaOx at a concentration of 250 μg/ml (66.7 μg/cm^2). Cells were exposed for 1, 3, 6, 12, 24, and 48 h for isolation of mRNA and for 24 h for enzyme-linked immunosorbant assay (ELISA) to determine the secretion of protein into the culture medium. Because cells are known to produce free radicals on exposure to CaOx crystals, we also investigated the effect of free radical scavenger catalase on the crystal-induced expression of MCP-1 mRNA and protein. Exposure of NRK52E cells to the crystals resulted in increased expression of MCP-1 mRNA and in increased production of the chemoattractant. Treatment with catalase had a negative effect on the increased expression of both MCP-1 mRNA and protein, which indicates the involvement of free radicals in the up-regulation of MCP-1 production. Results indicate that MCP-1, which is often associated with localized inflammation, may be one of the chemokine mediators associated with the deposition of various urinary crystals in the kidneys.

Chemokines are a superfamily of low-molecular-weight secretory proteins that function principally as stimulators of leukocyte recruitment and activation at the sites of inflammation.[77,78] There are four defined chemokine subfamilies based on their primary structure, C, CC, CXC, and CX3C. Most of the chemokines belong to the CC or CXC subfamily. Members of the CC chemokine subfamily, such as MCP-1, are chemotactic for monocytes and other leukocyte subsets and thus elicit an inflammatory and immune reaction. Most chemokines can be produced by a wide variety of cell types after proper stimulation with proinflammatory cytokines, or even by direct stimulation, and exert their effect on one or more target cell populations.

A diverse population of renal cells, including human proximal tubules, renal cortical epithelial cells, interstitial fibroblasts, and glomerular endothelial cells, express various cytokines and chemokines such as interleukin-10, MCP-1, interferon-induced protein-10 (IP-10), and regulated upon activation normal J cell activated and secreted (RANTES). Obstructed rat kidneys (hydronephrosis) show increased expression of mRNA of RANTES, macrophage inflammatory protein-1 (MIP-1), and MCP-1.[79] Production of MCP-1 by renal proximal tubular epithelial cells is up-regulated in various types of glomerular disease.[80] Similarly, glomerular endothelial cells also produce MCP-1 during glomerulonephritis.[81] Chemokine production by renal cells occurs by activation of gene transcription. Cultured renal cells secrete several chemokines. Results showed that approximately 70%–80% of monocyte chemotactic activity produced by cultured human mesangial cells,[82] renal cortical epithelial cells,[83] proximal tubular epithelial cells,[80] and bovine glomerular endothelial cells[81] is accounted for by MCP-1. MCP-1 has been found in cortical tubular cells in association with interstitial mononuclear infiltration associated with experimental obstruction.[84] Stretching of endothelial cells can also induce MCP-1 production.[85] These results suggest that increased tubular pressure during obstruction induces the epithelial cells to produce MCP-1 and may be the initial event in recruiting leukocytes to the interstitium. Production of MCP-1 is also influenced by the presence of reactive oxygen species. MCP-1 mRNA expression was increased in mouse mesangial cells in the presence of superoxide ions.[86] MCP-1 levels were elevated in the urine of patients with many glomerulonephropathies[87] and correlated with glomerular injury and glomerular monocyte infiltration. Transplant patients with rejection who did not respond to steroids had elevated MCP-1 in their urine, whereas those who responded showed a decline in MCP-1 levels.[88] Patients with active lupus nephritis showed higher urinary levels of MCP-1 than controls or patients with inactive disease.[89]

Additional observations

As discussed above, both Ox ions and CaOx crystals induce oxidative stress in the kidneys, a condition in which either the endogenous antioxidant defenses are depleted or more reactive oxygen species (ROS) such as superoxide and H_2O_2 are produced than can be dealt with. This oxidative stress initiates a cascade of events culminating in renal pathology (Fig. 2). Mitochondria have been shown to be a source of free radicals and ROS,[90,91] but the possibility of other sources has not been ruled out. NADPH oxidase is also a major source of ROS in the kidneys[92] and may be involved in the generation of ROS by renal epithelial cells exposed to CaOx crystals. We have shown a reduction in Ox-induced injury of NRK52E cells in the presence of diphenyleneiodonium chloride (DPI), an NADPH oxidase inhibitor (unpublished results).

ROS activate many signaling molecules such as protein kinase C (PKC), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) and transcription factors such as NF-κB and activated protein 1 (AP-1). Activation of these molecules leads to up-regulation of genes and proteins such as MCP-1, OPN, fibronectin, and TGF-β_1. Recent studies have provided evidence that both Ox and CaOx crystals selectively activate p38 MAPK signal transduction pathways.[93,94] In addition, p38 MAPK is essential for reinitiation of the induced DNA synthesis. Ox exposure also caused modest activation of JNK as determined by c-Jun phosphorylation. Apparently, renal epithelial response to Ox involves signal transduction via MAP kinases, similar to the cellular responses to many other challenges. Cytosolic phospholipase A_2 (cPLA2) is released on the activation of MAP kinases and translocated to the cell membrane. cPLA2 preferentially hydrolyses arachidonoyl phospholipids, generating a number of by-products, including arachidonic acid and lysophospolipids. Exposure of MDCK cells to Ox produces a time- and concentration-dependent increase in cPLA2 activity.[90] Inhibition of cPLA2 activity blocked the Ox-induced up-regulation of Egr-1, c-*jun*, and c-*myc* genes. Exposure of MDCK cells to Ox also increased the generation of ceramide, another signaling lipid, most probably through the activation of neutral sphingomyelinase. An inhibitor of cytoplasmic PLA2

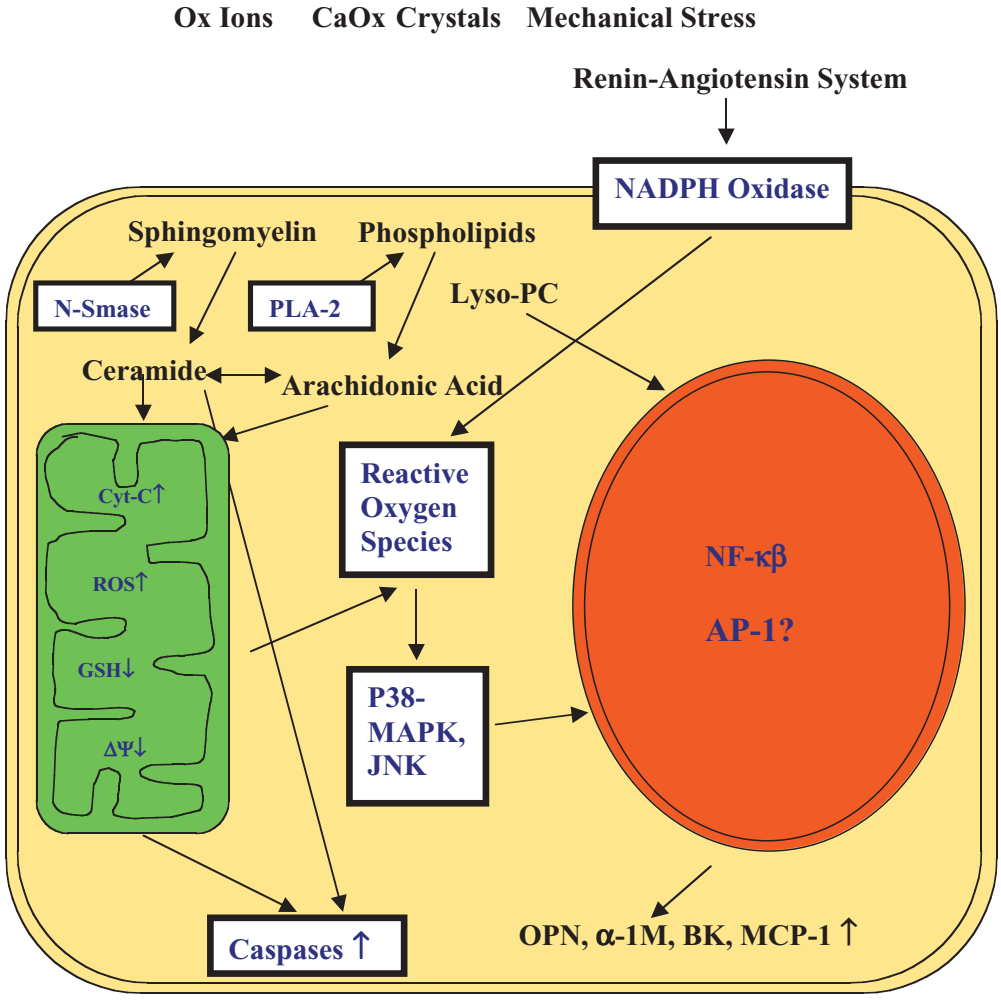

Fig. 2. The kidney produces all components of the renin–angiotensin system (RAS), and the intrarenal RAS plays a major role in renal disease progression.[148–149] Kidneys produce both angiotensinogen and angiotensin converting enzymte (ACE), and the juxtaglomerular apparatus is the main source of circulating renin. Renin catalyzes the production of angiotensin I (Ang I), which is converted to Ang II by the actions of ACE. Ang II acts through two receptors, types 1 (AT1) and 2 (AT2), and mediates many effects of the RAS, regulating numerous physiological reactions including salt and water balance, aldosterone release, and blood pressure. Oxidative stress plays a significant role in the proinflammatory effects of Ang II. Ang II is implicated in causing oxidative stress by stimulating membrane-bound NADH/NADPH oxidase, which leads to increased generation of superoxide.[150] ACE inhibitors and AT1 receptor blockers have been shown to provide protection against Ang II-induced fibrosis and oxidative stress. Kidneys are under oxidative stress when renal epithelial cells are exposed to high oxalate levels, CaOx crystals, and the mechanical stress associated with crystal deposition in the renal tubules. Reactive oxygen species (*ROS*) may be produced by the activation of NADPH oxidase located at the plasma membrane or by the activation of phospholipase A2 (*PLA-2*) and neutral sphingomyelinase (*N-Smase*) with effects on mitochondria through lipid products arachidonic acid, lyso-phosphatidylcholine (*Lyso-PC*), and ceramide. The production of ROS and cytochrome-C (*Cyt-C*) goes up. There is a reduction in mitochondrial membrane potential (ΔΨ) and reduced glutathione (*GSH*). These actions result in activation of caspases leading to cellular apoptosis. Reactive oxygen species also activate the p38 mitogen-activated protein kinase (*MAPK*) signal transduction pathways with influence over various transcription factors such as nuclear factor-κβ (*NF-κβ*) and activated protein 1 (*AP-1*). There is an increase in expression of immediate early genes and production of various crystallization modulators such as osteopontin (*OPN*), bikunin (*BK*), and α-1-microglobulin (*α-1M*) and chemoattractants such as monocyte chemoattractant protein 1 (*MCP-1*). *JNK*, c-Jun-N-terminal kinase

blocked ceramide production, indicating the involvement of the PLA2 pathway.

Calcium phosphate crystals

Human studies

Calcium phosphate crystals are encountered in the kidneys of patients with a variety of diseases, which lead to hypercalciuria. Elevated levels of calcium in the glomerular filtrate, tubular fluids, and urine raise the supersaturation with respect to CaP, thus increasing the chances for crystal nucleation and deposition in the kidneys. The most common causes include hyperparathyroidism and renal tubular acidosis. Some disorders are inherited, e.g., Dent's disease, which is caused by an inactivating mutation in CLC5, a voltage-gated chloride channel.[95] Other disorders result from complications arising from the use or abuse of medicines such as dexamethasone and furosemide.[96,97] Both medicines are given to very low birth weight premature

babies with respiratory problems. Adults also use furosemide for certain medical conditions, sometimes for weight control and edema. Long-term use of high doses of furosemide has been shown to cause nephrocalcinosis. During nephrocalcinosis, CaP crystals appear to form intracellularly inside mitochondria of the renal tubular epithelial cells.[98] Subsequently, crystal deposits are seen in the basement membrane as well as in the renal interstitium. Extensive nephrocalcinosis, or CaP crystal deposition, results in chronic inflammation and interstitial fibrosis, leading to tubulointerstitial disease and renal insufficiency.

It has also been proposed that a high level of phosphate is deleterious to the renal tubules, induces nephrocalcinosis, and is responsible for progressive decline in renal function in chronic renal failure.[99] CaP crystals form in the renal tubules as well as in the interstitium. Dietary phosphate restriction has a salutary effect on renal function.

Animal model studies

A number of techniques have been used to experimentally induce hypercalciuria in animal models, including the administration of vitamin D. When given with a hyperoxaluria-inducing agent such as ethylene glycol, dispensation of vitamin D resulted in deposition of CaOx crystals in the rat kidney.[100] Administration of 1,25-dihydroxycholecalciferol alone resulted in the formation of CaP concretions in the kidney.[101] Concretion formation was reduced by calcium antagonist 1,4 dihydronaphthyridine. Deposition of CaP resulted in impaired renal function. Other experimental methods to induce nephrocalcinosis include intraperitoneal injections of calcium glucanate or parathyroid hormone (PTH) in mice or rats.[102–104] Both methods showed crystal deposition in the cortical tubules. Calcium glucanate administration in mice resulted in CaP deposition in the epithelial basement membrane, whereas calcification in rats given PTH started inside the cellular mitochondria. Interestingly, administration of calcium antagonists prevented or reduced nephrocalcinosis induced by consumption of a high-cholesterol diet.[103,104] Dietary supplementation with essential fatty acids reduced calcium glucanate-induced renal calcification.[105] Reduction in calcific deposits improved renal function.[104,105]

The dietary calcium to phosphorus ratio appears to play a significant role in calcification, and female weanling rats are more susceptible to calcification than are mature females or weanling or mature males.[106] Diet-induced calcification is primarily seen in renal tubules of the corticomedullary junction. High phosphate levels play a significant role in the progression of renal failure and in CaP crystal deposition in the kidneys of uremic rats. Uremia was induced in male Sprague–Dawley rats by surgical removal of 70% of the functional renal mass. Increase in dietary phosphate levels created significant and progressive deterioration in renal function, which was associated with extensive cellular necrosis, tubular dilatation, interstitial inflammation, and nephrocalcinosis. Administration of 3-phosphocitric acid, which is known for its inhibitory activities against CaP crystallization, effectively reduced CaP crystal deposition in the kidney as well as reducing pathological changes in the kidney.[107] In another study 5/6 nephrectomized female Sprague–Dawley rats were given a high-phosphate diet with or without one of the phosphate binders: calcium carbonate or sevelamer hydrochloride. Both treatments improved serum phosphorus levels and reduced secondary parathyroidism, renal deposition of CaP, and tubulointerstitial fibrosis and hence preserved the renal function.[108]

Recently, knockout mice have been developed with potential for studies of CaP-induced inflammation of the kidneys. Targeted inactivation of *Npt2* in mice leads to severe renal phosphate wasting and hypercalciuria. Renal calcification was observed in both weanling and adult $Npt2^{-/-}$ mice but not in $Npt2^{+/+}$ mice.[109] Calcific deposits were pervasive throughout the kidneys. Osteopontin (OPN), a chemoattractant and mineralization modulator, was found to colocalize with calcification. In addition, renal OPN mRNA was significantly elevated in $Npt2^{-/-}$ mice. Renal calcification correlated not only with the absence of the *Npt2* gene but also with the genes responsible for the production and catabolism of 1,25-dihydroxyvitamin D. Disruption of the caveolin-1 gene impaired renal calcium absorption leading to hypercalciuria. Calcium phosphate crystals were seen in the urine of all male $Cav^{-/-}$ mice and frank stones were present in 13% of male $Cav^{-/-}$ mice.[110]

Three mice models of Dent's disease with disrupted or reduced expression of the *CLC-5* gene have also been recently developed. Transgenic mice with reduced *CLC-5* expression were developed by introduction of an antisense ribozyme targeted against *CLC-5.* These mice were markedly hypercalciuric but nephrocalcinosis was not observed and hypercalciuria could be abolished by dietary manipulation.[111] *CLC-5* knockout mice developed by deleting the genomic sequence showed low molecular weight proteinuria but no hypercalciuria.[112] Another mouse model of Dent's disease was developed by disrupting exon VI of the *CLC-5* gene.[95] These mice showed significant polyuria, hypercalciuria, and hyperphosphaturia; low molecular weight proteinuria; generalized aminoaciduria; and diffuse von-Kossa-positive nephrocalcinosis at the corticomedullary junction.

Tissue-culture studies

Based on our experience with CaOx crystal deposition in the kidneys and other studies of biomineralization, we hypothesized that CaP crystals can also injure renal epithelial cells and that injury will be mediated by free radical production. We exposed proximal-tubular-origin LLC-PK1 and collecting-duct-origin MDCK cell lines to various concentrations of calcium hydrogen phosphate dihydrate, or brushite (Br), crystals and investigated the effect on the cells by staining with trypan blue and by measuring the release of LDH into the medium as a indicator of injury.[113] To determine the involvement of reactive oxygen species, we also measured LDH release, trypan blue exclusion, and

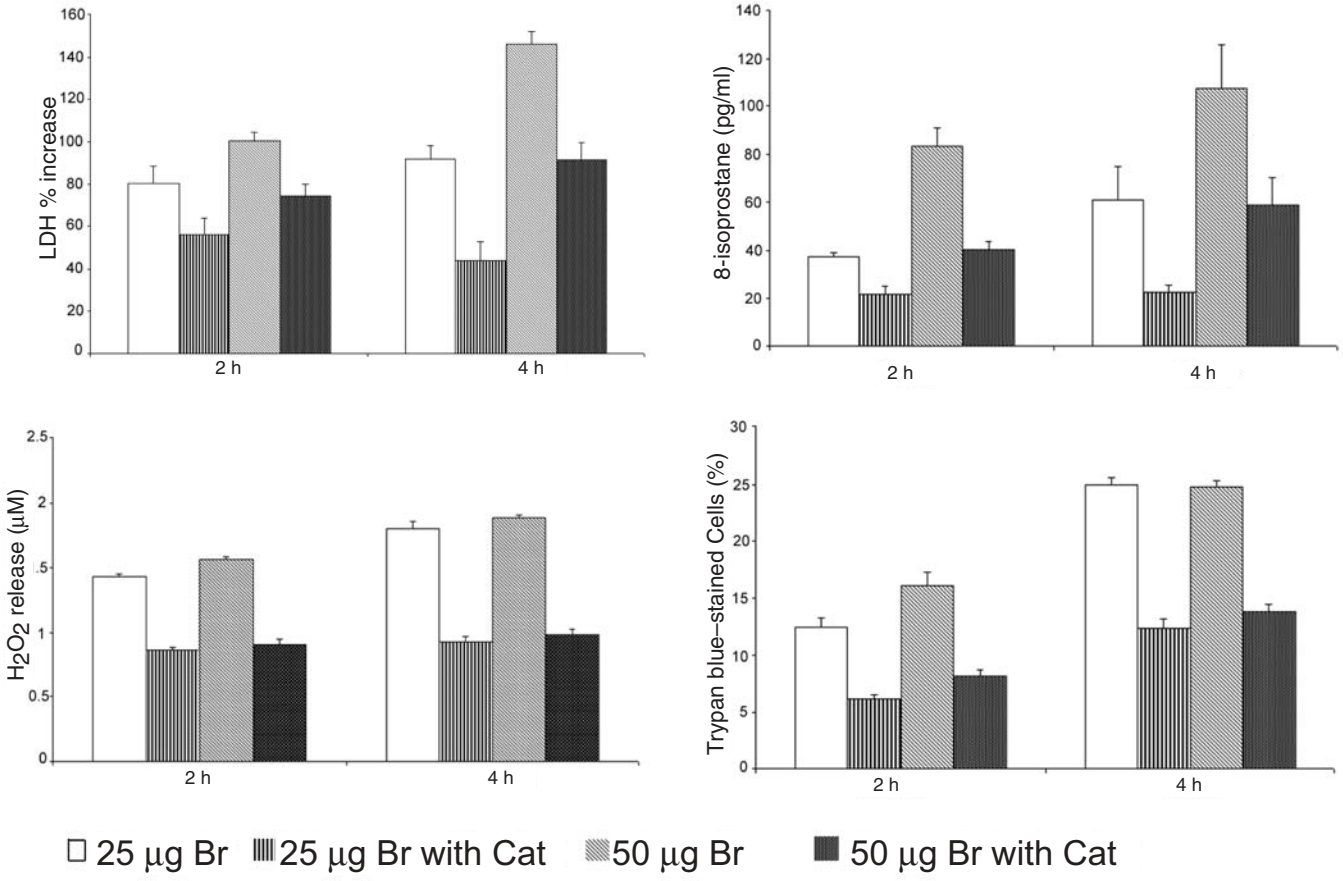

Fig. 3. *LLC-PK*$_1$ cells were exposed to two different concentrations of brushite (*Br*), i.e., 25 μg/cm^2 or 50 μg/cm^2, for 2 or 4 h in the presence or absence of antioxidant catalase (*Cat*). Markers of cell injury [trypan blue exclusion and lactate dehydrogenase (*LDH*) release] as well as oxidative stress, H_2O_2 production, and release of 8-isoprostane were all reduced in the presence of antioxidants

production of hydrogen peroxide (H_2O_2) and 8-isoprostane (8-IP) in the presence and absence of antioxidants catalase and superoxide dismutase (SOD). In both cell types, exposure to Br crystals was associated with LDH release as well as the production of H_2O_2 and 8-IP (Fig. 3). The presence of SOD and catalase reduced LDH release and reduced staining with trypan blue. Catalase was also associated with reduced production of H_2O_2 and 8-IP. Thus, results show that Br crystals are injurious to cells of both the proximal tubules and the collecting ducts and that the injury is mediated by reactive oxygen species.

In a separate experiment, we determined the induction of the chemokine MCP-1 in renal epithelial cells on exposure to Br crystals.[76] Confluent cultures of NRK52E cells derived from rat kidneys were exposed to Br crystals at a concentration of 250 μg/ml (66.7 μg/cm^2). The cells were exposed for 1, 3, 6, 12, 24, and 48 h for the isolation of mRNA and for 24 h for ELISA to determine the induction of the gene and the secretion of MCP-1 protein into the culture medium. Because renal epithelial cells are known to produce free radicals on exposure to both CaOx and Br crystals, we also investigated the effect of the free radical scavenger catalase on the Br-induced expression of MCP-1 mRNA and protein. Treatment with catalase had a negative effect on the increased expression of both MCP-1 mRNA and protein, which indicates the involvement of free radicals in the up-regulation of MCP-1 production. These results indicate that MCP-1, which is often associated with localized inflammation, may be one of the chemokine mediators associated with CaP-induced inflammation and fibrosis of the kidneys.

Additional observations

Deposition of calcium pyrophosphate dihydrate (CPPD) and a variety of other CaPs, including hydroxyapatite, carbonate apatite, octacalcium phosphate, and tricalcium phosphate, collectively termed basic calcium phosphate (BCP), causes many diseases of the joints.[114,115] BCP, and to some extent CPPD, crystals induce mitogenesis, stimulate production of prostaglandin E2 (PGE2), activate phospholipase C, promote the synthesis of metalloproteinases (MMPs), and induce proto-oncogenes c-*fos* and c-*myc*.[116–118] Induction of MMP-1 (collagenase) and MMP-3 (stromelysin) is dependent upon the p42/44 mitogen-activated protein kinase (MAPK) signal transduction pathway.[119,120] Another independent pathway involved is the calcium-dependent protein kinase C (PKC) signal transduction pathway.[121] BCP crystals not only up-regulate the pro-

duction of MMPs but also down-regulate the production of tissue inhibitors of metalloproteinase (TIMP).[122] Results show that calcium phosphate crystals activate p42/44 MAPK as well as calcium-dependent PKC pathways. Both these pathways lead to an up-regulation of the transcription factor AP-1, promoting mitogenesis as well as the synthesis of MMPs and interfering with the ECM deposition/degradation equilibrium.

Uric acid or urate crystals

Human studies

From 2% to 18% of the population of the west are hyperuricemic, i.e., their plasma urate levels are above 7 mg/dl, i.e., are in excess of the saturation value for urate at normal body temperature and blood pH. Uric acid precipitation is favored by the acidic pH of the distal renal tubules and collecting ducts. Three types of renal diseases can develop in hyperuricemic patients. Precipitation of uric acid crystals in the renal collecting ducts of leukemia and lymphoma patients undergoing chemotherapy leads to the first type, namely acute tubular obstructive disease.[123] The second is gouty nephropathy, which develops in patients with chronic hyperuricemia. Deposits of monosodium urate crystals develop in the collecting ducts and distal tubules as well as in the medullary interstitium. These interstitial deposits evoke an intense inflammatory reaction and are seen as tophi, masses of slender urate crystals surrounded by mononuclear cells and foreign body giant cells. Up to 40% of patients with gout may develop significant chronic renal disease and half of patients with gout show reduced glomerular filtration rate (GFR) levels. Autopsies of 79%–99% of patients with gout have been reported to show gouty nephropathies. The third renal disease, uric acid neophrolithiasis, is caused by hyperuricosuria.[124] Urinary pH below 5.5 is also a factor in uric acid nephrolithiasis because uric acid is insoluble in acidic urine. Uric acid or sodium acid urate crystals can also promote crystallization of calcium salts either through heterogeneous nucleation or salting out. As a result, up to 20% of calcium stone formers are hyperuricosuric.

Animal model studies

Hyperurecemia with or without uricosuria and intrarenal crystal deposition can be induced by the administration of the uricase inhibitor oxonic acid to rats.[125–127] Urate oxidase, or uricase (EC 1.7.3.3), is a purine metabolic enzyme that catalyzes the conversion of uric acid to allantoin in most mammals, except for humans and certain other primates. Administration to rats of a dose of 2% oxonic acid mixed with food induced hyperuricemia without the renal deposition of crystals, tubular obstruction, or interstitial inflammation. Hyperurecemia alone was sufficient to cause hypertension, intrarenal vascular disease, and renal tubular injury. Vascular injury was mediated by uric acid inducing vascular smooth muscle cell proliferation and activation of the renin–angiotensin system.[127–129] Feeding oxonic acid with uric acid produced hyperurecemia, uricosuria, and intrarenal deposition of urate crystals.[126] Renal deposits were both tubular and interstitial and consisted of amorphous and crystalline material. Epithelial cells were injured, showing signs of both cellular death and regeneration. The interstitium was enlarged and giant cells surrounded the crystals. There was a significant increase in the interstitial expression of α-smooth muscle actin (α-SMA), as determined by immunostaining using anti mouse α-SMA antibody. It has been proposed that crystals of uric acid, monosodium urate, or both precipitating in the tubular lumen erode through the tubular basement membrane and pass into the interstitium where they promote inflammation and fibrosis. Potassium citrate ameliorated the tubulointerstitial lesions of experimentally induced uric acid nephropathy in rats. These rats showed reduced renal tubular atrophy, interstitial fibrosis, and inflammatory infiltrate. There was reduced expression of α-SMA in the interstitium. Rats had reduced albuminuria and higher creatinine clearance.

To create a mouse model for hyperuricemia and gout, the urate oxidase gene was disrupted in the mouse by homologous recombination in embryonic stem cells.[130] Urate oxidase deficiency in mice caused pronounced hyperuricemia and urate nephropathy. Serum uric acid was tenfold higher in homozygous mice. There was generalized injury to all segments of the kidneys. Gross examination showed urate deposition starting as white streaks at the corticomedullary junction. Microscopic examination showed crystals blocking the dilated renal tubules. Renal epithelium was often focally denuded. Atrophy was evident in both tubules and glomeruli. Chronic inflammation was characterized by infiltration of lymphocytes and macrophages into the renal interstitium.

Tissue-culture studies

Interaction between renal epithelium and monosodium urate or uric acid crystals has also been investigated *in vitro*, in which cultures of MDCK cells were exposed to crystals.[131] Cells were exposed to crystals either in monolayer cultures or single-cell suspensions. Electron microscopic analysis of the exposed cells showed development of specific reaction sites on cell surfaces. Interaction began with attachment of crystals to cell surfaces. Crystals were endocytosed and later seen inside phagolysosomes. Crystals were also seen within the intercellular spaces. A number of enzymes, including lactate dehydrogenase (LDH), α-galactosidase, and β-glucuronidase and prostaglandin E2 (PGE2), were released by the MDCK cells exposed to crystals.

We recently demonstrated uric acid-induced LDH release, up-regulation of the MCP-1 gene, and increased production of MCP-1 by NRK52E cells. Results of our studies also showed that free radicals were involved in the induction of the MCP-1 gene because both the production of protein and expression of the gene were reduced in the presence of catalase.[76]

Additional observations

It is postulated that tubular epithelial cells react to the presence of uric acid or monosodium urate crystals by promoting their attachment and endocytosis. Association of crystals with phagolysosomes causes the release of lysosomal enzymes. Release of lysosomal enzymes and cytosolic LDH into the medium indicates cellular disruption. Later the crystals move into the interstitium where they induce a foreign body reaction and become surrounded by monocytes/macrophages. Observations that renal epithelial cells exposed to uric acid crystals can produce MCP-1, a major chemoattractant for monocytes and important in both vascular diseases and atherosclerosis, indicate that infiltration of the interstitium by the inflammatory cells may start even before crystals have reached there. Uric acid has also been shown to stimulate the synthesis of MCP-1 in vascular smooth muscle cells by activating p38 MAP kinase and nuclear transcription factors NF-κB and AP-1.[132] Additional observations of PGE2 release by both the leukocytes[133,134] and renal epithelial cells[131] on exposure to uric acid or urate crystals suggest the possibility of local vasodilatation, which will assist the movement of monocytes and other inflammatory cells to the site of crystal deposition.

Soluble uric acid stimulates human monocytes to produce tumor necrosis factor-α (TNF-α), intereleukin-1β (IL-1β), and IL-6.[132] Circulating levels of TNF-α are markedly increased in mice on induction of sustained hyperuricemia.[135] The interaction of neutrophils with monosodium urate crystals causes the release of lysosomal enzymes[136] and reactive oxygen species[137–139] and the expression of inflammatory mediators including cyclooxegenase-2 and cytokine TNF-α, IL-1, IL-6, and IL-8.[140–144] IL-8 induction was mediated through extracellular signal-regulated kinase-1 (ERK-1)/ERK-2 signaling and AP-1 and NF-κβ transcriptional activation.[145] Src family tyrosine kinase signaling plays a significant role in this crystal-induced IL-8 expression.[146] Monosodium urate crystals also stimulate the macrophages to produce metalloproteinases, including MMP-9, either directly or through the production of cytokine TNF-α.[147] Inhibiting ERK-1/ERK-2 can abolish urate-induced MMP-9 induction in the macrophages.

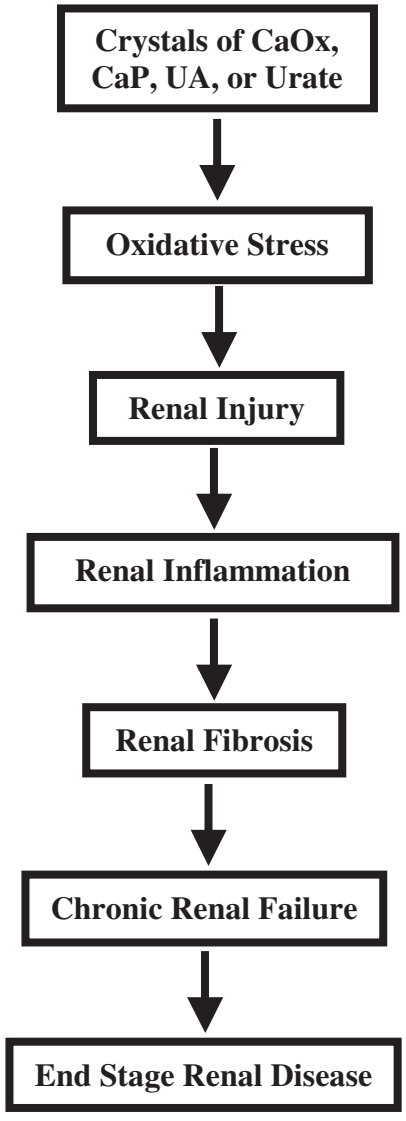

Fig. 4. Schematic presentation of the events involving crystal-induced injury leading to inflammation and fibrosis. *CaOx*, calcium oxalate; *CaP*, calcium phosphate; *UA*, uric acid

Concluding remarks

The urine present in the renal tubules often becomes supersaturated with slightly soluble salts such as CaOx and CaP. When supersaturation is high enough and lasts long enough, crystals nucleate and are either excreted as crystalluria or deposited in the renal tubules. Even certain drugs taken at high concentrations can crystallize in the kidneys. Tubular crystals migrate to the interstitium either through the cells, by being endocytosed at the luminal surface and exocytosed at the basolateral side, or perhaps through the intercellular spaces. Sometimes, as happens in nephrocalcinosis, crystals may also form in the cells, most likely in mitochoindria, or even in the epithelial basement membrane. Intracellular crystals can be taken into the phagolysososmes. Thus crystals come into direct contact with both the tubular epithelial cells and cells present in the renal interstitium. Most, but not all, crystals are injurious to renal tubular cells as well as to other cells and invoke an inflammatory response. In most cases, reactive oxygen species are produced and become involved. Like foreign bodies, crystals may themselves stimulate inflammatory cell infiltration. Obstruction of renal tubules by crystals may cause mechanical stress and injury. Tubular epithelial cells, interstitial resident cells, and inflammatory cells release a variety of cytokines, chemokines, mitogens, and growth factors, promoting further inflammation. Many of the factors stimulate transformation of fibroblasts into myofibroblasts. Synthesis of extracellular matrix is increased, leading to fibrosis. Thus high Ox and uric acid levels and the formation of CaOx, CaP, uric acid, or urate crystals can injure cells and induce

inflammation followed by fibrosis, which leads to chronic and eventually end-stage renal disease (Fig. 4).

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